Complete degradation of anthracene by Manganese Peroxidase

in organic solvent mixtures

G. Eibes, T. Lú-Chau, G. Feijoo, M.T. Moreira* and J.M. Lema

Dept. of Chemical Engineering. School of Engineering, University of Santiago de Compostela. Santiago de Compostela. E-15782, Spain.

*Author for correspondence (Fax: 34-981-528050; tmoreira@usc.es)

Abstract

The goal of this study is the development of a system based on the use of the ligninolytic enzyme Manganese Peroxidase (MnP) for the degradation of polycyclic aromatic hydrocarbons (PAHs), of which anthracene was selected as an example. A main problem of these compounds is their poor solubility in aqueous media. Therefore, the addition of different water miscible organic solvents (acetone, methyl-ethyl-ketone, methanol and ethanol) was considered as a previous step to increase the bioavailability of anthracene. Due to the maximal solubilisation of anthracene and the minimum loss of MnP activity, acetone was selected as the optimal cosolvent, allowing to enhance 140-fold the anthracene solubility for an acetone concentration of 36% (v/v).

The *in vitro* degradation of anthracene by MnP was investigated for different concentrations of the main cofactors and substrates that affect the catalytic cycle of MnP (Mn^{2+}, H_2O_2 and organic acids) as well as for other environmental parameters (temperature, air/oxygen atmosphere and light source). The system attained a nearly complete degradation of anthracene, around 100%, after 6 hours of operation under optimal conditions.

Key words: anthracene, biodegradation, Manganese Peroxidase, miscible organic solvents, solubility, stability

1. 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. These compounds are hydrophobic with low water solubility, thus they are easily adsorbed onto organic matter as soils and sediments. Besides, their recalcitrant behaviour greatly hampers their naturally biological degradation [1, 2].

Among other possibilities, an environmentally friendly approach for PAHs degradation could be based on the use of white rot fungi, which are known to degrade a great variety of complex compounds due to their complex enzymatic system [3]. Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) are extracellular peroxidases produced by white rot fungi and the onset of their production is associated to secondary metabolism conditions in response to nutrient depletion [4]. In particular, manganese and nutrient nitrogen have been shown to have strong regulating effects [5]. The ligninolytic system is nonselective, consequently other aromatic substrates, such as PAHs are potentially oxidized and biodegraded by white rot fungi [6, 7, 8]. The catalytic action of these enzymes 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 [9, 10]. However, a wider application of these enzymes is hindered by the fact that enzymes work properly in aqueous media, where nonpolar compounds as polyaromatics present a very low solubility.

An increased solubilisation of polyaromatics in aqueous media would have beneficial effects on the potential degradation of these compounds [11, 12, 13]. A good

approach to enhance PAHs solubility in several orders of magnitude is the addition of water-miscible cosolvents or surfactants [14, 15, 16]. These latter compounds may present a low solubilisation of PAHs and partial inhibition of the ligninolytic activity [17]. Although enzymatic catalysis in organic solvents is considered a promising approach for solving environmental problems, most of the available work is related to hydrolytic enzymes, applied for synthesis of organic compounds [18, 19]. The potential of using more complex enzymes such as ligninolytic enzymes produced by white rot fungi, which require specific environmental conditions for the activation of their catalytic cycle, is almost untapped [8].

The goal of this work is the evaluation of a system based on the use of MnP for the degradation of a PAH model compound, anthracene, in water-miscible organic solvents. Anthracene, a three-ring PAH, was chosen due to its low aqueous solubility (0.07 mg/L [20]) and this compound has been proved to be a substrate of ligninolytic peroxidases [6]. Enzymatic degradation was selected as an alternative to bacterial processes because the biological degradation usually requires long periods of treatment (from 2 to 4 weeks) and presents lag phases (2 days) till the degradation begins [21, 22].

The initial stage of the process was the selection of the most appropriate cosolvent from a list of four relatively safe, easily available and fairly inexpensive chemicals: acetone, methyl-ethyl-ketone (MEK), methanol and ethanol. The influence of the solvent on MnP activity was used as a criterion for this selection. In a second stage, the optimisation of the degradation process was conducted taking into account specific physico-chemical factors which may directly affect the activation of the MnP catalytic cycle and the degradation rate of anthracene: (a) the concentration of cofactors and substrates required for the action of MnP (Mn^{2+}, H_2O_2, organic acids) [23, 24] and

(b) operating parameters such as temperature, light source and maintenance of air or oxygen atmosphere [25].

2. Materials and methods

2.1. Chemicals

Anthracene and anthraquinone were obtained from Janssen Chimica (99% purity). Acetone, methanol and ethanol were purchased from Panreac (chemical purity); methyl-ethyl-ketone was supplied by Sigma-Aldrich (99.5% purity).

2.2. MnP production

MnP was obtained from two metabolically distinct white-rot fungi, *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) and *Bjerkandera* sp. BOS55 (ATCC 90440), with some different catalytic properties, the latter presenting a superior resistance against high concentrations of H_2O_2 [26]. *P. chrysosporium* was cultured in 250-mL Erlenmeyer flasks on the N-limited BIII medium [27]. *B.* sp. BOS55 was grown in a 10-L fermenter (Braun-Biotech International) on skimmed cheese whey medium [28]. Once the peak production of MnP was detected, the fermentation was stopped. Crude enzyme was concentrated by ultrafiltration using a 10-kDa cut-off type YM-10 membrane (Amicon), and then it was centrifuged for 10 min at 20,000 × *g*.

2.3. MnP activity assays

MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) spectrophotometrically at 30ºC (Cecil CE 7200, UK). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM DMP, 1 mM $MnSO_4$, and up to 600 μL of supernatant in a total volume of 1 mL. The reaction was initiated by adding 0.4 mM H_2O_2. One MnP activity unit was defined as the amount of

enzyme transforming 1 μmol DMP per minute [29].

2.4. Anthracene solubility assays

The solubility of anthracene was determined in 20-mL aliquots containing 25 mg anthracene (final concentration of 1.25 mg/L) with different concentrations of solvent ranging from 1% to 100%. The aliquots were placed in 100-mL Erlenmeyer flasks sealed with teflon plugs, in triplicate, equilibrated for 24 h on a shaker (150 rpm) at 20ºC or 30ºC. Afterwards, the 20-mL assays were filtered through a Millex-LCR$_{13}$ cartridge (Millipore Corp.), with a pore diameter of 0.45 μm and analysed by high-pressure liquid chromatography (HPLC).

2.5. Effect of water: solvent mixtures on MnP stability

The stability of crude MnP from cultures of *B.* sp. BOS55 and *P. chrysosporium* was evaluated in water: solvent mixtures by the determination of the peroxidase titers of MnP at periodic intervals. The assay conditions evaluated included: two temperatures (20ºC and 30ºC), a solvent concentration attaining the solubilisation of 10 mg/L of anthracene according to the results from the anthracene solubility assays and 10 mM sodium malonate (pH 4.5) in a total volume of 10 mL.

2.6. Anthracene biodegradation assays

Oxidation of anthracene was carried out in 100-mL Erlenmeyer flasks, sealed with teflon plugs, with magnetic stirring at room temperature, i.e. 23ºC (except when indicated). The reaction mixture (50 mL) consisted of acetone 36% (v:v), anthracene (5 mg/L) and MnP (200 U/L) with different concentrations of the main cofactors and

substrates reported for MnP (Mn^{2+}, H_2O_2 and organic acid: malonic, oxalic, citric and tartaric acid). Mn^{2+} concentration was assayed at 20 μM and 100 μM, while H_2O_2 was added continuously at three addition rates: 1, 5 and 25 μmol/L·min. In the experiments where the effect of the organic acid was considered, malonic acid concentration ranged from 1 mM to 30 mM. Experiments with oxalic, citric and tartaric acid at similar concentrations were also conducted. The possible effects of other environmental parameters, such as temperature, light and oxygen atmosphere, on the degradation of anthracene were also investigated. The influence of temperature was evaluated in assays performed at 23ºC, 30ºC and 40ºC. An oxygen atmosphere was also investigated by flushing industrial oxygen at periodic intervals (3 min every 30 min). Samples were withdrawn periodically to determine anthracene and anthraquinone concentrations by HPLC as described above, and the evolution of MnP activity was spectrophotometrically determined. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel using boiled MnP. No change in anthracene concentration after 6-8 h of incubation was observed in any controls (data not shown).

2.7. Analytical determinations

A HP 1090 HPLC, equipped with a diode array detector monitoring the absorbance at 253 nm, a 4.6 × 200 mm Spherisorb ODS2 reverse phase column (5 μm; Waters) and a HP ChemStation data processor were used for determining the anthracene concentration. The injection volume was set at 10 μL and the isocratic eluent (80% acetonitrile and 20% water) was pumped at a rate of 0.4 mL/min.

3. Results

3.1. Solubility of anthracene in water: solvent mixtures

The solubility of anthracene in four water miscible solvents: acetone, methyl-ethyl-ketone (MEK), ethanol and methanol, was determined at 20ºC (Figure 1) and 30ºC. Concentrations higher than 70% acetone dissolved anthracene completely (1.25 g/L) while the alcohols attained lower solubilities, only being equivalent at 100% solvent. The addition of MEK provided the highest anthracene solubilities in a concentration range between 10 and 30% (v:v) in comparison with the other solvents. However, higher concentrations of MEK resulted in the formation of two differentiated phases: aqueous and non-aqueous, which impeded the utilisation of MEK as a water miscible solvent.

Table 1 shows the solvent concentrations for the solubilisation of 1, 10 and 100 mg/L of anthracene at 20 and 30ºC. The solubilisation at 30ºC was slightly more beneficial for all cosolvents since it implied a reduction between 7-12% of the total addition of the organic solvent in comparison with that required for 20ºC. The minimum concentrations of the organic solvents required to attain a solubility of 10 mg/L, which is a 140-fold increase of the anthracene solubility in water at 20ºC (0.07 mg/L [20]), were: 27% MEK, 36% acetone, 44% ethanol and 55% methanol (Table 1).

3.2. Stability of MnP in solvent: water mixtures

The effect of solvent: water mixtures on the activity and stability of crude MnP from *B.* sp. BOS55 and *P. chrysosporium* were evaluated. MnP activity was determined instantaneously after mixing the solvent mixtures with MnP. Acetone: water mixtures

maintained the enzymatic activity at values near 100%, whereas proportions of methanol higher than 50% caused a sharp decay of the initial activity of MnP from *B.* sp. BOS55 (Figure 2A). Ethanol caused a slight decline of MnP activity, being more evident for ethanol proportions higher than 50%. MEK addition was found to have a negligible effect on the range considered (1-30%). Similar results were obtained for MnP from *P. chrysosporium*, where methanol exerted the most detrimental effect on MnP activity (Figure 2B).

In order to determine the effect of the presence of solvent on MnP stability, the residual enzymatic activity was periodically checked in prolonged incubations of MnP in the water: solvent mixtures (Figures 3A and 3B). The concentrations of solvents used for incubations were the necessary to dissolve 10 mg/L of anthracene at 20ºC (bold values in Table 1).

The inactivation strength of MnP from *B.* sp. BOS55 in decreasing order is methanol, ethanol, MEK and acetone (Figure 3A). Furthermore, a control assay performed in the absence of solvent maintained its initial MnP activity after 24 h. Control and acetone mixture followed similar trends and thus, at the end of the experiment, acetone mixture activity was 97% of the control activity. On the contrary, a remarkable deactivation of the enzyme was found in methanol: water mixtures. This instability is time-dependent and gradual, leading to an irreversible loss of enzymatic activity after only 20 min. At 30ºC the rate of inactivation was higher in all cases (data not shown) and the deactivating action of solvents followed a similar trend.

Similar assays were carried out to determine the stability of MnP from *P. chrysosporium* cultures (Figure 3B), which was proved to be more extensively affected than MnP incubations from *B.* sp. BOS55. Methanol also exerted a large inactivation,

being already evident in the first minutes of incubation. Acetone mixture turned out to be the best solvent, in terms of enzyme inactivation, although a pronounced decrease in comparison with that of the control (48 %), was observed.

3.3. In vitro *degradation of anthracene*

To maximize *in vitro* degradation of anthracene by MnP, different concentrations of the main cofactors and substrates reported for MnP (Mn^{2+}, H_2O_2 and malonic acid) were evaluated, the results being summarised in Table 2.

3.3.1. Effect of H_2O_2

In this series of experiments, a progressive reduction of the H_2O_2 addition rate was considered, from 25 to 1 μmol/L·min, for constant concentrations of malonic acid (1 mM) and Mn^{2+} (100 μM). A slower H_2O_2 addition rate had as a consequence a lower enzyme deactivation and only 8 U/L·h were consumed when 1 μmol H_2O_2 /L·min was applied. However, in terms of effectiveness, the best results were obtained at 5 μmol H_2O_2/L·min, with a maximal anthracene degradation of 9.8%, which implies an efficiency of 0.059 μmol anthracene degraded per unit of consumed MnP.

3.3.2. Effect of Mn^{2+}

Taking into account that Mn^{2+} directly affects the catalytic properties of MnP [30], two Mn^{2+} concentrations of 20 and 100 μM were evaluated for their effect on the anthracene degradation. In terms of anthracene degradation, there was statistically no significant difference between the two Mn^{2+} concentrations considered (9.8 ± 0.8 for a

Mn^{2+} concentration of 100 μM and 10.0 ± 2.4 for a Mn^{2+} concentration of 20 μM). For a practical application of this process, the lower concentration of Mn^{2+} was considered as the most adequate since possible discharges to water courses remain below the compulsory limits (36 μM).

3.3.3. Effect of organic acids

Malonic acid concentration was the main factor affecting the efficiency of the process (Table 2). Although the increase of the concentration led to a higher activity loss, the efficiency was improved up to 0.083 μmol anthracene/U MnP due to the superior degradation achieved, 43.3%, when 20 mM malonic acid was applied.

Other organic acids as oxalic, tartaric and citric, were also considered and similar assays were performed (Figure 4). Regarding to the anthracene degradation, the best result corresponded to 20 mM malonic acid (43.3%), followed by oxalic (32.6%). Tartaric acid seemed not to affect the MnP catalytic cycle, attaining similar degradations as observed without organic acid, and surprisingly, the addition of citric acid (both 10 and 20 mM) caused a reduction on the degradation extent (2.9 and 3.8%, respectively). Taking the loss of MnP activity into consideration, the addition of any organic acid increased MnP consumption. Oxalic acid 20 mM caused the greatest activity loss, leading to a total inactivation of MnP after 90 min. Tartaric and citric acid, in both concentrations, affected MnP activity in a similar way (activity loss around 50 U/L·h).

3.3.4. Effect of environmental parameters

Other parameters such as temperature and working under air or oxygen atmosphere were evaluated. As it can be seen in Table 3, dissolved oxygen (up to 25 mg/L in the reaction media) improved the anthracene degradation (50.5%) and anthraquinone production (19.0%) whereas the enzymatic activity loss was not affected. On the other hand, the increase of temperature to 30ºC led to a reduction of the anthracene degradation (34.9%), as well as a greater activity loss (83 U/L·h). Operation at 40ºC provoked a very severe activity loss (MnP was totally inactivated after 1h reaction), being therefore the oxidation of anthracene very small (5.5%).

Experiments in complete darkness were also performed to check the effect of the presence of light on the anthracene oxidation. It was observed that the extent of anthracene degradation was slightly lower in darkness (83% of that in presence of light), whereas no changes in activity loss were observed (data not shown).

3.3.5. Complete degradation of anthracene

So far, experiments to determine the optimal conditions for the *in vitro* oxidation of anthracene, have been conducted for 2 hours. In order to quantify the maximum extent of anthracene degradation, the operation was prolonged until complete oxidation. The degradation profile of 5 mg anthracene/L (28 μM) in a medium containing 36% acetone (v:v), malonic acid 20 mM, Mn^{2+} 20 μM, continuous addition of H_2O_2 at 5 μmol/L·min working under oxygen atmosphere is shown in Figure 5. The anthracene degradation was nearly complete after 6 hours. During the first 2 hours of the experiment, a marked activity loss and anthracene degradation were observed. A parallel experiment was carried out under an air atmosphere instead of oxygen, attaining in this case, a nearly complete oxidation of anthracene (98%) after 8 h (data not shown).

4. Discussion

The development of an efficient degradation system for polyaromatics based on the use of peroxidases *in vitro* requires their increased bioavailability by using cosolvents.

The use of water miscible organic solvents is preferred to hydrophobic ones considering that enzymes in nearly dry conditions have to be solubilised by modification with amphipathic compounds, lipids or surfactants [31, 32, 33]. Furthermore, diffusional resistance for substrates and products across the water-organic solvent interface in organic solvents may be a major problem [34].

The choice of an organic solvent for a given reaction should be based on three factors: i) the ecological toxicity of the solvent; ii) the effects of the solvent on the reaction (including solubility of the substrate); and iii) the effect of the solvent on the biocatalyst stability, since the solvent can affect the hydration shell of the enzyme molecule, necessary to maintain the native conformation [35].

Several authors studied the solubility of anthracene in organic solvents and in binary mixtures [36, 37, 38], but there is little information about water: miscible solvent mixtures [8]. MEK: water mixtures were only considered in the range of 1 to 30% MEK, which was the boundary conditions for the formation of two phases: organic and aqueous. Acetone permitted the total solubilisation of anthracene up to 1 g/L, followed by ethanol and methanol. While ethanol and methanol negatively affected MnP activity, acetone had no visible deactivation effect on MnP.

In monophasic systems, the enzymatic activity loss has been mainly attributed to the fact that water molecules in the enzyme are stripped away or replaced by solvent

molecules causing deformation and denaturation of the enzyme [39, 40]. Laane *et al.* [41] also found a quantitative correlation between the hydrophobicity of the solvent and the activity retention of the biocatalyst. Therefore, solvents with high values of partition coefficient between water and *n*-octanol (K_{ow}) are more favourable for preserving enzymatic activity. Methanol, the most hydrophilic solvent (log K_{ow}: -0.72), caused stronger inactivation of MnP than ethanol (log K_{ow}: -0.19), acetone (log K_{ow}: -0.16) and MEK (log K_{ow}: 0.37). Whereas MEK, the solvent with highest hydrophobicity, caused a higher inactivation of the enzyme than acetone. Therefore, not only hydrophobicity but other characteristics of solvents, such as hydrogen bonding, anion stabilisation and free energy of solvatation may also influence the enzyme stability [42, 43]. The stability of MnP in acetone mixtures is very high since incubations of enzyme in medium containing 90% of acetone for 24 h confirmed that MnP was scarcely deactivated (data not shown).

4.1. In vitro degradation of anthracene

There are several studies of *in vitro* oxidations of polyaromatics (anthracene, phenanthrene, pyrene) with crude or purified MnP [8, 44, 45]. The reported assays were performed on a very small scale (1-5 mL) and only a limited degradation yield was achieved (Table 4). Our results compare very favourably with those of the cited reports. The lower efficiencies achieved may be due to either some compounds lacking in the amounts required or to the non-optimised physicochemical conditions, both of which are necessary for the enzymatic action.

By improving the understanding of the main factors affecting anthracene degradation, an efficient treatment based on the use of free MnP may be defined. The

action of MnP depends on the combined action of several compounds, referred as substrates, cofactors and mediators, which initiate, participate and allow the completion of the catalytic cycle. Therefore, for optimizing the catalytic action of the enzyme, special attention was paid to study the influence of the following main factors: H_2O_2 and Mn^{2+} concentrations, organic acids and other operating parameters such as temperature and oxygen atmosphere .

The continuous addition of H_2O_2 at a controlled flow (5 μmol/L·min) permitted the progressive participation of H_2O_2 in the catalytic cycle through a suitable regeneration of the oxidised form of the enzyme, minimising the peroxide-dependent inactivation of the peroxidase [29].

Organic acids are required in the catalytic cycle of MnP because they facilitate the release of Mn^{3+} from the active site and also stabilize this species in aqueous solution [24, 46]. In addition, Kuan *et al.* [30] reported that complexed Mn^{2+} is the preferred substrate for the oxidised form of MnP compound II. Our results confirm that the concentration of the organic acid (e.g. malonic) is decisive on the action of the enzyme: on the one hand, degradation extent is improved, but on the other hand, activity loss also increases. Oxalic and malonic acids have been shown to be oxidatively decarboxylated by Mn^{3+} [47], generating a carbon dioxide anion radical which permits the endogenous formation of H_2O_2 via Mn^{2+} and a superoxide radical. The resulting accumulation of H_2O_2 may explain the greatest activity loss for both acids at high concentrations, specially oxalate which produces higher H_2O_2 concentrations [48]. Regarding to the other organic acids, it is possible that the binding of tartaric and citric acid (C_4 and C_6, respectively) to the enzyme is sterically hindered, being, therefore, the extent of degradation even lower than the corresponding to the reaction without exogenous

organic acid. The crude MnP contains lactic acid in a concentration of 1 mM from the fermentation medium, which would be enough to permit a low degradation extent.

Oxygen atmosphere increases the anthracene oxidation. This fact which has been observed in degradation of azo dyes in water may be attributed to the catalase-type activity of MnP [49]. MnP releases atomic oxygen which could be directly used for the degradation of anthracene. In this case, it is interesting to see that the maximum degradation rate is coincident with the highest dissolved oxygen concentration in the medium (27.9 mg/L).

The degradation of anthracene outcomes into its total decomposition to the dead-end product: the anthraquinone [3, 50]. The degradation mechanism, probably arising via one-electron oxidative pathway, has a large complexity with the generation of intermediate compounds such as anthrol and anthrone [51]. The apparent discrepancy between the expected ratio 1:1 of anthraquinone and anthracene and that obtained in this experimental work, around 1:2, indicates the presence of relative amounts or these or other intermediate compounds. In fact, the final step to anthraquinone is likely to be limiting the overall reaction rate of the process, as we determined an increase of the anthraquinone concentration around 10% in samples measured after 24 h. In this sense, ongoing research has as an objective the deeper knowledge of the degradation mechanism and kinetics and the way to enhance the rate of the whole process. Moreover, future work will be also focused on the biological degradation of anthraquinone by bacterial populations. Therefore, the overall process will be considered as a combination of an *in vitro* enzymatic system in the initial stage of degradation and a bacterial biological treatment to complete the process.

5. Conclusions

The proposal of the enzyme-based oxidation for the initial degradation of anthracene, as a model compound representative of recalcitrant polyaromatics, is the main focus of this work. Initially, a previous physical solubilisation of anthracene by exogenous addition of a cosolvent such as acetone (36%) was found to be important (it enhances 143-fold the anthracene solubility). Besides, the acetone in these conditions did not affect significantly the MnP activity. The operating conditions of the enzyme-based system were also evaluated to maximise the *in vitro* degradation of anthracene. The system attained a nearly complete degradation of anthracene, around 100%, after 6 hours of operation under optimal conditions.

Acknowledgements

This work was funded by the Spanish Commission of Science and Technology (CYCIT –Project PPQ2001-3063) and Gemma Eibes would like to express her gratitude to the Spanish Ministry of Science and Technology for her financial support (BES-2002-2809).

References

[1] Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 1992;3:351-368.

[2] Shuttleworth KL, Cerniglia CE. Environmental aspects of PAH biodegradation. Appl Biochem Biotechnol 1995;54:291-302.

[3] Field JA, de Jong E, Feijoo G, de Bont JAM. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. Appl Environ Microbiol 1992;58:2219-2226.

[4] Bumpus JA, Steven DA. Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: Involvement of the ligning degrading system. Bioessays 1987;6:166-170.

[5] Kirk TK, Farrell RL. Enzymatic "combustion": The microbial degradation of lignin. Annu Rev Microbiol 1987;41:465-505.

[6] Hammel KE, Kalyanaraman B, Kirk TK. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J Biol Chem 1986;261:16948-16952.

[7] Vázquez-Duhalt R, Westlake DWS, Fedorak PM. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl Environ Microbiol 1994;60:459-466.

[8] Field JA, Vledder RH, vanZeist JG, Rulkens WH. 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 1996;18:300-308.

[9] Brodkorb TS, Legge RL. Enhanced biodegradation of phenanthrene in oil

tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. Appl Environ Microbiol 1992;58:3117-3121.

[10] Meulenberg R, Rijnaarts HHM, Doddema HJ, Field JA. Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. FEMS Microbiol Lett 1997;154:45-49.

[11] Cerniglia CE, Heitkamp MA. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. In: Varanasi U, editor. Metabolism polycyclic aromatic hydrocarbons in the aquatic environment. Boca Raton: CRC Pres, 1984. p. 41-68.

[12] Bumpus JA. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 1989;55:154-158.

[13] Kilbane JJ. Extractability and subsequent biodegradation of PAHs from contaminated soil. Water Air Soil Pollut 1997;104:285-304.

[14] Field JA, Boelsma F, Baten H, Rulkens WH. Oxidation of anthracene in water/solvent mixtures by the white-rot fungus, *Bjerkandera* sp strain BOS55. Appl Microbiol Biotechnol 1995;44:234-240.

[15] Lee PH, Ong SK, Golchin J, Nelson GL. Use of solvents to enhance PAH biodegradation of coal tar-contaminated soils. Water Res 2001;35:3941-3949.

[16] Zheng Z, Obbard JP. Oxidation of polycyclic aromatic hydrocarbons (PAH) by the white rot fungus, *Phanerochaete chrysosporium*. Enzyme Microb Technol 2002;31:3-9.

[17] Kotterman MJJ, Rietberg HJ, Hage A, Field JA. Polycyclic aromatic hydrocarbons oxidation by the white rot fungus *Bjerkandera* sp. strain BOS55 in the presence of nonionic surfactants. Biotechnol Bioeng 1998;57:220-227.

[18] Zaks A, Klibanov AM. Enzymatic catalysis in nonaqueous solvents. J Biol Chem 1988;263:3194-3201.

[19] Klibanov AM. Improving enzymes by using them in organic solvents. Nature 2001;409:241-246.

[20] Mackay D, Shiu WY. Aqueous solubility of polynuclear aromatic hydrocarbons. J Chem Eng Data 1977;22:399-402.

[21] Bouchez M, Blanchet D, Vandecasteele JP. The microbial fate of polycyclic aromatic hydrocarbons: carbon and oxygen balances for bacterial degradation of model compounds. Appl Microbiol Biotechnol 1996;45:556-561.

[22] Moody JD, Freeman JP, Doerge DR, Cerniglia CE. Degradation of phenantrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. Appl Environ Microbiol 2001;67:1476-1483.

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

[24] Martinez AT. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme Microb Technol 2002;30:425-444.

[25] Mielgo I, López C, Moreira MT, Feijoo G, Lema JM. Oxidative degradation of azo dyes by manganese peroxidase under optimized conditions. Biotechnol Prog 2003;19:325-331.

[26] Palma C, Moreira MT, Feijoo G, Lema JM. Enhanced catalytic properties of MnP by exogenous addition of manganese and hydrogen peroxide. Biotechnol Lett 1997;19:263-267.

[27] Tien M, Kirk TK. Lignin peroxidase of *Phanerochaete chrysosporium*.

Methods Enzymol 1988;161:238-249.

[28] Moreira MT, Palma C, Mielgo I, Feijoo G, Lema JM. *In vitro* degradation of a polymeric dye (Poly R-478) by manganese peroxidase. Biotechnol Bioeng 2001;75:362-368.

[29] Moreira MT, Feijoo G, Sierra Alvarez R, Lema J, Field JA. Biobleaching of oxygen delignified kraft pulp by several white rot fungal strains. J Biotechnol 1997;53:237-251.

[30] Kuan IC, Johnson KA, Tien M. Kinetic analysis of manganese peroxidase. J Biol Chem 1993;268:20064-20070.

[31] Khmelnitsky YL, Levashov AV, Klyachko NL, Martinek K. Engineering biocatalytic systems in organic media with low water content. Enzyme Microb Technol 1988;10:710-724.

[32] Dordick JS. Enzymatic catalysis in monophasic organic solvents. Enzyme Microb Technol 1989;11:194-211.

[33] Vazquez-Duhalt R, Fedorak PM, Westlake DWS. Role of enzyme hydrophobicity in biocatalysis in organic solvents. Enzyme Microb Technol 1992;14:837-841.

[34] Ogino H, Ishikawa H. Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng 2001;91:109-116.

[35] Bell G, Halling PJ, Moore BD, Partridge J, Rees DG. Biocatalyst behaviour in low-water systems. Trends Biotechnol 1995;13:468-473.

[36] Powell JR, McHale MER, Kauppila ASM, Acree WE, Flanders PH, Varanasi VG, Campbell SW. Prediction of anthracene solubility in alcohol + alkane solvent mixtures using binary alcohol + alkane VLE data. Comparison of

Kretschmer-Wiebe and mobile order models. Fluid Phase Equil 1997;134:185-200.

[37] Hansen HK, Riverol C, Acree WE. Solubilities of anthracene, fluoranthene and pyrene in organic solvents: comparison of calculated values using UNIFAC (Dortmund) models with experimental data and values using the mobile order theory. Can J Chem Eng 2000;78:1168-1174.

[38] Jouyban A, Khoubnasabjafari M, Chan HK, Clark BJ, Acree WE. Solubility prediction of anthracene in mixed solvents using a minimum number of experimental data. Chem Pharm Bul 2002;50:21-25.

[39] Schulze B, Klibanov AM. Inactivation and stabilization of subtilisins in neat organic solvents. Biotechnol Bioeng 1991;38:1001-1006.

[40] Gorman LAS, Dordick JS. Organic solvents strip water off enzymes. Biotechnol Bioeng 1992;39:392-397.

[41] Laane C, Boeren S, Hilhorst R, Veeger C. Optimization of biocatalysis in organic media. In: *Stud Org Chem*, (Laane, C., Tramper, J., Lilly, M. D., eds), Elsevier, Amsterdam, 1987, 65-84.

[42] Gorjup B, Lampic N, Penca R, Perdih A, Perdih M. Solvent effects on ligninases. Enzyme Microb Technol 1999;25:15-22.

[43] Yoshida S, Chatani A, Honda Y, Watanabe A, Kuwahara M. Reaction of manganese-dependent peroxidase from *Bjerkandera adusta* in aqueous organic media. J Molec Catal B: Enzymatic 2000;9:173-182.

[44] Sack U, Hofrichter M, Fritsche W. Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Microbiol Lett 1997;152:227-234.

[45] Gunther T, Sack U, Hofrichter M, Latz M. Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductases. J Basic Microbiol 1998;38:113-122.

[46] Banci L, Bertini I, Dal Pozzo L, del Conte R, Tien M. Monitoring the role of oxalate in manganese peroxidase. Biochemistry 1998;37:9009-9015.

[47] Van Aken B, Agathos SN. Implication of manganese (III), oxalate, and oxygen in the degradation of nitroaromatic compounds by manganese peroxidase (MnP). Appl Microbiol Biotechnol 2002;58:345-351.

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

[49] López C, Moreira MT, Feijoo G, Lema JM. Dye decolourisation by manganese peroxidase in an enzymatic membrane bioreactor. Biotechnol Prog 2004;20:74-81.

[50] Hammel KE, Green B, Gai WZ. Ring fission of anthracene by a eukaryote. Proc Natl Acad Sci U S A 1991;88:10605-10608.

[51] Haemmerli S, Lignin peroxidase and the ligninolytic system of *Phanerochaete chrysosporium*, Ph.D. dissertation. Swiss Federal Institute of Technology, Zurich, Switzerland. 1988. p. 49-61 .

Table 1. Solvent concentration required for the solubilisation of 1, 10 and 100 mg/L of anthracene

Solvent	T (ºC)	Solvent concentration (%)		
		1 mg/L	10 mg/L	100 mg/L
MEK	20	17	**27***	ND
	30	14	24	ND
Acetone	20	21	**36**	53
	30	19	33	49
Ethanol	20	31	**44**	64
	30	28	41	60
Methanol	20	37	**55**	76
	30	32	51	67

ND: not determined

*Bold type values represent the values selected for the following experiments

Table 2. Effect of operational parameters on the anthracene degradation by MnP in 2-h reactions

Malonate (mM)	Mn^{2+} (μM)	H_2O_2 (μmol/L·min)	Anthracene degradation (%)	Anthraquinone production (%)	Activity loss (U/L·h)	Efficiency (μmol/U)
1	100	25	8.8 ± 0.2	1.3 ± 0.3	60 ± 2	0.022
1	100	5	9.8 ± 0.8	1.6 ± 0.5	29 ± 7	0.059
1	100	1	3.3 ± 1.6	0.6 ± 0.3	8 ± 4	0.056
1	20	5	10.0 ± 2.4	2.4 ± 0.6	33 ± 3	0.047
10	20	5	28.5 ± 3.3	9.2 ± 1.3	65 ± 6	0.068
20*	**20**	**5**	**43.3 ± 3.0**	**11.8 ± 0.4**	**70 ± 5**	**0.083**
30	20	5	37.5 ± 1.5	10.7 ± 0.5	88 ± 5	0.077

*Bold type values represent the values selected for the following experiments

Table 3. Effect of parameters on the anthracene degradation by MnP in 2 h reactions

Parameters	Anthracene degradation (%)	Anthraquinone production (%)	Activity loss (U/L·h)
Air	43.3	11.8	70
Oxygen	50.5	19.0	73
23ºC	43.3	11.8	70
30ºC	34.9	12.4	83
40ºC	5.5	0	200

Table 4. Degradation rate of anthracene in organic solvents: water mixtures

Solvent	Mediating agent	Degradation rate of anthracene (μmol/L·h)	Reference
40% acetone	-	0.96	[7]
5% DMF[a]	-	0.33	[41]
5% DMF[a]	-	0.70	[42]
5% DMF	5 mM GSH[b]	1.15	[41]
5% DMF	5 mM GSH[b]	2.34	[42]
36% acetone	-	4.40	This paper

[a] dymethylformamide

[b] glutathione

CAPTIONS TO FIGURES

Figure 1. Anthracene solubility at 20ºC in solvent: water mixtures. Symbols: methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

Figure 2. Inactivation of MnP from *Bjerkandera* sp. BOS55 (A) and *Phanerochaete chrysosporium* (B) in solvent: water mixtures at different concentrations. Symbols: methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△).

Figure 3. Stability of MnP incubations in solvent: water mixtures at room temperature. MnP from *Bjerkandera* sp. BOS55 (A) and *Phanerochaete chrysosporium* (B). Symbols: control (×), methyl-ethyl-ketone (●), acetone (■), ethanol (◇), methanol (△)

Figure 4. Effect of different organic acids on the anthracene degradation (A) and MnP activity consumption (B) in 2-h reactions. Symbols: control (■), malonic (□), oxalic (■), tartaric (■) and citric acid (■)

Figure 5. Time course of anthracene degradation in 6-h reaction. Symbols: MnP activity (○), Anthracene (■), Anthraquinone (▲)

Figure 1

A

B

Figure 2

A

B

Figure 3

A

B

Figure 4

Figure 5