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Operation of a two-phase partitioning bioreactor for the oxidation of anthracene by the enzyme manganese peroxidase

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Abstract

A study was conducted to determine the potential of a two-phase partitioning bioreactor (TPPB) for the treatment of a poorly soluble compound, anthracene, by the enzyme manganese peroxidase (MnP) from the fungus *Bjerkandera* sp. BOS55. Silicone oil was used as the immiscible solvent, which contained anthracene at high concentrations. The optimization of the oxidation process was conducted taking into account the factors which may directly affect the MnP catalytic cycle (the concentration of H_2O_2 and malonic acid) and those that affect the mass transfer of anthracene between the organic and the aqueous phase (solvent and agitation speed). The main objective was carried out in terms of improved efficiency, i.e., maximizing the anthracene oxidized per unit of enzyme used. The TPPB reached nearly complete oxidation of anthracene at a conversion rate of 1.8 mg l^{-1} h^{-1} in 56 h, which suggests the application of enzymatic TPPBs for the removal of poorly soluble compounds.

Keywords: Biphasic reactor; Anthracene; Enzymatic oxidation; Manganese peroxidase

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants released into the environment, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. Thus, soils from gasworks sites and carbochemical plants as well as refineries and filling stations are often contaminated with PAHs. There is toxicological concern about the presence of PAHs in the environment, since some have been shown to have mutagenic and carcinogenic properties and 16 are listed on the US Environmental Protection Agency as priority pollutants (Keith and Telliard, 1979).

The natural biodegradation of PAHs is mainly restricted by two factors: (i) the low water solubility of the PAHs and (ii) their hydrophobicity. Therefore, these types of compounds tend to accumulate on the organic matter in soil and thus, their desorption from soil limits their availability to microorganisms for biodegradation (Cerniglia, 1992; Shuttleworth and Cerniglia, 1995). The use of an organic solvent has been proposed to resolve this hitch (Jimenez and Bartha, 1996; Kilbane, 1997; Lee et al., 2001).

Among other possibilities, an environmentally friendly approach for PAH degradation could be based on the use of white rot fungi, which are known to degrade a great variety of compounds due to their complex enzymatic system (Field et al., 1992). Lignin peroxidase (LiP) and manganese peroxidase (MnP) are extracellular peroxidases produced by white rot fungi and the onset of their production is associated with secondary metabolism conditions in response to nutrient depletion (Bumpus and Steven, 1987). The ligninolytic system is non-selective, consequently aromatic substrates, such as PAHs are potentially oxidized and biodegraded by white rot fungi (Hammel et al., 1986;

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Vázquez-Duhalt et al., 1994; Field et al., 1996). Quinones are the main products generated during the enzymatic oxidation of PAHs and they are more polar and water soluble, and therefore more susceptible to further degradation by indigenous bacteria present in soils and sediments (Brodkorb and Legge, 1992; Meulenberg et al., 1997).

Oxidation of anthracene by the ligninolytic enzyme MnP has been studied in a one-phase reactor in medium containing a miscible organic solvent (Eibes et al., 2005). Monophasic reactions, based on miscible organic solvent:water mixtures, do not present diffusional resistance for substrates and products. In the mentioned report acetone was used in a concentration of 36% (v/v) to dissolve 5 mg l^{-1} of anthracene. Under optimum conditions the oxidation rate was 0.83 mg l^{-1} h^{-1} with MnP losses of 30 U l^{-1} h^{-1}, so the ratio between oxidized anthracene and activity consumption was 0.028 mg U^{-1}. One of the problems associated to this aqueous system is the impossibility of increasing anthracene concentration over 5 mg l^{-1} for 36% acetone. If the amount of acetone in the mixture is increased, a higher inactivation of the enzyme would occur.

In order to oxidize higher concentrations of anthracene *in vitro* by MnP, the addition of a second immiscible phase in a two-phase partitioning bioreactor (TPPB) was considered. TPPB design requires a critical consideration: the solvent selection, which greatly influences the mass transfer and oxidation rates. The selected solvent should be non-toxic for the enzyme, it should possess suitable physical and chemical properties (be immiscible, non-volatile, etc.), and be inexpensive and readily available (MacLeod and Daugulis, 2003). In TPPBs, the substrate diffuses from the water-immiscible phase to the aqueous phase, and the enzyme degrades the substrate at the interface and/or in the aqueous phase. The mass transfer rate is favoured by an increased surface area for partitioning, therefore, the rate of biodegradation in a TPPB is governed by the size of the interface between the two liquid phases (Köhler et al., 1994; Ascón-Cabrera and Lebeault, 1995).

The use of TPPBs has allowed the biological treatment of many toxic and recalcitrant pollutants, such as PAHs, at unprecedented loads and rates. Janikowski et al. (2002) have successfully used this technology to degrade anthracene and other PAHs in biphasic reactors with *Sphingomonas aromaticivorans* and dodecane as the organic phase. The volumetric degradation rate of anthracene was 5.5 mg l^{-1} h^{-1} after approximately 30 h. However, the application of TPPBs for the *in vitro* oxidation of PAHs is still lacking.

This paper deals with the oxidation of anthracene by the enzyme MnP in a TPPB. The reactions involved in the process are shown in Fig. 1. The enzyme MnP is present in the aqueous phase with the cofactors and substrates required for the catalytic cycle. The anthracene molecules transferred from the organic to the aqueous phase are oxidized by the Mn^{3+} ions generated during the catalytic cycle. The products formed, anthraquinone mainly (Eibes et al., 2006), can be transferred to the organic phase. The operation of this enzymatic reactor allows working with high concentration of anthracene and the reuse of the enzyme in several batches. The optimization of the oxidation process was conducted taking into account the following specific factors: (a) those which may affect the mass transfer of anthracene: solvent and agitation speed and (b) those parameters which may directly affect the MnP catalytic cycle: the concentration of H_2O_2 and malonic acid. This system is, to our knowledge, the first attempt to degrade a PAH enzymatically in a biphasic reactor.

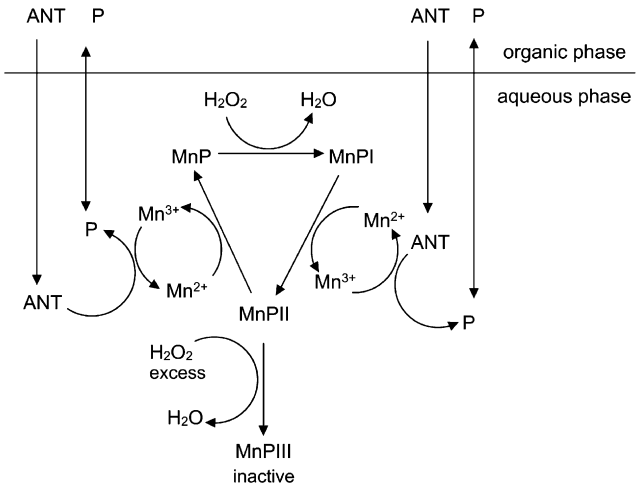

Fig. 1. Scheme of the physico-chemical and enzymatic mechanisms involved in the degradation of anthracene (ANT) by MnP in a TPPB. The Mn^{3+} ions formed in the catalytic cycle of MnP oxidize the molecules of ANT present in the aqueous phase to form the products (P) which transfer to the organic phase according to the thermodynamic equilibrium.

2. Materials and methods

2.1. Chemicals

Anthracene (99% purity) as well as silicone oil 200 FLUID 20 cSt and all other chemicals used were obtained from Sigma–Aldrich.

2.2. 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), and was then centrifuged for 10 min at 20 000 × *g*. Neither LiP nor Laccase activities were detected in any of the separated fractions during the procedure.

2.3. MnP activity assays

MnP activity was determined spectrophotometrically (Cecil CE 7200, UK) at 30 °C 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 49600 M^{-1} cm^{-1}. MnP assays were carried out with 200 μl sodium malonate (250 mM, pH 4.5), 50 μl 2,6-DMP (20 mM), 50 μl $MnSO_4$ (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 (4 mM), ending up with a volume of 1 ml. One unit of activity was considered as the amount of enzyme which oxidizes 1 μmol per min.

2.4. Effect of silicone oil on MnP activity

Experiments with 10% silicone oil in the absence of anthracene and without the addition of hydrogen peroxide were performed to evaluate the effect of the solvent at three agitator speeds. The aqueous phase consisted on 33 mM sodium malonate, 33 μM Mn^{2+} and 400 U l^{-1} of MnP in a total volume of 100 ml. Level 1 of agitator speed formed few medium droplets (visually >5 mm diameter), however, agitation at level 2 created droplets of smaller diameter (1–5 mm). Level 3 of agitator speed produced a complete dispersion (<1 mm). Samples were withdrawn periodically in order to measure the enzymatic activity in aqueous layer.

2.5. PAH oxidation assays in bottles

Oxidation of PAHs was carried out in 500-ml glass bottles, with magnetic stirring at room temperature, i.e. 23 °C. The reaction mixture (100 ml) consisted of silicone oil (10 ml) saturated with anthracene (≈360 mg l^{-1}). The aqueous phase, 90 ml, consisted of 33 μM Mn^{2+}, 33 mM malonic acid and the continuous addition of 5 μM min^{-1} H_2O_2 (except when indicated). Samples were withdrawn periodically, centrifuged for 15 min at 3400 rpm to separate the two phases, and the disappearance of anthracene in the organic phase was determined by fluorescence spectroscopy. Anthracene concentration in the aqueous phase was assumed to be negligible (water solubility of anthracene: 0.07 mg l^{-1} (Mackay and Shiu, 1977)). Fluorescence spectra were collected using a QuantaMaster QM1 fluorescence spectrometer (Photon Technology International, London, Ontario, Canada), equipped with a 75 W Xenon arc lamp, Czerney-Turner excitation and emission monochromators. Excitation and emission slits were set to 2 nm bandpass for all measurements. A solution sample holder was used to hold the quartz cuvettes in the path of the excitation radiation. The quartz cuvettes used were type 3H, with a path length of 10 mm, obtained from NSG Precision Cells, (Farmingdale, New York, USA). In order to be in the linear range of detection for anthracene, all samples taken from organic layer were diluted by a factor of 10000 in anhydrous ethanol. The detection conditions were: $\Delta\lambda = 125$ nm, peak maximum = 377 nm and integration area = 360–390 nm.

The changes in MnP activity in the aqueous phase were spectrophotometrically determined, and pulses of MnP were added to maintain an activity in the reactor greater than 100 U l^{-1}. To verify that removal took place due only to an enzymatic oxidation, controls were run in parallel in the absence of MnP.

2.6. PAH oxidation assays in BIOSTATQ

The experiments with pH control were carried out in a BIOSTAT®Q reactor (B. Braun-Biotech International, Melsungen, Germany). It was equipped with pH, temperature and pO_2 sensors and a magnetic agitator. The temperature was set to 25 °C and pH was controlled at 4.5 by pumping HCl (1 M) or malonic acid (250 mM). Agitation rates varied from 200 to 300 rpm. The total reaction volume was 250 ml, and the organic phase (25 ml silicone oil) saturated with anthracene. The aqueous phase (225 ml) consisted of 33 μM Mn^{2+}, sodium malonate and the continuous addition of H_2O_2. Anthracene in the organic phase was diluted by a factor of 100, and analyzed by a HP 1090 HPLC, equipped with a diode array detector monitoring the absorbance at 254 nm, a 4.6 × 200 mm Spherisorb ODS2 reverse phase column (5 μm; Waters) and a HP ChemStation data processor. The injection volume was set at 10 μl and the isocratic eluent (80% acetonitrile and 20% water) was pumped at a rate of 1 ml min^{-1}. The concentration in the aqueous phase was considered to be negligible. Anthraquinone was the only oxidation product identified.

Aqueous samples were used to determine MnP activity spectrophotometrically and malonate concentration by HPLC. Pulses of MnP were added in order to maintain the activity in the reactor higher than 100 U l^{-1}. Malonic acid concentration was determined by a HP 1090 HPLC with a refractive index detector, using sulphuric acid as mobile phase (0.6 ml min^{-1}) and a Aminex-87H BioRad column (BioRad Laboratories, Madrid). The injection volume was set at 20 μl.

3. Results

3.1. Hydrogen peroxide optimization

The parameters affecting the catalytic cycle of MnP were investigated to optimize the anthracene oxidation in TPPBs operated with silicone oil. Hydrogen peroxide, the promoter of the catalytic cycle of MnP, was added continuously through a peristaltic pump avoiding a high concentration in the reactor, which would cause MnP inactivation. Different hydrogen peroxide addition rates were assayed: 1, 5, 15 and 25 μM min^{-1} and the anthracene oxidation was evaluated as well as MnP loss rate and the

Table 1
Experiments at different addition rates of hydrogen peroxide

H_2O_2 ($\mu mol\ l^{-1}\ min^{-1}$)	Anthracene degradation rate ($mg\ l^{-1}\ h^{-1}$)	MnP activity loss rate ($U\ l^{-1}\ h^{-1}$)	Efficiency ($mg\ U^{-1}$)
1	0.16	3.4	0.047
5	**0.38**	**8.4**	**0.046**
15	0.28	17.4	0.016
25	0.27	17.1	0.016

efficiency, in terms of anthracene oxidized per unit of activity used. Table 1 shows that the higher the hydrogen peroxide addition was, the higher activity loss but not the oxidation rate. 1 and 5 $\mu M\ min^{-1}$ of hydrogen peroxide attained similar efficiencies (0.047 and 0.046 $mg\ U^{-1}$, respectively) but the anthracene oxidation rate for 1 $\mu M\ min^{-1}$ was 2.4-fold lower, therefore, 5 $\mu M\ min^{-1}$ was considered an adequate value of hydrogen peroxide addition.

The pH of the reaction was 4.5 and it was maintained with no significant change over the course of 30 h (Fig. 2a). At that time, the pH started to increase, reaching a maximum of 8 after 70 h for all experiments. The faster the hydrogen peroxide rate was, the faster the pH increased. Values of pH higher than 6 have been shown to be responsible of a marked MnP inactivation (Mielgo et al., 2003).

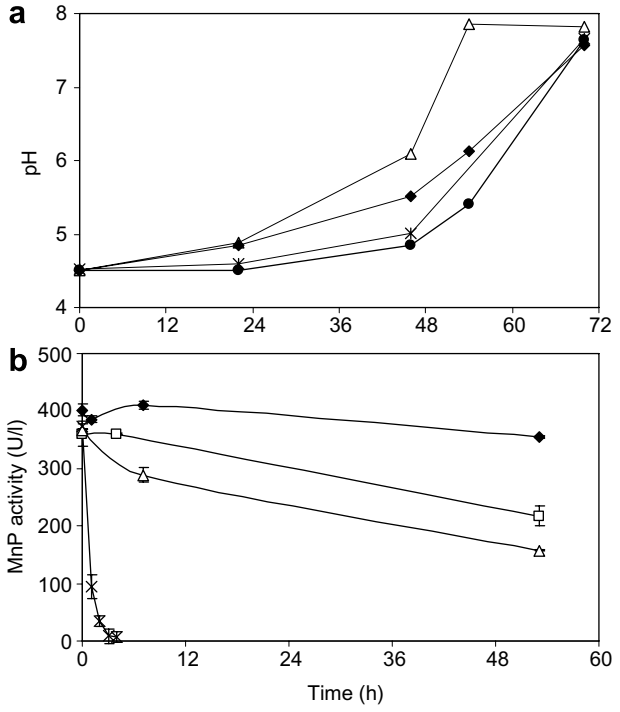

Fig. 2. (a) pH profiles at different hydrogen peroxide addition rates: (△) 25, (♦) 15, (✳) 5 and (●) 1 $\mu mol\ l^{-1}\ min^{-1}$; (b) effect of the agitation rate on the MnP activity: (♦) no agitation, (□) level 1 (mean droplet diameter around 0.5–1 cm), (△) level 2 (mean diameter < 0.5 cm) and (×) complete agitation (homogeneous phase).

3.2. Sodium malonate concentration and pH control

In order to avoid the detrimental effect of the pH increase, the concentration of the buffer, sodium malonate, was studied with concentrations ranging from 10 to 66 mM (Table 2; experiments 1–4). The increase of the concentration of the buffer should regulate the pH to a larger extent, and hence, the activity consumption should be lower. When sodium malonate concentration increased from 50 to 66 mM (experiments 3 and 4), the enzymatic loss also increased: from 8.4 to 11.8 $U\ l^{-1}\ h^{-1}$, which was not desirable. Bearing in mind the efficiency, the best values were obtained with 33 or 50 mM of malonate (0.046 $mg\ U^{-1}$). The addition of higher concentrations of buffer did not decrease the enzymatic consumption; on the contrary, it caused higher MnP losses. Moreover, the pH increased to 8 after 70 h of reaction even with the higher concentration of sodium malonate. The lower activity loss was obtained in experiment 1, utilizing 10 mM of malonate (6.8 $U\ l^{-1}\ h^{-1}$), although in that case, the anthracene oxidation rate was also the lowest (0.29 $mg\ l^{-1}\ h^{-1}$) and the removal of anthracene stopped after 47 h of reaction (36% removal) in spite of enzyme and hydrogen peroxide in the medium.

The following experiments were performed with pH control, and the concentration of malonate was determined in order to check if it was a limiting factor. pH was regulated at 4.5 by adding HCl (1 M) whenever it was required (Table 2 – experiment 5). The effect of controlling pH with HCl led to a slight diminution of MnP consumption rate in comparison with experiment 2, where pH was not controlled (7.3 and 7.7 $U\ l^{-1}\ h^{-1}$, respectively). However, the oxidation rate did not undergo great changes (0.37 and 0.36 $mg\ l^{-1}\ h^{-1}$, respectively). Regarding to the concentration of malonate in the reactor, it continuously decreased during the 72 h of reaction at a rate of 39 $mmol\ h^{-1}$. Malonate is an essential compound in the catalytic cycle of MnP, and therefore, its presence on the reaction medium has to be maintained. For that reason, control of pH was carried out by the addition of malonic acid: 0.25 M

Table 2
Experiments at different malonate concentration and pH

Experiment	pH	Malonate (mM)	Degradation rate ($mg\ l^{-1}\ h^{-1}$)	Activity loss rate ($U\ l^{-1}\ h^{-1}$)	Efficiency ($mg\ U^{-1}$)
1	Free	10	0.29	6.8	0.042
2	Free	33	0.36	7.7	0.046
3	Free	50	0.38	8.4	0.046
4	Free	66	0.39	11.8	0.033
5	4.5[a]	33	0.37	7.3	0.050
6	4.5[b]	33	0.41	7.5	0.055
7	**4.5[b]**	**10**	**0.42**	**5.4**	**0.079**
8	4.5[b]	5	0.42	5.7	0.074

[a] pH controlled with HCl.
[b] pH controlled with malonic acid.

Table 3
2^2 fractional experiment matrix and experimental results

	A	*B*	Agitation (rpm)	Silicone oil (%)	Percentage of degrad (time)	Degrad rate (mg l^{-1} h^{-1})	Activity loss rate (U l^{-1} h^{-1})	Efficiency (mg U^{-1})
1	−1	−1	200	10	92 (72 h)	0.42	5.1	0.083
2	−1	1	200	30	43 (72 h)	0.62	4.5	0.139
3	1	−1	300	10	97 (55 h)	0.61	7.4	0.083
4	**1**	**1**	**300**	**30**	**89 (56 h)**	**1.76**	**7.3**	**0.243**
5	0	0	250	20	90 (56 h)	1.19	6.8	0.175
6	0	0	250	20	92 (56 h)	1.21	6.4	0.187

(experiment 6). An increase in the oxidation rate was observed in comparison with experiment 2 (from 0.36 to 0.41 mg l^{-1} h^{-1}) but the loss of MnP activity remained practically the same (7.6 and 7.5 U l^{-1} h^{-1}, respectively). Trying to decrease the enzymatic loss to a larger extent, the initial concentration of malonate was reduced again to 10 mM, but in this case, by controlling pH (experiment 7). As was expected, the enzymatic consumption decreased (5.4 U l^{-1} h^{-1}), not only in comparison with experiment 6 but also with experiment 1, where pH was uncontrolled. The oxidation rate, 0.042 mg l^{-1} h^{-1}, was similar to that obtained in experiment 6, but much higher than experiment 2: 1.45-fold. Hence, the efficiency, 0.079 mg U^{-1}, was 1.44-times greater than in experiment 6 and 1.88-times higher than experiment 1. Finally, the initial malonate concentration was decreased to 5 mM (experiment 8), but there was no improvement in the efficiency of the system (0.074 U mg^{-1}) because the MnP consumption was not decreased. Therefore, the conditions selected for the following experiments were: 10 mM malonate, malonic acid as agent for controlling pH and addition of H_2O_2 at a rate of 5 μM min^{-1}.

3.3. Silicone oil concentration and agitation speed

In order to favour the transfer of anthracene to the aqueous phase and the kinetics of the enzymatic reaction, the effect of the agitation rate and the ratio of silicone oil: aqueous phase were evaluated. As both variables are likely to be interdependent, a 2^2 experiment design was considered to optimize the efficiency of the system (Box et al., 1978).

First, the effect of different values of agitation rates on the MnP activity was evaluated to select an adequate operational range of agitation. A non-stirred control and three different levels were assayed (Fig. 2b). Level 1 caused the formation of few solvent droplets dispersed on the water phase with a diameter between 5 and 10 mm. At level 2 the number of droplets increased and the diameter diminished (<5 mm). Finally, a higher agitation produced a visually homogeneous phase (<1 mm) which could be related to an agitation speed higher than 500 rpm. This strong agitation resulted in a complete inactivation of the enzyme after only 3 h, while MnP activity after 53 h at level 1 and level 2 was maintained at 61% and 44%, respectively, when compared with the control experiment.

Considering both factors, interfacial surface and enzyme deactivation, the values of agitation considered in the experimental design were 200 and 300 rpm. Moreover, the percentage of silicone oil was assessed at 10% and 30%. Four experiments at the conditions determined by the limits of the range considered as well as two experiments in the centre of the region of interest (250 rpm and 20% silicone oil) were carried out. The anthracene oxidation rate, the activity loss rate and the efficiency corresponding to each experiment are shown in Table 3.

It is important to highlight that, increasing either the silicone oil fraction or the agitation rate, favour the anthracene oxidation rate, with both parameters having a similar effect (compare experiments 1–2, 1–3 and 2–4, 3–4). However, the increase in agitation led to a marked increase in the MnP activity consumption (around 4.8 U l^{-1} h^{-1} for 200 rpm and 7.3 U l^{-1} h^{-1} for 300 rpm). Even so, the highest efficiency was obtained at 300 rpm and 30% silicone oil, where a nearly complete oxidation was achieved after 56 h (experiment 4). The profile of removal is shown in Fig. 3. The experimental results were adjusted to a response surface represented by Eq. (1).

$$Z_i = a_i + b_i X + c_i Y + d_i XY \quad (1)$$

where "*X*" and "*Y*" are the dimensionless agitation rate and silicone oil fraction, respectively, and the subindex (*i*)

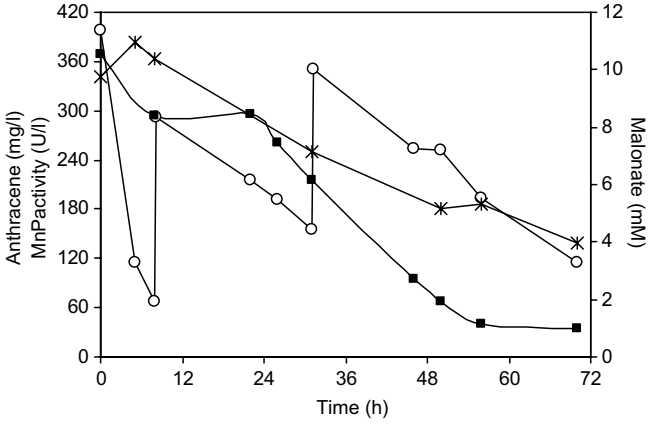

Fig. 3. Oxidation of anthracene in a TPPB with 30% (v/v) silicone oil as organic phase, 10 mM malonate in the aqueous phase, the continuous addition of 5 μmol l^{-1} min^{-1} H_2O_2 and the control of pH by the addition of malonic acid. Symbols: (■) anthracene concentration in the organic phase, (○) MnP activity and (✳) malonate concentration in the aqueous phase.

Table 4
Regression coefficients of the 2^2 factorial experimental design

	Constant	Agitation	Silicone oil	Agitation · silicone oil
Degradation rate	0.969	0.333	0.337	0.239
Activity loss rate	6.24	1.26	–	–
Efficiency	0.152	0.026	0.054	0.026

–: No significance.

indicated the type of objective function (Z_i) considered: oxidation rate, activity loss rate or efficiency. The coefficients for the three objective functions are shown in Table 4. A confidence level of 90–95% was considered to determine the significance of the coefficients.

In the case of oxidation rate, both agitation rate and silicone oil fraction had a similar weight in the equation and the combined effect had two-thirds of that (coefficients: 0.33, 0.34 and 0.24, respectively). Regarding activity loss rate, only the agitation parameter had a significant effect. The increase of the silicone oil volume did not provoke a modification of the enzymatic deactivation rate. Moreover, the efficiency was mainly dependent on the ratio silicone oil:aqueous phase, the higher volumes of silicone oil leading to higher values of efficiency. Both the agitation and the combined effect had similar weigh (coefficients: 0.026 and 0.026) and represented around half of that of the fraction of solvent (0.054). Fig. 4 shows the response surface for the efficiency.

In order to improve the results in terms of efficiency, additional experiments were carried out on the line representing the steepest ascent of the function on the best point of the surface. The parametric representation of that line is indicated by Eq. (2):

$$X = 0.55 \cdot s + 1$$
$$Y = 0.84 \cdot s + 1 \quad (2)$$

where "s" conditions the length of the movement from the base point, in that case (+1, +1).

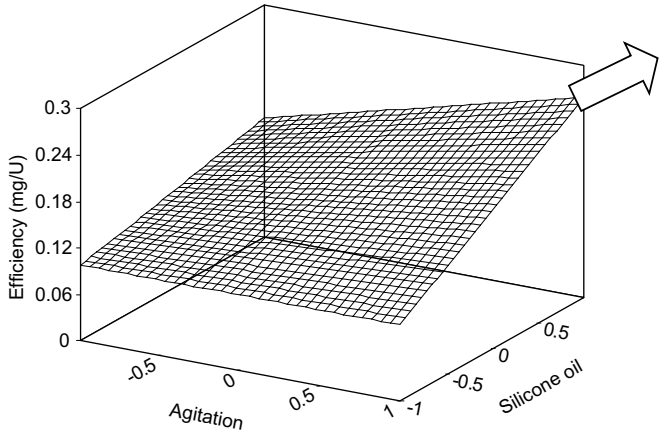

Fig. 4. Response surface for the efficiency as a function of dimensionless agitation rate and silicone oil fraction. The arrow represents the path of the steepest ascent.

Although different "movements" were performed from the base point, considering a golden section protocol (Rudd and Watson, 1968) none of them improved the results obtained in experiment number 4, with an agitation rate of 300 rpm and a fraction of silicone oil:aqueous phase of 30%.

4. Discussion

The present work was initiated to assess the applicability of enzymatic two-phase reactors for the removal of recalcitrant and poor-soluble compounds. Anthracene was selected as the model compound due to its low water solubility: 0.07 mg l^{-1} (Mackay and Shiu, 1977). The organic phase functioned as a reservoir of the pollutant, delivering anthracene to the aqueous phase where the enzyme MnP and the cosubstrates performed the oxidation. We chose silicone oil as the solvent because it has already been successfully used in TPPBs with various microorganisms for the degradation of PAHs (Bouchez et al., 1997; Marcoux et al., 2000; Muñoz et al., 2003) due to its hydrophobicity, biocompatibility, chemical stability, and resistance to hydrolytic and oxidative breakdown as discussed by Ascón-Cabrera and Lebeault (1993). Also, some solvents, such as hydrocarbons, might sequester the PAHs, thus decreasing their solubilisation in the aqueous phase and their availability to the microorganisms or enzymes (Efroymson and Alexander, 1995; Muñoz et al., 2003).

By improving the understanding of the main factors affecting the enzymatic oxidation of anthracene, 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 to as substrates, cofactors and mediators, which initiate, participate and allow the completion of the catalytic cycle. The optimization of these and other parameters for the removal of anthracene in monophasic systems was studied in a previous work (Eibes et al., 2005). There, the most important factors which affected the efficiency of the process were the hydrogen peroxide addition rate and the concentration of the organic acid (sodium malonate), which were evaluated in the present work. The results obtained here for the optimization of hydrogen peroxide are in agreement with those obtained in the monophasic system. The continuous addition of H_2O_2 at a controlled flow (5 μM min^{-1}) permitted the progressive participation of H_2O_2 in the catalytic cycle through a suitable regeneration of the oxidized form of the enzyme, minimizing the peroxide dependent inactivation of the peroxidase (Moreira et al., 1997).

Organic acids are required in the catalytic cycle of MnP because they facilitate the release of Mn^{3+} from the active site and also for the stabilisation of these species in aqueous solution (Banci et al., 1998; Martínez, 2002). The concentration of malonic acid was demonstrated to be decisive for the removal of anthracene; on the one hand, the oxidation extent is improved, but on the other hand, activity loss also increases. The presence of malonate on the medium has to be maintained, because a lack of it during the

process provokes a rapid decrease of the reaction rate, as happened when the initial concentration of malonate was 10 mM and no addition of malonic acid was provided. The disappearance of sodium malonate in the reactors could be explained by the fact that it has been shown to be oxidatively decarboxylated by Mn^{3+} (Van Aken and Agathos, 2002), 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 at high concentrations of sodium malonate. Moreover, the radical species and peroxides formed during this process are highly reactive and can be used by MnP in a partly autocatalytic process, which may explain the improvement of the degradation rate (Hofrichter et al., 1998).

The availability of poorly soluble compounds is usually a limitation which can be resolved favouring the mass-transfer rate. The principal approach to increase the mass-transfer to the aqueous phase is by enhancing the solubilisation or dissolution rates. This can be achieved by increasing the total surface area between the substrate and the aqueous phase, which is often accomplished by increasing the agitation (Déziel et al., 1999) or the dispersed phase volume (Prokop and Erikson, 1972). Optimal interfacial areas lead to optimal enzymatic activities (through equilibrated coupling of the substrate transfer rate and the substrate uptake rate) (Ascón-Cabrera and Lebeault, 1995). The volumetric liquid–liquid interfacial area, a ($m^2\ m^{-3}$), can be calculated as (Bailey and Ollis, 1986):

$$a = \frac{6 \cdot \varphi}{d_{sm}} \tag{3}$$

where d_{sm} ($m^3\ m^{-2}$) is the Sauter mean (surface averaged) droplet diameter and ϕ corresponds to the dispersed-phase volume fraction, which is the ratio of the organic phase volume to the total liquid volume. The equation shows that the interfacial area increases with a decrease in the mean drop size and with an increased phase ratio. However, it is also known that drop diameters have a tendency to increase with an increase in the phase ratio (Prokop and Erikson, 1972). Ascón-Cabrera and Lebeault (1993) have studied the effect of variations of the organic phase volume (8.3–83% v/v silicone oil) on the interfacial area and observed maximal values between 20% and 40% and agitation rates between 400 and 700 rpm. The optimal values of the organic phase volume agree with the optimal value obtained in this work: 30% v/v. However, in our work, the agitation rates were not increased to those values, because high values of agitation rate (above 500 rpm) had a significant detrimental effect on the MnP activity. Shear-induced inactivation of MnP from *Bjerkandera* sp. BOS55 was considered negligible under vigorous magnetic stirring and operational time below 4 d (data not shown). Inactivation of enzymes can be caused by dissolved solvent molecules, and/or by contact with the interface (Ross et al., 2000). In the present case, silicone oil is insoluble in water, therefore the interfacial mechanism likely dominates. In emulsion reactors the observed rate of enzyme inactivation is function of interfacial tension, liquid density difference, dispersed phase fraction, mixing intensity and reactor geometry (Walstra, 1993). In the system considered in this work, the main factor affecting the inactivation of the enzyme was the rise in agitation rate, which increased the interfacial area where the enzymes adsorb and subsequently unfold. The increase in agitation also favoured the desorption of the inactivated enzyme from the interface (Baldascini and Janssen, 2005).

Increasing the dispersed phase ratio and the agitation rate, led to higher values of oxidation rates. Hence, mass transfer processes limited the whole reaction process at the lower values of the conditions studied. Considering the removal of anthracene by MnP in monophasic systems the mean oxidation rate was 0.78 $mg\ l^{-1}\ h^{-1}$ and the maximum oxidation rate was 1.35 $mg\ l^{-1}\ h^{-1}$ (Eibes et al., 2005) which is below the value obtained in this work for 300 rpm and 30% silicone oil (1.76 $mg\ l^{-1}\ h^{-1}$). In the first case there were no mass transfer limitations, and the concentration of anthracene, 5 $mg\ l^{-1}$, was much higher than that in TPPB, thus the oxidation rates should be higher. The explanation could lie in the production of the radical species and peroxides mentioned previously. The length of the experiments in TPPBs was around 10-times longer than in monophasic reactors; moreover, in the present work there was a continuous addition of malonic acid, and therefore the concentration of decomposition products from the acid was much higher for TPPBs, which could lead to higher oxidation rates.

Previous studies on non-aqueous enzymatic catalysis have been primarily focused on biotransformation reactions for chemical production or purification, while applications for remediation of environmental pollutants have been largely ignored (Wang et al., 1999). The results achieved in this work, show great promise for the application of enzymatic TPPBs for the elimination of sparingly soluble compounds. The oxidation rate of 1.8 $mg\ l^{-1}\ h^{-1}$ obtained here is 3-times lower than the value obtained in cultures of *Sphingomonas* by Janikowski et al. (2002). But it is noteworthy that the use of enzymatic reactors is simpler and the operational requirements are lower. Moreover, the reuse of silicone oil and enzyme was shown to be feasible as it was demonstrated in experiments in which the silicone oil depleted in anthracene was separated from the aqueous phase, re-contaminated with the PAH and returned to the aqueous phase in a further batch experiment (data not shown). Further research on the physico-chemical and enzymatic mechanisms involved in the TPPB is required to select the most appropriate solvent and to scale up the reactor.

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