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Strategies for the design and operation of enzymatic reactors for the degradation of highly and poorly soluble recalcitrant compounds

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Abstract
The presence of recalcitrant compounds in both wastewaters and soils is an important environmental problem. Oxidative enzymes from white-rot fungi have been successfully utilised for the *in vitro* degradation of xenobiotics, such as the azo dye Orange II and the polycyclic aromatic hydrocarbon anthracene (compounds with high and low solubilities, respectively). Two different reactor configurations are proposed: (i) an enzymatic membrane reactor for the treatment of soluble compounds, consisting of a continuous stirred tank reactor coupled to an ultrafiltration membrane to facilitate the retention and recycling of enzyme; and (ii) a two-phase enzymatic reactor for the degradation of poorly soluble compounds, consisting of an immiscible solvent, which contains the contaminant at high concentrations, and the aqueous phase containing the enzyme and cofactors involved in the catalytic cycle. In this paper, factors affecting the design and operation of both systems are discussed, and experimental results concerning the efficiency and stability of the processes are presented.

Keywords: *Enzymatic membrane reactors, two-phase partitioning bioreactors, recalcitrant compounds, anthracene, Orange II, manganese peroxidase*

Features of enzymatic reactors

Numerous advantages arise from the use of enzymes rather than microorganisms for environmental purposes: (i) enzymes can be active under a wider variety of conditions, such as pH, ionic strength or temperature; (ii) higher pollutant concentrations can be maintained in enzymatic reactors with no inhibitory problems; (iii) operational times are reduced with no lag period due to microbial growth; (iv) media composition is simpler and enzymatic requirements are low provided the enzyme can be reused; (v) control of the process is easier; and (vi) no sludge is produced. However, the cost of enzyme production and its sensitivity to changes in environmental conditions must be taken into account when favouring the efficiency of the enzymatic process.

We have studied the feasibility of using enzyme reactors for the degradation of soluble and poorly soluble recalcitrant contaminants. Some typical examples of soluble compounds are nitroaromatic explosives, phenols and dyes, such as the azo dye Orange II, a recalcitrant compound commonly present in industrial wastewaters. Typical examples of poorly soluble contaminants are polycyclic aromatic hydrocarbons (PAHs), pesticides, and polychlorinated biphenyls (PCBs). Their high hydrophobicity makes these compounds environmentally persistent. Anthracene, a tricyclic PAH, was selected as a model compound in this class.

Factors to consider for the operation of enzymatic reactors

Selection of the enzyme

The enzyme used as a catalyst for the degradation of recalcitrant compounds should exhibit high oxidation and ionisation potential and non-specific activity, thereby providing the ability to degrade a broad range of compounds. As the enzyme–substrate interaction may be constrained by the large size of

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the enzyme, the use of diffusible enzymes or related mediators is desirable. Moreover, extracellular enzymes are preferred since their production is easier and cheaper. All these characteristics are fulfilled by the ligninolytic enzyme manganese peroxidase (MnP).

Catalytic cycle of the enzyme

MnP was first discovered in *Phanerochaete chrysosporium*, and is produced by a number of white-rot fungi (Tien & Kirk 1988). The MnP catalytic cycle is similar to that of other peroxidases and involves a 2-electron oxidation (Figure 1). The initial oxidation of MnP by H_2O_2 produces an intermediate compound that promotes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} is stabilised by organic acids, and the Mn^{3+}-organic acid complex acts as a strong diffusing oxidiser (oxidation potential: 1.54 V) (Kuan & Tien 1993). Excess H_2O_2 promotes enzyme inactivation. The enzyme is able to degrade a variety of complex compounds (Kuan et al. 1993; Martínez 2002).

The catalytic cycle is affected by the presence of different cosubstrates and cofactors, as well as environmental reaction conditions (Wariishi et al. 1988). These not only affect the stoichiometry of the reaction, but also the kinetics and stability of the enzyme. Two main goals have to be achieved: (i) adequate conversion, which may be determined by environmental restrictions; and (ii) high efficiency, defined as the amount of substrate degraded per unit of enzyme consumed during the reaction.

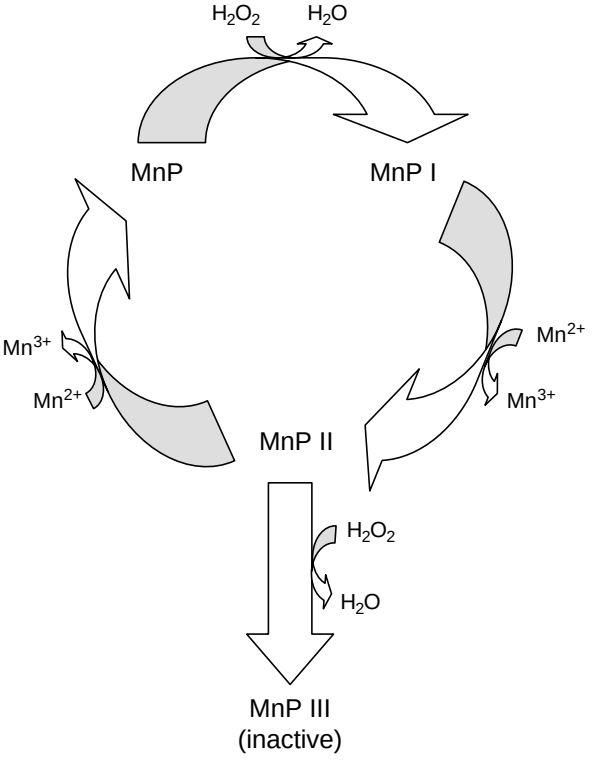

Figure 1. Scheme of the catalytic cycle of MnP.

H_2O_2 is directly involved both in the activation of the catalytic cycle at balanced concentrations, and the deactivation of the enzyme at high concentrations (Timofeevski et al. 1998). A low concentration of H_2O_2 would result in kinetic or even stoichiometric limitations, while the enzyme activity would be protected against inactivation (Mielgo et al. 2003a). Organic acids also present a similar mixed effect; a high concentration favours chelation of Mn^{3+}, but may affect the stability of the enzyme (Hofrichter et al. 1998), probably due to endogenous formation of H_2O_2 during decarboxylation (Schlosser & Hofer 2002; Van Aken & Agathos 2002).

Operational parameters

The hydraulic retention time (HRT), loading rate and environmental factors (temperature, pH) were the most significant operational parameters affecting the continuous process. HRT is related to the desired conversion (degradation) of pollutant and kinetics, which is directly affected by the pollutant concentration. Thus, a higher loading rate could lead to a faster but less efficient process. Increasing temperature could favour the reaction rate, but it may also increase enzyme deactivation. An appropriate temperature must be selected when trying to find a compromise between both aspects, as well as when considering economical factors. Operational pH must also be controlled at values near the optimal range. In the case of MnP, pHs lower than 4 and higher than 6 should be avoided.

Modelling and control of enzymatic reactors

When changes in the influent flow or the pollutant concentration occur, one of the main aspects to consider for proper continuous operation of an enzyme reactor is the system dynamics. The concept, design and operation of an efficient control system require a good kinetic model. In the case of MnP, the substrate to be oxidised (pollutant) is not the primary substrate for the enzyme (which is H_2O_2), although the degradation rate is greatly influenced by the concentration of the substrate and, thus, this parameter must be considered as a variable (López et al. 2007). Furthermore, the study of reactor behaviour in unsteady-state conditions will be indicative of system stability as it depicts system resistance against alterations.

Retention of the enzyme

To minimise losses, retention of the enzyme in the reactor is a priority when dealing with the design and operation of an enzyme reactor. When the pollutant is highly soluble in water, recovery of the enzyme from the effluent requires an additional system, whereas for low solubility compounds, the enzyme is trapped in the aqueous phase. Next, we discuss different strategies depending on the solubility of the pollutants.

Reactors treating highly soluble compounds. The configurations of enzymatic reactors treating soluble compounds can be classified according to the manner in which the enzyme is retained: (i) immobilised onto a support, forming larger structures that can be retained due to their size, or (ii) free in solution, being retained by a membrane.

Immobilisation of the enzyme onto a support is usually complex and increases processing costs. To improve the economical feasibility of immobilised enzyme reactors, a number of requirements should be met: the specific activity of the derivative (units of enzyme per gram of support) should be as high as possible; the support should have good mechanical resistance and minimum interaction with the substrates or products. Previous studies have demonstrated that a support based on glutaraldehyde-activated agarose was suitable for the immobilisation of MnP (Mielgo et al. 2003b). The immobilised enzyme was applied for the degradation of the dye Orange II in a continuous stirred tank reactor. However, there were some operational difficulties in this configuration: high activity loss during immobilisation, lower reaction rates, more complex control, adsorption of the dye onto the support and, particularly, the inability to replace the deactivated immobilised enzyme with fresh enzyme.

The second option was a continuous stirred tank reactor where the soluble enzyme was retained by means of an ultrafiltration membrane coupled to the reactor (Figure 2A) (López et al. 2004a). The main advantages of this configuration are: (i) operation with free enzyme, avoiding limitations of mass

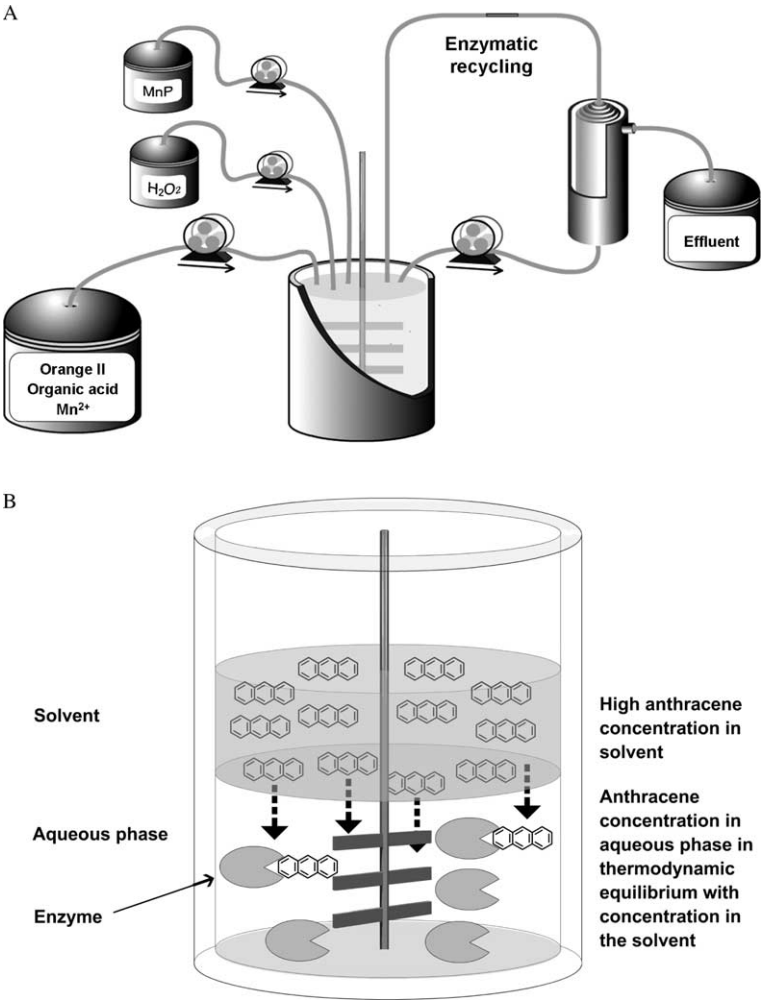

Figure 2. Schematic diagrams of (A) an enzymatic membrane reactor; (B) an enzymatic biphasic reactor.

transfer and, consequently, low kinetic rates; (ii) retention of non-biodegradable molecules with high molecular weight; (iii) ability of the products of degradation to cross the membrane, being discharged in the effluent; and (iv) simple operation.

Reactors treating poorly soluble compounds. The *in vitro* degradation of poorly soluble compounds is limited by their enzyme availability. The addition of miscible cosolvents is a good approach to increase the solubility by several orders of magnitude. Eibes et al. (2005) established a system for the degradation of anthracene by MnP in a medium containing a mixture of acetone:water (36% v:v). In this work, 5 mg L^{-1} of anthracene were degraded after 6 h operation under optimal conditions. However, this system presented a number of limitations: (i) the concentration of anthracene in the medium was limited by the amount of co-solvent; (ii) higher amounts of solvent resulted in greater inactivation of the enzyme; (iii) the enzyme could not be recycled; and (iv) the reuse of the solvent would require a separation process.

To overcome these limitations, a completely different option, a two-phase partitioning bioreactor (TPPB), was considered (Figure 2B). In TPPBs, a solvent immiscible in the water phase with an appropriate partition coefficient is added. The substrate is almost completely dissolved in the organic phase, from which it diffuses to restore equilibrium, as the enzyme degrades the pollutant in the aqueous phase (Vrionis et al. 2002). TPPBs have been successfully used for the biological treatment of toxic and recalcitrant pollutants, such as PAHs. Janikowski et al. (2002) performed the degradation of anthracene and other PAHs in biphasic reactors in cultures of *Sphingomonas aromaticivorans* and dodecane as the organic phase.

The selection of the appropriate solvent is critical for the design of TPPBs because it greatly influences mass transfer and, consequently, degradation rates. The selected solvent should be inexpensive, readily available, and exhibit suitable physical and chemical properties (be immiscible, non-volatile, etc.) (Déziel et al. 1999; Marcoux et al. 2000; Villemur et al. 2000; MacLeod & Daugulis 2003). Furthermore, when dealing with enzymatic reactors, the potential interaction between the solvent and enzyme is critical. It is important that the solvent is not a substrate of the enzyme (MacLeod & Daugulis 2003) and its effect on enzymatic activity is as low as possible (Ross et al. 2000). The partition coefficient should enable the system to achieve the highest possible concentration of substrate in the aqueous phase. Solvents with high partition coefficients can sequester the target compound, thus limiting its biodegradation rate (Efroymson & Alexander 1995).

The substrate transfer rate from the water-immiscible to the aqueous phase is another essential factor, and has to be enhanced in order not to limit the overall degradation rate. Mass transfer 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 & Lebeault 1995). The interfacial area is defined by Equation 1:

$$a = \frac{6\phi}{d_{sm}} \quad (1)$$

where ϕ is the proportion of the organic phase in the reactor and d_{sm} is the Sauter mean diameter of the solvent drops. Therefore, increasing the proportion of organic solvent or decreasing the diameter of the drops by increasing the agitation speed would augment the interfacial area.

In vitro degradation of Orange II in an enzymatic membrane reactor

Optimisation of the parameters involved in the catalytic cycle

The effect of different variables involved in the MnP catalytic cycle on the degradation of the azo dye, Orange II (Table I), was studied in discontinuous experiments (Mielgo et al. 2003a). The most remarkable results were observed with the addition of H_2O_2: continuous pumping of H_2O_2 with a rate equal to the rate of H_2O_2 consumption in the enzymatic reaction (34 μmol H_2O_2 L^{-1} min^{-1} (Moreira et al. 2001)), improved degradation with very low enzymatic deactivation. Once all the parameters and conditions were optimised, an initial Orange II concentration of 100 mg L^{-1} was degraded up to 90% after 10 min with minimum consumption of enzyme (Mielgo et al. 2003a).

Table I. Optimisation of reaction conditions for the discontinuous degradation of Orange II by MnP.

Parameter	Range	Optimal value
Organic acid	Oxal-Malon-Acet	Acetate
[acid]	1–50 mM	1 mM
$[Mn^{2+}]$	0–1000 μM	33 μM
H_2O_2 addition	FB-continuous	Continuous
$[H_2O_2]$	50–200 μmol L^{-1} min^{-1}	50 μmol L^{-1} min^{-1}
[MnP]	50–200 U L^{-1}	200 U L^{-1}
pH	4.5–8	4.5
T	20–30°C	30°C

V = 25 mL; initial Orange II concentration = 100 mg L^{-1}.

Optimisation of the operational parameters of the enzymatic membrane reactor

The effect of operational parameters on the efficiency of an enzymatic membrane reactor degrading an effluent containing 100 mg L^{-1} of the soluble dye Orange II was studied (López et al. 2004a).

Two strategies were considered to replace the enzyme consumed during the reaction: (i) the stepwise addition of enzyme when the activity fell below 100 U L^{-1}, which was considered the minimum level necessary to degrade the dye (Mielgo et al. 2003a); (ii) continuous pumping of the crude enzyme into the vessel to maintain a constant level of activated enzyme into the reactor (López et al. 2004a). Experiments E1 in Figure 3 show that the continuous addition of MnP increased the efficiency to 2.58 mg of Orange II degraded per unit.

Experiments E2 in Figure 3 show that 225 U L^{-1} of enzyme enhanced the degradation compared to 125 U L^{-1} with no higher consumption of enzyme, as the enzyme requirement was higher at the beginning of the reaction, but the loss per hour was similar. Addition of 50 μmol H_2O_2 L^{-1} min^{-1}, selected from discontinuous assays, favoured the kinetics of the process and allowed a low HRT (20 min) resulting in an Orange II loading rate (OLR) of 0.3 g L^{-1} h^{-1}. However, it resulted in activity loss >30 U of MnP L^{-1} h^{-1}. To decrease the enzyme loss, the H_2O_2 addition rate would have to be decreased and HRT increased to maintain high conversion. Experiments E3 depicted in Figure 3 performed at a HRT of 45 min and 15 μmol H_2O_2 L^{-1} min^{-1} achieved nearly 90% degradation and increased efficiency to 12.2 mg U^{-1}. Finally, experiments were performed to determine the best HRT required to achieve significant decolourisation. Although a HRT of 90 min resulted in higher decolourisation, the loading rate decreased to 0.067 g Orange II L^{-1} h^{-1}, decreasing efficiency. Hence, a HRT of 60 min was adequate for the continuous treatment of Orange II under these conditions.

Control systems

Experience of the Orange II degradation process led us to consider two different control systems: (i) a feed-back system based on the measurement of dissolved oxygen (DO) in the reaction vessel, or (ii) a feed-forward system based on a dynamic model including kinetics and hydraulics.

Monitoring the DO concentration during the different decolourisation assays enabled us to assess the influence of a variety of operational variables. The DO concentration was modified when we changed the H_2O_2 addition rate. The constant DO concentration attained in the different steady states depended on the OLR applied (López et al. 2004a). This suggests that DO is an indicative parameter of the behaviour of the reaction, as it is generated in the reaction between H_2O_2 and Mn^{3+} (Martínez et al. 1996), and consumed during the degradation of Orange II (López et al. 2004b). A high level of DO indicates an excess of H_2O_2 supply over oxygen utilisation in the enzymatic reaction.

The Orange II concentration and H_2O_2 addition rate were considered the variables essential for defining the kinetic model (López et al. 2007). The resulting equation followed a Michaelis–Menten model with respect to the Orange II concentration and a first-order linear dependence with respect to the H_2O_2 addition rate (Equation 2).

$$r = \frac{r_m S}{K_M + S}(K_{H_2O_2} + Q_{H_2O_2}) = \frac{0.33S}{58.2 + S}(2.4 + Q_{H_2O_2}) \quad (2)$$

The kinetics were combined with the hydraulics of the system, and the resulting dynamic model was successfully validated by comparing experimental

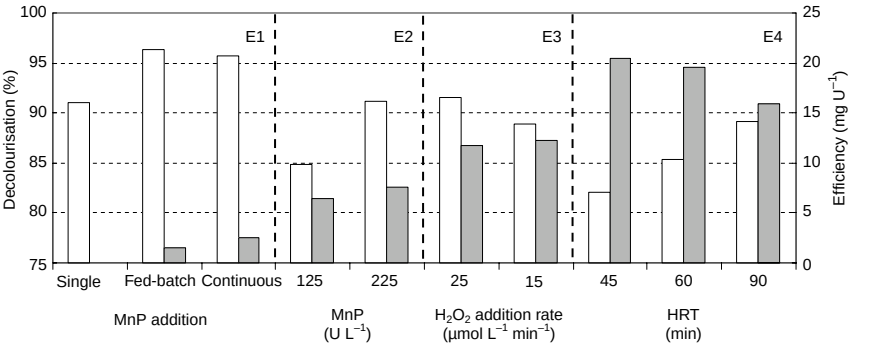

Figure 3. Comparative parameters of different strategies for the degradation of Orange II in an enzymatic membrane reactor: percentage of decolourisation (white bars) and efficiency (grey bars). Concentrations in the influent: Orange II 100 mg L^{-1}, malonate 1 mM, Mn^{2+} 33 μM. Experimental conditions are summarised in Table II.

Table II. Experimental conditions for assays E1–E4 in Figure 3.

Experiment	MnP (U L^{-1})	H_2O_2 addition rate (μmol L^{-1} min^{-1})	Organic acid	HRT (min)
E1	–	50	Acetic	60
E2	–	50	Acetic	20
E3	200	–	Acetic	45
E4	200	15	Oxalic	–

data with model predictions in different assays under steady-state conditions (Figure 4A).

Finally, an unsteady-state continuous process was performed to assess system stability and verify the control systems based on both the DO concentration and dynamic model (Figure 4B). The initial conditions were similar to previous assays; once steady-state was reached, perturbations of Orange II concentration, HRT, H_2O_2 addition rate or temperature were performed over 1 h periods. The system recovered the steady state within 3 h after the end of each alteration. DO followed the same behaviour as determined in previous experiments. This verified the fact that it could be used as a good control parameter. The dynamic model predicted the Orange II concentration in the effluent, not only during the steady states, but also when deviations in stationary conditions occurred.

In vitro degradation of anthracene in TPPBs

Optimisation of the operation of TPPBs must address the following: (i) selection of an appropriate solvent; (ii) study of the parameters involved in the catalytic cycle of the enzyme; (iii) enhancement of the mass transfer of the substrate from the organic phase; and (iv) model and control of the process.

Selection of the solvent

Several solvents, including mineral and vegetable oils, alcohols, alkanes, ketones and esters, were considered due to their high boiling points, low water solubility, low cost, lack of toxicity and

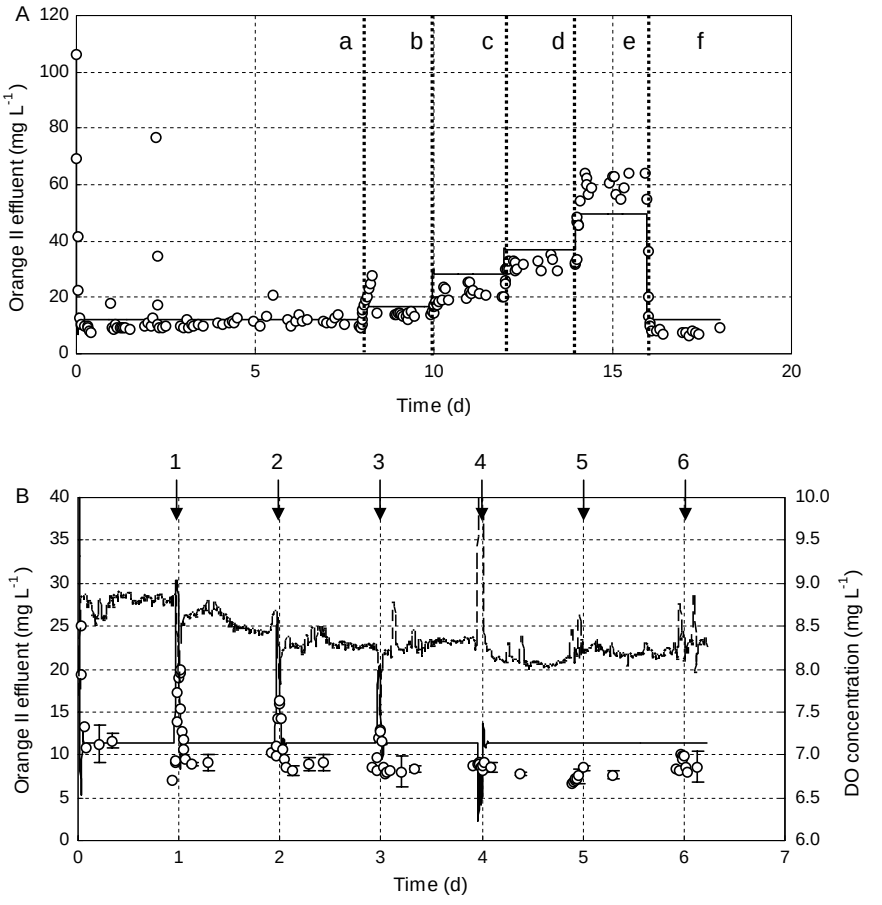

Figure 4. Experimental (○) and simulated data (—) of Orange II concentration in continuous assays. Initial conditions: Orange II 100 mg L^{-1}; malonate 1 mM; Mn^{2+} 33 μM; MnP 200 U L^{-1}; HRT 60 min; H_2O_2 addition rate 15 μmol L^{-1} min^{-1}; pH 4.5. (A) Steady-state operation at different Orange II concentrations in the influent: (a) 100 mg L^{-1}; (b) 150 mg L^{-1}; (c) 200 mg L^{-1}; (d) 250 mg L^{-1}; (e) 300 mg L^{-1}; (f) 100 mg L^{-1}. (B) Non-steady state operation with 1-h overloads: (1) Orange II 200 mg L^{-1}; (2) HRT 30 min; (3) H_2O_2 7.5 μmol L^{-1} min^{-1}; (4) H_2O_2 30 μmol L^{-1} min^{-1}; (5) T 20°C; (6) T 30°C; (–) dissolved oxygen.

commercial availability. The partition coefficients (K_{SW}) were evaluated for each solvent (Table III). The values of log K_{SW} obtained ranged from 3.7 (silicone oil) to 5.2 (undecanone). We avoided the highest values of K_{SW} and selected two solvents for further study: silicone oil, with the minimum log K_{SW} of 3.7, and dodecane, with an intermediate log K_{SW} value of 4.5.

The second factor in the selection of the solvent was its interaction with the enzyme. The enzyme was exposed to different interfacial areas by modifying the agitation rate (Figure 5). Under similar agitation rates, silicone oil formed a higher interfacial area due to its lower interfacial tension (20 and 53 mN m^{-1} for silicone oil and dodecane, respectively). This showed that, even at higher interfacial areas, enzyme inactivation in silicone oil was lower.

Both factors, partition coefficient and enzyme inactivation, were more favourable in the case of silicone oil. Consequently, silicone oil was selected for subsequent experiments.

Optimisation of the parameters involved in the catalytic cycle

As described for the degradation of Orange II, the main factors involved in the catalytic cycle of MnP were evaluated for the degradation of anthracene (Eibes et al. 2007). Figure 6 describes the efficiency, in terms of anthracene degraded per unit of enzyme consumed, for experiments at different hydrogen peroxide addition rates, malonate concentrations and pH control.

The highest efficiencies were obtained at H_2O_2 addition rates of 1 and 5 μmol L^{-1} min^{-1}. The efficiency decreased with higher addition rates due to enzyme inactivation as a result of excess H_2O_2. The concentration of the organic acid had a similar effect: higher concentrations resulted in greater enzyme inactivation and lower efficiency. Finally, control of pH was evaluated because an increase in pH was observed during the reaction, reaching values close to 8 after 70 h of operation. Although the pH was initially maintained at 4.5 by the addition of HCl, addition of malonic acid to control

Table III. Log K_{SW} of 15 different solvents.

Solvent	Log K_{SW}	Solvent	Log K_{SW}
Silicone oil	3.7	Triacetin	4.8
Paraffin oil	4.3	Olive oil	4.9
Sunflower oil	4.3	Corn oil	4.9
Oleic alcohol	4.4	Ethyl acetate	5.0
Decanol	4.4	Biodiesel	5.0
n-Hexadecane	4.5	Marc olive oil	5.0
Dodecane	4.5	Undecanone	5.2
Engine oil	4.6		

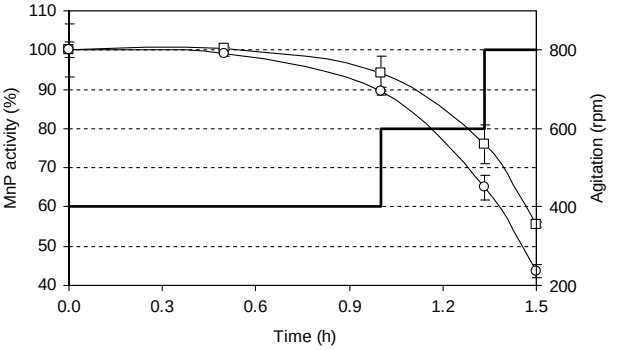

Figure 5. Effect of agitation (—) on MnP activity in media with dodecane (○) or silicone oil (□).

the pH was considered as the concentration of sodium malonate decreased in the reactor. The efficiency of the experiment with 10 mM sodium malonate was increased 1.9-fold relative to the experiment with no pH control.

Optimisation of the mass transfer coefficients

Enhancement of substrate transfer rate was achieved by increasing the volume of silicone oil and the agitation speed. Both factors favoured diffusion from the organic phase because they increased the interfacial area, but they also affected enzymatic activity. A 2^2 experimental design was considered to optimise the system efficiency. The ranges evaluated were 200–300 rpm and 10–30% of silicone oil (v:v), and the response surface corresponding to efficiency is represented by Equation 3 (Eibes et al. 2007):

$$EF = 0.152 + 0.026\ AG + 0.054\ SO + 0.026\ AG\ SO \quad (3)$$

where EF denotes system efficiency, AG denotes the agitation rate, and SO denotes the fraction of silicone oil (both factors are normalised). The equation shows that increasing both the volume fraction of silicone oil and the agitation speed led to higher efficiencies with a maximum occurring at 300 rpm and 30% silicone oil.

Model of the process

The equation describing substrate concentration in the aqueous phase (S_w) is given by:

$$\ln (S^* - S_W) = \ln S^* - k_L a\ t \quad (4)$$

where S^* is the equilibrium concentration of the substrate in the aqueous phase and $k_L a$ is the mass transfer coefficient. The determination of substrate concentration in the aqueous phase enabled us to find the mass transfer coefficient for each condition of agitation speed and volume fraction of solvent (Table IV). The data show large increases in $k_L a$

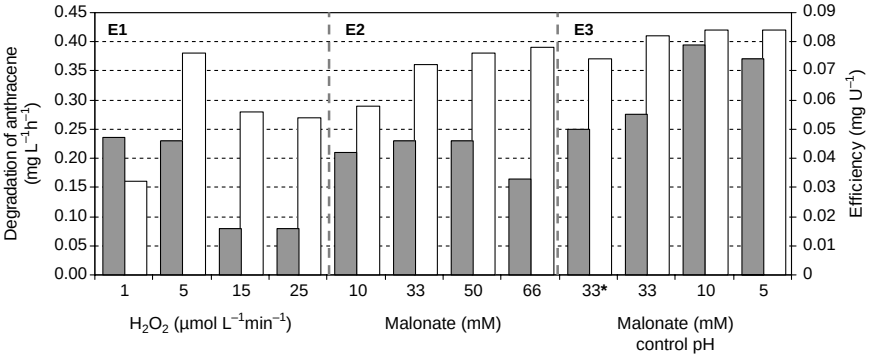

Figure 6. Degradation of anthracene (grey bars) and different efficiency values (white bars) obtained for each condition of hydrogen peroxide addition (E1), initial malonate concentration with no pH control (E2) and initial malonate concentration with pH control via the addition of HCl (*) or malonic acid (E3).

especially in a narrow range of agitation speed (200–250 rpm). This effect was more pronounced when low volumes of silicone oil were present. These results agree with the conclusions obtained above, which suggest an optimised mass transfer rate at 300 rpm and 30% silicone oil. The values of $k_L a$ were fitted to a surface, and, thus, related to the agitation and fraction of solvent through an empirical correlation.

The equation, which describes the behaviour of anthracene in the organic phase (S_S) considering the mass transfer and the degradation kinetics (as a pseudo-first order (Eibes et al. 2006)), is shown in Equation 5:

$$\ln S_S = \ln S_{S0} - \frac{k_L a}{k_{sw}} \left(\frac{k_{cat}}{k_L a + k_{cat}} \right) \frac{V_w}{V_S} \; t \qquad (5)$$

The partition coefficient of anthracene in silicone oil (k_{sw}) had been determined previously ($k_{sw} = 5012$; Table III) and the mass transfer coefficient ($k_L a$) was correlated with the operational parameters, as described above. Finally the kinetic constant k_{cat} was determined from batch experiments. The model was validated by comparing experimental data with model predictions in experiments at different agitation speeds and fractions of silicone oil (Figure 7). As presented previously, the highest degradation rate was obtained at 300 rpm and 30% silicone oil (v:v), oxidising 90% of anthracene present in the organic phase after 56 h.

Table IV. Values of $k_L a$ obtained for experiments at different agitation rates and volumes of silicone oil.

	$k_L a$ (min^{-1})		
Agitation speed (rpm)	10% silicone oil	20% silicone oil	30% silicone oil
50	0.01	0.02	0.12
150	0.10	0.36	0.36
200	0.27	0.30	0.68
250	2.99	2.26	3.14
350	3.29	3.30	3.30

Conclusions and perspectives

Enzymatic membrane reactors are a promising technology, as they are easy to operate and control. Degradation processes can be conducted continuously for more than 20 days without membrane cleaning or replacement. Furthermore, enzyme reactors are versatile since there are a wide variety of commercially available membrane shapes, materials and modules. The selection process must primarily consider the characteristics of the effluent and the enzyme molecular weight.

The use of a two-phase partitioning bioreactor, which utilises a second immiscible phase for enzymatic degradation of poorly soluble compounds, is advantageous because of its simple operation due to the easy recovery and reuse in subsequent experiments of the solvent after substrate depletion. Although *a priori* mass transfer could be considered a limitation of this system, selection of the appropriate solvent and optimising the conditions to give

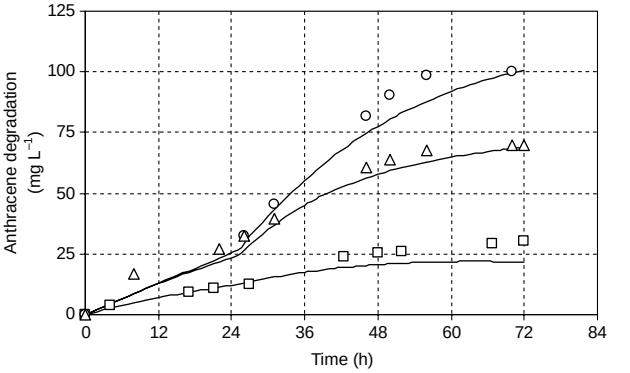

Figure 7. Experimental and fitted data of assays at different agitation rates and volumes of solvent: 200 rpm–10% (□), 250 rpm–20% (○), 300 rpm–30% (Δ).

maximum efficiency enabled us to obtain unprecedented degradation rates.

The challenges for those processes are now focused on development and implementation of a control system and further scale-up to industrial scale.

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References

Ascón-Cabrera MA, Lebeault JM. 1995. Interfacial area effects of a biphasic aqueous/organic system on growth kinetic of xenobiotic-degrading microorganisms. Appl Microbiol Biotechnol 43:1136–1141.

Déziel E, Comeau Y, Villemur R. 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation 10:219–233.

Efroymson RA, Alexander M. 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. Environ Sci Technol 29:515–521.

Eibes G, Lu Chau T, Feijoo G, Moreira MT, Lema JM. 2005. Complete degradation of anthracene by manganese peroxidase in organic solvent mixtures. Enzyme Microb Technol 37:365–372.

Eibes G, Cajthaml T, Moreira MT, Feijoo G, Lema JM. 2006. Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere 64:408–414.

Eibes G, Moreira MT, Feijoo G, Daugulis AL, Lema JM. 2007. Operation of a two phase partitioning bioreactor for the degradation of anthracene by the enzyme manganese peroxidase. Chemosphere 66:1744–1751.

Hofrichter M, Ziegenhagen D, Vares T, Friedrich M, Jäger MG, Fritsche W. 1998. Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide. FEBS Lett 434:362–366.

Janikowski TB, Velicogna D, Punt M, Daugulis AJ. 2002. Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas* sp. Appl Microbiol Biotechnol 59:368–376.

Köhler A, Schüttoff M, Bryniok D, Knackmuß HJ. 1994. Enhanced biodegradation of phenanthrene in a biphasic culture system. Biodegradation 5:93–103.

Kuan IC, Tien M. 1993. Stimulation of manganese peroxidase activity: A possible role for oxalate in lignin biodegradation. Proc Natl Acad Sci USA 90:1242–1246.

Kuan IC, Johnson KA, Tien M. 1993. Kinetic analysis of manganese peroxidase. The reaction with manganese complex. J Biol Chem 268:20064–20070.

López C, Moreira MT, Feijoo G, Lema JM. 2004a. Dye decolorization by manganese peroxidase in an enzymatic membrane bioreactor. Biotechnol Prog 20:74–81.

López C, Valade AG, Combourieu B, Mielgo I, Bouchon B, Lema JM. 2004b. Mechanism of enzymatic degradation of the azo dye Orange II determined by *ex situ* ^{1}H nuclear magnetic resonance and electrospray ionization–ion trap mass spectrometry. Anal Biochem 335:135–149.

López C, Moreira MT, Feijoo G, Lema JM. 2007. Dynamic model of an enzymatic membrane reactor treating xenobiotic compounds. Biotechnol Bioeng, DOI: 10.1002/bib.21311.

MacLeod CT, Daugulis AJ. 2003. Biodegradation of polycyclic aromatic hydrocarbons in a two-phase partitioning bioreactor in the presence of a bioavailable solvent. Appl Microbiol Biotechnol 62:291–296.

Marcoux J, Déziel E, Villemur R, Lépine F, Bisaillon JG, Beaudet R. 2000. Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. J Appl Microbiol 88:655–662.

Martínez AT. 2002. Molecular biology and structure–function of lignin-degrading heme peroxidases. Enzyme Microb Technol 30:425–444.

Martínez MJ, Böckle B, Camarero S, Guillén F, Martínez AT. 1996. MnP isoenzymes produced by two *Pleurotus* species in liquid culture and during wheat-straw solid-state fermentation. ACS Symp Ser 655:183–196.

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

Mielgo I, Palma C, Guisán JM, Fernández-Lafuente R, Moreira MT, Feijoo G, Lema JM. 2003b. Covalent immobilisation of manganese peroxidases (MnP) from *Phanerochaete chrysosporium* and *Bjerkandera* sp. BOS55. Enzyme Microb Technol 32:769–775.

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

Ross AC, Bell G, Halling PJ. 2000. Organic solvent functional group effect on enzyme inactivation by the interfacial mechanism. J Mol Catal B: Enzym 8:183–192.

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

Tien M, Kirk TK. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol 161:238–249.

Timofeevski SL, Reading NS, Aust SD. 1998. Mechanisms for protection against inactivation of manganese peroxidase by hydrogen peroxide. Arch Biochem Biophys 356:287–295.

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

Villemur R, Déziel E, Benachenhou A, Marcoux J, Gauthier E, Lépine F, Beaudet R, Comeau Y. 2000. Two-liquid-phase slurry bioreactors to enhance the degradation of high-molecular-weight polycyclic aromatic hydrocarbons in soil. Biotechnol Prog 16:966–972.

Vrionis HA, Kropinski AM, Daugulis AJ. 2002. Enhancement of a two-phase partitioning bioreactor system by modification of the microbial catalyst: Demonstration of concept. Biotechnol Bioeng 79:587–594.

Wariishi H, Akaleswaran L, Gold MH. 1988. Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of oxidized states and the catalytic cycle. Biochemistry 27:5365–5370.