6.6. Application of enzymatic reactors for the degradation of highly and poorly soluble recalcitrant compounds

Juan Manuel Lema, Full Professor, Deptartment of Chemical Engineering, School of Engineering, Universidad de Santiago de Compostela, Spain. Phone: 34-98-1563100 Ext: 16793, FAX: 34-98-1528050; e-mail: jmlema@usc.es

Gemma Eibes, Carmen López, M. Teresa Moreira, Gumersindo Feijoo, Deptartment of Chemical Engineering, School of Engineering, Universidad de Santiago de Compostela, Spain.

6.6.1. Potential application of oxidative enzymes for environmental purposes

The discharge of recalcitrant compounds in the environment represents an important ecological concern since their complex structure and low bioavailability confer resistance for biodegradation and difficult their transformation in conventional sewage treatment plants (STP). Advanced oxidation processes such as ozonation, Fenton treatment or UV exposure, have arisen as possible alternatives to treat particular effluents before entering a STP (Chen et al. 2007; Lee et al. 2007; Suárez et al. 2007). Other possibility is based on biotechnological approaches, which consider the use of bacterial or fungal cultures to carry out their transformation. In particular, the utilization of white-rot fungi may be valuable, due to their ability to oxidize and decompose very complex and strong structures as lignin. The action of these fungi is attributed to extracellular oxidases and peroxidases, the most outstanding being lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac).

Degradation of organopollutants by fungal cultures has been carried out successfully at bench and pilot scale, preferentially in batch or semi-continuous operations (Alleman et al. 1995; Fujita et al. 2002; Mielgo et al. 2002). Continuous treatment systems are desirable alternatives to increase process efficiency. A modified conventional configuration (fixed-bed bioreactor) coupled with a pulsation system has proved to assure efficient and prolonged operation of the bioreactor (Mielgo et al. 2002). The feasibility of this approach was evaluated in the degradation of three different dyes: anthraquinone type (Poly R-478), azo type (Orange II) and phtalocyanine type (Reactive Blue 98). This configuration provided good oxygen transfer, controlled mycelia extension by pneumatic pulsation and prevented preferential paths. By modifying the operating conditions of this system related to Mn^{+2} and hydrogen peroxide (H_2O_2) concentrations, temperature and oxygen supply, the decolorizing system was robust and flexible enough to maintain fairly high efficiency of removal (80-98%), even when working at conditions far apart from the optimal. However, the application of microbial reactors presented as drawbacks the operation at hydraulic retention times of about 1 day, the addition of nutrients and aeration and the necessity of maintaining sterile conditions.

Under a practical point of view, the potential use of enzymes for environmental purposes appears as a solution to overcome these drawbacks. Both strategies are compared in Table 6.6.1, being enzymatic treatment more advantageous than fungal degradation in many aspects. Several works report the ability of different enzymes for the oxidation of organopollutants (Table 6.6.2). However, the application of an enzymatic system for degradation of recalcitrant compounds is a scarcely explored alternative mainly due to the cost of the enzyme.

Table 6.6.1

Table 6.6.2

6.6.2. Requirements for an efficient catalytic cycle

The first step to develop an enzymatic reactor requires a wide knowledge of enzyme behavior, regarding the substrates and cofactors involved in the catalytic cycle. In whole fungal cultures, these compounds are endogenously produced and their necessary replacement takes place naturally, whereas in enzymatic processes their addition is required to effectively complete the catalytic cycle. Let us consider MnP as a selected example. It has a similar catalytic cycle than other peroxidases, involving a 2-electron oxidation (Fig. 6.6.1). The initial oxidation of MnP by H_2O_2 leads to an intermediate compound which promotes oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} is stabilized by organic acids and the Mn^{3+}-organic acid complex formed acts as an active diffusing oxidizer (1.54 V) that attacks organic molecules non-specifically at locations remote from the enzyme active site (Kuan and Tien 1993). It is important to highlight that an excess of H_2O_2 can cause irreversible enzyme inactivation.

Fig. 6.6.1

In natural environments, fungi adapt their degradative capacity to the slowest reaction step. In wood decay, the microorganism uses Mn^{2+} present in wood and produces H_2O_2 endogenously by extracellular oxidases. In contrast, the *in vitro* application of MnP requires addition of Mn^{2+}, H_2O_2 and organic acid not only to assure the completion of the catalytic cycle but also to enhance reaction kinetics and enzyme stability. Mn^{2+} affects positively the reaction rate, though this beneficial effect is greatly dependent on the H_2O_2 concentration (Palma et al. 1997). H_2O_2 has a dual effect, as it is directly involved both in the activation of the catalytic cycle at a balanced concentration and deactivation of the enzyme at a high concentration (Timofeevski et al. 1998). On the contrary, a low concentration of H_2O_2 implies kinetic or even stoichiometric limitations, while the enzyme activity is protected from inactivation (Mielgo et al. 2003). Organic acids provoke a similar cross effect: a high concentration favors the chelation of Mn^{3+} but it may affect the stability of the enzyme (Hofrichter et al. 1998). This effect could likely be due to the endogenous formation of H_2O_2 during the decarboxylation of sodium malonate (van Aken and Agathos 2002). Not only a high reaction rate is desirable, but also a minimum loss of enzymatic activity.

When the practical application of the enzyme for the transformation of a substrate is considered, an important goal is to attain the best efficiency, defined as the maximum amount of substrate degraded per minimum units of enzyme inactivated during the reaction.

6.6.3. Enzymatic reactor configurations

The choice of the enzyme reactor configuration depends on the properties of the reaction system. For each class of biotransformation, the medium in which conversion takes place is dependent on the properties of reactants and products. The reaction components may have high or low aqueous solubility, which will greatly affect the reactor selection. When the xenobiotic compound is highly soluble in water, the choice of the enzyme reactor is apparently simple, focusing the design on the retention of the enzyme. However, for poorly soluble compounds, bioavailability of the xenobiotic is the most restrictive step for degradation. Systems to improve the interaction between the enzyme or its related mediator and the substrate have to be applied. Among several possibilities, Table 6.6.3 shows different reactor configurations to treat highly and poorly soluble compounds, which are discussed below.

Table 6.6.3

6.6.3.1. Highly soluble compounds

The design of enzymatic reactors for the degradation of highly soluble compounds may consider the use of a semi permeable membrane for the separation of the enzyme and products or substrates (enzyme membrane reactors) or the immobilization of the enzyme in a support (immobilized enzyme reactors).

- *Membrane reactors*

The system is provided with a membrane of suitable molecular cut-off, which acts as a selective barrier for the retention of the enzyme. Permeable substrates and products are taken out from the reaction mixture by the action of a gradient (chemical potential, pressure) through the membrane. Based on the combination of membranes and enzyme reactors two main configurations are considered, as shown in Fig. 6.6.2. In the first configuration, the enzyme may be immobilized by covalent binding between an activated group of the membrane and a functional group of the protein (Guisán et al. 1997), adsorbed by Van der Waals or electrostatic forces (Lante et al. 2000) or entrapped or encapsulated onto the matrix during the membrane preparation process (Isono and Nakajima 2000). The alternative configuration is based on the selective extraction of substrates from the influent by specific membranes. Once the substrate reacts with the enzyme, products diffuse back through the membrane to reach the effluent (Prazeres and Cabral 1994). The main drawbacks of this system are the requirement of low molecular weight substrates and the diffusion of compounds through the membrane, which

usually slows down the kinetics of the process (Kelsey et al. 1990). In direct contact enzyme reactors, the substrate is fed directly to the compartment of the enzyme, enabling a direct contact between them. The most common system consists of a traditional stirred tank reactor combined with a membrane separation unit (Prazeres and Cabral 1994). The retained enzyme is recycled back to the reactor, whereas the degraded products are able to pass through the membrane. The main advantages of this configuration are: i) operation with free enzyme, avoiding mass transfer limitations; ii) retention of non-biodegradable molecules with high molecular weights; iii) ability of the degraded products to cross the membrane, being discharged in the effluent; and iv) easy operation.

Fig. 6.6.2

- *Immobilized enzyme reactors*

References about immobilized enzyme reactors for environmental purposes are scarce (Katchalski-Katzir and Kraemer 2000). The concept is based on the immobilization of the enzyme onto a support by covalent binding or ionic interaction. The feasibility of the immobilized enzyme reactors is determined by the following requirements: i) the specific activity of the derivative (units of enzyme per g of support) should be as high as possible; ii) the support or membrane should be applied with a secondary function, such as the separation of substrates or products, and iii) the support should have good mechanical resistance and minimum interaction with the substrates or products. Additionally, the immobilization process should be simple and inexpensive.

Different reactor configurations are proposed for the use of immobilized enzymes (Fig. 6.6.3): A) stirred tank reactors; B) fixed bed reactors; and C) fluidized bed reactors. The selection of the best option depends on the type of support and reaction kinetics. However, very often the activated supports do not present adequate characteristics for the performance of a fixed or fluidized bed reactor and, therefore, the development of stirred tank reactors based on immobilized enzyme or even, sequential stirred tank reactors appear as the more feasible option.

Fig. 6.6.3

6.6.3.2. Poorly soluble compounds

- *Solvents and surfactants*

The *in vitro* degradation of poorly soluble compounds is limited by the extent to which they are available to the enzymes, which could lead to biocatalytic conversions at low rates. The addition of solubilizing agents, such as surfactants or solvents, might be considered to overcome this limitation. Although the use of surfactants can exert a deleterious effect on enzyme activity and

the solubilization is rather limited, they have been frequently applied to increase the availability of certain substrates and in some cases they have been found to enhance the oxidation via formation of peroxyl radicals (Kotterman et al. 1998). Enzymatic catalysis in organic solvents has undergone rapid expansion in the last decades and, although believed to be a promising approach in decontamination, most of the work reported is related to hydrolytic enzymes. The potential of using more complex enzymes as ligninases, which require specific substrates and cofactors for the catalytic cycle, is almost untapped (Field et al. 1996). The addition of water miscible solvents (cosolvents) can enhance the solubility of compounds by several orders of magnitude, (Eibes et al. 2005; Eibes et al. 2006). However, the selection of the cosolvent requires special attention, because hydrophilic solvents have a great tendency to strip bound water away from enzyme molecules leading to a rapid inactivation (Klibanov 2001). Moreover, the solvent and enzyme recovery has to be considered to make this process economically feasible.

The use of a biphasic medium to carry out bioconversions at higher overall concentrations has clear advantages for those reactions where one or more reaction components are poorly water-soluble. The principle of a biphasic biocatalytic process is based on the addition of a virtually water-immiscible organic solvent to an aqueous phase (at concentrations well above the aqueous phase saturation limit) containing the biocatalyst to create a biphasic reaction medium (Fig. 6.6.4). When the substrate is hydrophobic, the organic phase will be initially rich in substrate which will then distribute into the aqueous phase at a concentration defined by its partition coefficient. Once in the aqueous phase, the enzyme will degrade the substrate, this being the driving force for the subsequent substrate spread out of the organic phase in order to maintain a thermodynamic equilibrium. Depending upon their solubility, degradation products may remain in the aqueous phase or partition into the organic phase.

The selected solvent should have suitable physical and chemical properties (be immiscible, non-volatile, etc.), and be inexpensive and readily available (Déziel et al. 1999; MacLeod and Daugulis 2003; Marcoux et al. 2000). Furthermore, the possible interaction between the solvent and the enzyme has to be considered. It is important that the presence of the solvent does not interfere with the degradation of the target substrate (MacLeod and Daugulis 2003) and its effect on the enzyme activity be as low as possible (Ross et al. 2000). The development of solvent-resistant enzymes will facilitate the application of two-liquid phase biocatalysis.

Fig. 6.6.4

- *Factors affecting reactor efficiency* The application of two phase partitioning bioreactors (TPPBs) presents some difficult biochemical engineering challenges including the selection of an appropriate reaction medium, reactor design and operating parameters. Drawbacks arising from the use of biphasic reactors may be related to the non-direct contact between the enzyme and the substrate, which may imply diffusional resistance. The substrate transfer rate from the water-immiscible phase to the aqueous phase (Eq. 6.6.1) is another critical factor and it has to be enhanced so as not to limit the overall degradation rate.

$$J_S = k_L a \cdot (s_w^* - s_w) \qquad \text{Eq. 6.6.1}$$

where J_S is the flow of the substrate (M/h); k_L is the mass transfer coefficient ($m \cdot h^{-1}$); a is the specific interfacial area ($m^2 \cdot m^{-3}$); and (s_w* - s_w) is the difference between the equilibrium and the actual substrate concentration in the aqueous phase (M). Mass transfer coefficient is dependent on the system geometry, the physical properties of medium (solvent viscosity, density, interfacial tension, substrate diffusivity…) and flow dynamics (Welty et al. 1984). Apart from these solvent properties, the partition coefficient of the substrate in the solvent is another factor which has to be taken into account. Solvents with high partition coefficient can sequester the target compound, thus reducing its biodegradation rate (Efroymson and Alexander 1995). Therefore, low values of partition coefficients are preferred in order to achieve high concentration of the substrate in the aqueous phase.

An increased surface area enables higher substrate transfer rate; therefore, the rate of biodegradation in a TPPB may be governed by the interfacial area (Köhler et al. 1994), as defined by Eq. 6.6.2:

$$a = \frac{6 \cdot \varphi}{d_{sm}} \qquad \text{Eq. 6.6.2}$$

where φ is the proportion of the organic phase in the reactor and d_{sm} is the Sauter mean diameter of the solvent drops, these parameters being very important when selecting the operating conditions.

6.6.4. Modeling of enzymatic reactors

Mathematical models, especially when coupled with computer techniques, are a very effective tool in searching for optimal operating conditions in the design, operation and control of enzyme reactors. The study of a reliable model for the enzyme reaction system is of significant

importance for the industrial applications of the biocatalyst. The model has to be effective in a wide range of values of the process variables.

The most important characteristics of an enzyme are the basic data about the kinetics of the catalyzed process and knowledge about the factors that affect the kinetic properties. The following step for further developing the model is to find the relationship between the reaction rates and the reactor configuration. The above-mentioned formal kinetic model for the overall reaction rate and the balance equations for a reactor configuration are used to predict the process behavior and to give a rule for optimizing efficiency (see Chapter 5). To validate the kinetic model, the data from batch reactor experiments should be in good agreement with simulated data obtained by means of numerical integration. Fig. 6.6.5 represents the scheme followed to obtain the model for an enzyme reactor operation.

Furthermore, the study of the reactor behavior in unsteady-state conditions will be indicative of the stability of the system as it shows its resistance against alterations, such as changes in the influent flow or pollutant concentration. The dynamic model of a particular process is an essential tool for developing an effective control strategy.

Fig. 6.6.5

6.6.5. Case studies

Two different reactor configurations were studied in order to evaluate the removal of a highly soluble compound, the azo dye Orange II and a poorly soluble compound, the polycyclic aromatic hydrocarbon (PAH), anthracene.

6.6.5.1. *In vitro* degradation of Orange II in an enzyme membrane reactor

The degradation of the dye Orange II was carried out in an enzyme reactor consisting of a continuous stirred tank reactor (CSTR) coupled with a membrane to recover the enzyme (Fig. 6.2.B). From an economic point of view, a compromise has to be made between the productivity, the conversion and the loss of enzyme activity. Before using a continuous enzymatic membrane reactor for the degradation of compounds, the optimization of both the parameters involved in the catalytic cycle of the enzyme and the operational conditions of the reactor must be considered; besides, the development of a control system is very useful for the simulation and optimization of the process.

- *Optimization of the parameters involved in the catalytic cycle*

The effect of the variables involved in the catalytic cycle of the enzyme MnP was evaluated in batch experiments. Addition rate of H_2O_2, concentration of Mn^{2+}, pH and MnP activity were investigated. Results are presented in Fig. 6.6.6 (Mielgo et al. 2003). The purpose was to obtain conditions to attain high decolorization rate of Orange II with low consumption of enzyme. Initial MnP activity for the experiments was 200 U/L unless indicated. When H_2O_2 was continuously pumped at an addition rate of 34 μmol H_2O_2/L·min, almost complete degradation (93%) was obtained after 10 min of reaction, while in the case of fed-batch operation only 76% degradation was reached after 60 min (Fig. 6.6.6 A). Therefore, continuous addition of H_2O_2 was considered for the following experiments. Only 33 μM of Mn^{2+} was required for Orange II decolorization and no further improvement was attained at higher concentrations (Fig. 6.6.6 B). Values of pH around 4.5 were adequate for an efficient operation (Fig. 6.6.6 C). Concerning the enzyme, 150 U/L of MnP was determined to be the minimum activity required to reach a good decolorization after 1 h (Fig. 6.6.6 D). The optimization of all parameters permitted to reach 90% degradation of 100 mg/L of Orange II after 10 min with minimum inactivation of enzyme.

Fig. 6.6.6

- *Optimization of the operational parameters of the enzymatic membrane reactor*

Based on the optimization of the conditions attained in discontinuous experiments (Mielgo et al. 2003), the degradation of the dye was performed in a continuous enzyme reactor (López et al. 2004). Different experiments were planned to maximize process efficiency, defined as the ratio between degradation rate and enzymatic consumption.

Three strategies for enzyme addition were considered (Fig. 6.6.7): i) Single initial addition of MnP; ii) Stepwise addition of enzyme every hour; iii) Continuous pumping of the enzyme into the vessel in order to maintain a relatively constant level of activity (López et al. 2004). Continuous addition of enzyme implied high decolorization, low MnP activity loss and the highest efficiency of the process. Using this strategy of MnP addition, different flow rates of H_2O_2 were evaluated: 15, 25 and 50 μmol/L·min, finding that a compromise solution should be selected depending on the particular objectives: maximizing decolorization or minimizing enzymatic deactivation.

Fig. 6.6.7

- *Modeling*

Deep knowledge of the enzymatic reaction is necessary for a proper selection of the variables that should be considered in the reaction model. In this case, two variables were selected: Orange II concentration, as the dye is the substrate to be oxidized, and H_2O_2 addition rate, as the primary

substrate of the enzyme (López et al. 2007). The performance of some discontinuous experiments at different initial values of both variables resulted in the definition of a kinetic equation, defined using a Michaelis-Menten model with respect to the Orange II concentration and a first-order linear dependence relative to H_2O_2 addition rate (Eq. 6.6.3).

$$r = \frac{r_m \cdot s}{K_M + s}\left(K_{H_2O_2} + Q_{H_2O_2}\right) = \frac{0.33 \cdot s}{58.2 + s}\left(2.4 + Q_{H_2O_2}\right) \qquad \text{Eq. 6.6.3}$$

where r is the degradation rate (μM/min); r_m is a constant dimensionless value; s is the Orange II concentration (μM); K_M is the Michaelis constant (μM); K_{H2O2} is the constant for the H_2O_2 addition rate (μM/min) and Q_{H2O2} is the H_2O_2 addition rate (μM/min).

A dynamic model was defined considering the kinetic equation and the hydraulics of the enzymatic membrane reactor. This model was validated comparing experimental data with model predictions at different experiments in steady-state conditions. Even when some modifications were performed, as changes in the Orange II concentration in the feed, the control system was able to predict the Orange II concentration in the reactor (Fig. 6.6.8).

Fig. 6.6.8

The study of the behavior of the reactor in unsteady-state conditions was useful, not only to validate the dynamic model, but also to obtain information about the system. Fig. 6.6.9 shows the results of an experiment where some perturbations of the steady state were performed at 1-h intervals. Orange II hydraulic retention time (HRT) and H_2O_2 addition rate were modified in order to cause a destabilization of the system, which recovered steady state in less than 3 h after the end of each alteration. As it can be observed, the dynamic model successfully simulated the behavior of the system.

Fig. 6.6.9

The dynamic model was validated both in steady and unsteady-state conditions, which is quite interesting in case that a control based on feed-forward strategy is applied. The prediction of the final concentration of Orange II from the initial data would allow the system control to modify the flow rate of MnP, Orange II or H_2O_2, in order to adapt the conditions to the desired final value.

Dissolved oxygen (DO) concentration in steady state was observed to be dependent of the experimental conditions (López et al. 2004). DO decreases when there is an unbalance between H_2O_2 addition rate and organic loading rate (OLR) (Fig. 6.6.9). This suggests DO as a suitable parameter for monitoring the reaction behavior and a control variable to apply a feed-back control system (López et al. 2007).

6.6.5.2. *In vitro* degradation of anthracene in TPPBs

The degradation of a poorly-soluble compound, anthracene, was carried out in a TPPB by the oxidative action of MnP. When dealing with biphasic reactors, the selection of the appropriate solvent is the first step in the optimization of the process. As well as with soluble compounds, the parameters involved in the catalytic cycle should be studied but also other operational parameters affecting mass transfer of the substrate. Finally, a model of the process will help to understand the whole process and to choose the most adequate operational parameters.

- *Selection of the solvent*

Two factors must be considered to select an adequate solvent: partition coefficient of anthracene in solvent/water medium and stability of enzyme in biphasic systems. A list of solvents presenting high boiling point, low water solubility, low cost, lack of toxicity and commercial availability was first considered to determine the partition coefficient of anthracene (K_{SW}) (Fig. 6.6.10). Solvents presenting high values of K_{SW} were avoided, since the degradation rate might be reduced due to the diminution of anthracene concentration in aqueous phase. Both silicone oil, with the minimum log K_{SW} 3.7, and dodecane, with an intermediate value of log K_{SW} 4, were selected for enzyme inactivation studies. Organic solvents can exert a deleterious effect on the biocatalyst, which may be due to the interaction with dissolved solvent molecules or with the interface between the aqueous and organic phases (Ross et al. 2000). Silicone oil and dodecane are nearly insoluble in water with high hydrophobicity values (log K_{OW} 6.6 and 11 for dodecane and silicone oil, respectively), being therefore the main mechanism of interfacial interaction. Different interfacial areas were produced by modifying agitation in the presence of silicone oil and dodecane. Under the same agitation rate, silicone oil formed higher interfacial areas than dodecane due to its lower interfacial tension (20 and 53 mN/m for silicone oil and dodecane, respectively). The inactivation rates for silicone oil and dodecane were 6.7 and 11.8 U/L·h at 400 rpm; 61 and 81 U/L·h at 600 rpm; and 138 and 143 U/L·h at 800 rpm, respectively, dodecane causing higher enzymatic inactivation at all agitation rates. In consequence, even at higher interfacial areas, enzyme inactivation in silicone oil was lower. As silicone oil had the lowest partition coefficient and led to lower inactivation rates of the enzyme, it was selected for the following experiments.

Fig. 6.6.10

- *Optimization of the parameters involved in the catalytic cycle*

The effects of the main factors involved in the catalytic cycle of MnP on the degradation of anthracene were studied (Eibes et al. 2007). Fig. 6.6.11 shows the degradation rate and

efficiency, in terms of milligrams of anthracene degraded per unit of enzyme inactivated, for experiments at different H_2O_2 addition rate, malonate concentration and pH control.

Regarding H_2O_2 addition rate, similar efficiencies were obtained at 1 and 5 μmol/L min, but the highest degradation rate was obtained at 5 μmol/L min. Higher addition rates decreased the efficiency due to the inactivation caused by H_2O_2 excess. A similar effect was observed for the concentration of the organic acid, being necessary to reach a compromise solution between enzyme inactivation and degradation efficiency. Next, pH control was considered because a pH increase was observed along the reaction, reaching values closer to 8 after 70 h of operation. The addition of malonic acid to control the pH at 4.5 was evaluated, since the concentration of sodium malonate was observed to decrease in the reactor. Efficiency was then increased 1.9-fold compared to the experiment with no pH control.

Fig. 6.6.11

- *Optimization of the mass transfer coefficients*

Two factors were considered to affect substrate transfer rate: fraction of silicone oil and agitation rate. Diffusion from the organic phase was favored by increasing both factors because they increase interfacial area (Eq. 6.6.2), but this may also affect enzymatic activity. Since both variables, solvent fraction and agitation rate, are likely to be co-dependent, a 2^2 experiment design was considered to optimize the efficiency of the system (Eibes et al. 2007). The ranges considered were 200-300 rpm and 10-30% of silicone oil (v:v) and the response surface corresponding to efficiency (EF) is represented by Eq. 6.6.4:

$$\eta = 0.152 + 0.026 \cdot \upsilon + 0.054 \cdot \sigma + 0.026 \cdot \upsilon \cdot \sigma \qquad \text{Eq. 6.6.4}$$

being η the efficiency of the system, υ the agitation rate and σ the fraction of silicone oil (all factors normalized).

The equation shows that efficiency was mainly dependent on the ratio of the organic and aqueous phases and higher fractions of silicone oil led to higher efficiency values. Both agitation and the combined effect had similar relative weights in the equation, both being positive. The highest efficiency was obtained at 300 rpm and 30% silicone oil, where a nearly complete oxidation was achieved after 56 h. Although different experiments were performed following the pathway of the steepest ascent, considering a golden section optimization protocol, none of them improved efficiency.

-*Modeling of the process*

Process modeling has to consider the two major aspects involved: i) mass transfer of anthracene and ii) enzymatic kinetics and thus the coefficients for each mechanism of the proposed model were evaluated. In order to quantify mass transfer coefficients, anthracene

balance in the aqueous phase in the absence of enzymatic reaction has to be considered, being the substrate concentration in the aqueous phase given by Eq. 6.6.5:

$$\ln\left(s^* - s_w\right) = \ln s^* - k_L a \cdot t \qquad \text{Eq. 6.6.5}$$

The substrate concentration was measured in the aqueous phase for a period of time, thus enabling to find the mass transfer coefficient (k_La) for each condition of agitation rate and fraction of solvent. Fig. 6.6.12 shows the experimental k_La values for the conditions evaluated, presenting a great increase in a short range of agitation rates (200-250 rpm), being more pronounced when low fractions of silicone oil were present. Although mass transfer coefficients were maximized at 250 rpm for all the evaluated fractions of silicone oil, the experimental results of anthracene degradation indicated that 300 rpm and 30% silicone oil are the optimal conditions.

The values of k_La were fitted to a surface (r^2 = 0.986) represented in Fig. 6.6.12 and thus related to the agitation rate (υ) and the fraction of silicone oil (σ) through an empiric correlation with five parameters (Eq. 6.6.6).

$$k_L a = b + c \cdot \sigma + d \cdot \left(0.5 + \frac{\arctan\left(\frac{(\upsilon - e)}{f}\right)}{\pi} \right) \qquad \text{Eq. 6.6.6}$$

Fig. 6.6.12

In order to obtain the catalytic coefficient, both balances in organic and in aqueous phase were considered. The enzymatic degradation of anthracene by MnP was considered as pseudo-first order kinetics with an autocatalytic effect due to the presence of the degradation products (Eq.6.6.7). Quinones, which are the main degradation products of PAHs, can act as electron carriers as described by Méndez-Paz et al (2005), thus accelerating the overall degradation.

$$r_S = \left(\alpha + \beta \cdot p\right) \cdot s \qquad \text{Eq. 6.6.7}$$

Including the kinetics onto the mass balance of anthracene in the aqueous phase and substituting in the organic phase balance, Eq. 6.6.8 is finally obtained which describes the behavior of anthracene in the organic phase (s_S) for each hydrodynamic condition:

$$\frac{ds_S}{dt} = -\frac{k_L a}{k_{sw}} \cdot \frac{V_w}{V_s} \cdot \left(s_S - \frac{k_L a \cdot s_S}{k_L a + \alpha + \beta \cdot \frac{s_{S0} - s_S}{\frac{V_w}{V_s} + k'_{sw}}} \right) \qquad \text{Eq. 6.6.8}$$

The partition coefficient of anthracene in silicone oil was previously determined (k_{sw}=5012, Fig. 6.6.10), the mass transfer coefficient is given by the empiric correlation previously obtained (Eq. 6.6.6) and the kinetic constants α and β were estimated by using the method of least squares from the experiments at different agitation rates and fractions of silicone oil. The experimental data of anthracene degraded along the reactor operation and the fitted model for four different hydrodynamic conditions are plotted in Fig. 6.6.13. The highest degradation rate was obtained at 300 rpm and 30% silicone oil (v/v), oxidizing 90% of anthracene present in the organic phase after 56 h. Taking into account the efficiency function (η), the path of the steepest ascent for agitation was 0.55·s +1, and for silicone oil volume 0.84·s + 1. The final experiment was carried out considering a step s = 2.4, which meant 365 rpm and 50% (v/v) silicone oil. The degradation rate obtained, 1.29 mg/L_R·h, was 1.36-fold lower than that of the optimal conditions. The activity loss was slightly higher, 7.8 U/L_R·h and the efficiency, 0.166 mg/U, represented 73% of that obtained at 300 rpm and 30% (v/v) silicone oil.

Fig. 6.6.13

6.6.6. Conclusions and perspectives

Ligninolytic fungi are able to oxidize dyes and other soluble compounds, achieving high degrees of degradation. The main problem of fungal treatments is the requirement of sterile conditions and high HRT (12-24 h). On the contrary, enzyme reactors present several advantages, such as the possibility to work at much lower HRT, due to the fast kinetics of the enzymatic reaction. Thus, high conversions and degradation efficiencies are expected.

The application of enzymes in continuous processes can be performed in non sterile conditions, and allows working with high loads of enzymes in the reactor, to maintain a stable enzyme activity, to reduce the risk of product inhibition and to minimize costs, energy and waste products. Enzyme membrane reactors are proposed as a promising technology, as they are very easy to operate and control, and the degradation process can be carried out continuously during more than 20 days with no cleaning or membrane change. Furthermore, the enzymatic reactors are quite versatile, since there is a wide variety of membrane shapes, materials and modules commercially available. The selection process must mainly consider the characteristics of the effluent and the enzyme molecular weight.

The addition of a second immiscible phase for the enzymatic degradation of poorly-soluble compounds provides several advantages, such as a simpler operation, mainly due to the easy

recovery of the solvent depleted of substrate and its reuse in subsequent operations. Mass transfer could be considered *a priori* as a limitation for this system and to be the determinant of lower efficiencies. However, the selection of the appropriate solvent, as well as the determination of the adequate conditions which lead to the maximum efficiency allowed us to obtain unprecedented degradation rates in enzyme reactors.

Enzyme reactors appear as an attractive technology for the degradation of hardly biodegradable compounds, which has to be further optimized and tested at higher scale. Increasing the enzyme efficiency (mass of pollutants/units of enzyme deactivated), improving the reliability in continuous systems and implementing control strategies for a more stable operation, are some of the goals to be attained in the near future.

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Tables

Table 6.6.1. Main features of fungal and enzymatic reactors for environmental purposes

Fungal reactors	Enzymatic reactors
Autocatalytic	Need of cofactors and mediators
Mineralization in most cases	Biotransformation to intermediates
Inhibition by pollutants	Higher tolerance to pollutants
Longer treatment time required (d)	Shorter treatment time (h)
Difficult control	Easier and simpler control
Sterile conditions	Non-sterile conditions
Higher background experience	Cost of enzymes

Table 6.6.2. *In vitro* degradation of organopollutants by oxidative enzymes

Organopollutant	Enzyme	Source	Reference
Trinitrotoluene	MnP	*Nematoloma forwardii*	Scheibner and Hofrichter (1998)
	MnP	*Phlebia radiata*	van Aken et al. (1999)
Organochlorines	LiP, MnP	*Phanerochaete chrysosporium*	Valli et al. (1992)
Polychlorinated biphenyls	Lac	*Trametes versicolor*	Dec and Bollag (1995); Roper et al. (1995)
Bleach-plant effluent	MnP	*P. chrysosporium*	Jaspers et al.(1994)
	Lac	*T. versicolor*	Archibald et al. (1990)
Synthetic dyes	LiP	*P. chrysosporium*	Cripps et al. (1990); Ollikka et al. (1993)
	MnP	*B. adusta* *P. chrysosporium*	Heinfling et al. (1998)
	MnP	*B. sp BOS55*	Mielgo et al. (2003), López et al. (2004)
	HRP*	Commercial	Bhunia et al. (2001)
Polycyclic aromatic hydrocarbons (PAHs)	LiP	*P. chrysosporium*	Bumpus (1989); Hammel et al. (1986)
	LiP	*N. forwardii*	Günther et al. (1998)
	MnP	*P. chrysosporium*	Bogan and Lamar (1995); Bogan et al. (1996)
	MnP	*N. forwardii*	Günther et al. (1998)
	Lac	*T. versicolor*	Johannes et al. (1996); Majcherczyk et al. (1998)
	Lac	*Coriolopsis gallica*	Pickard et al. (1999)

* Horseradish peroxidase

Table 6.6.3. Possible configurations of enzymatic reactors treating highly or poorly soluble compounds

Substrate	Key factor	Alternative	Configuration	References
Highly soluble (dyes, phenols, nitroaromatic compounds…)	Retention of the enzyme	Enzyme recovery by means of a membrane	Monophasic reactor coupled to a membrane	Basheer et al. (1993); López et al. (2002); Pasta et al. (1999)
		Immobilization of the enzyme	Monophasic reactor with enzyme immobilized onto a support	Grabski et al. (1998); Hublik and Schinner (2000); Krastanov (2000); Sasaki et al. (2001)
Poorly soluble (PAHs, pesticides, polychlorinated byphenils…)	Availability of the pollutant	Addition of a surfactant	Emulsion reactor with free enzyme	Bogan and Lamar (1995); Moen and Hammel (1994); Steffen et al. (2003)
		Addition of a miscible solvent	Monophasic reactor with free enzyme	Eibes et al. (2006); Field et al. (1996); Rodakiewicz-Nowak and Jarosz-Wilkolazka (2007)
		Addition of an immiscible solvent	Biphasic reactor with free enzyme	Eibes et al. (2007)

Figure Captions

Fig. 6.6.1. Scheme of the catalytic cycle of MnP

Fig. 6.6.2. Schematic diagrams of different configurations of enzymatic membrane reactors: (A) stirred tank reactor with enzyme immobilized or retained by a membrane; (B) stirred tank reactor coupled to ultrafiltration membrane

Fig. 6.6.3**.** Schematic diagrams of different configurations of immobilized enzyme reactors: (A) stirred tank reactors , (B) fixed bed reactor and (C) fluidized bed reactor

Fig. 6.6.4. Scheme of the enzymatic biphasic reactor for the degradation of poorly soluble compounds

Fig. 6.6.5. Flow chart of the process to obtain the model of enzymatic reactors

Fig. 6.6.6. Orange II decolorization in batch reactors after 10 min (grey bars) or 60 min (white bars) at different strategies of H_2O_2 addition (A), Mn^{2+} concentration (B), pH (C) and initial MnP activity (D). Basic conditions: MnP 200 U/L, H_2O_2 addition rate 1.36 μmol/min, Mn^{2+} 33 μM and pH 4.5

Fig. 6.6.7. Orange II decolorization (white bars) and efficiency (grey bars) in the enzymatic membrane reactor at different strategies of MnP addition. Continuous addition of MnP was performed at 50, 25 and 15 μmol H_2O_2/L·min

Fig. 6.6.8. Experimental (○) and simulated data (—) of Orange II concentration in a continuous experiment at different Orange II concentrations in the influent: days 0-4: 100 mg/L; days 4-6: 150 mg/L; days 6-8: 200 mg/L; days 8-10: 250 mg/L; days 10-12: 300 mg/L and days 12-14: 100 mg/L. Concentrations in the influent: malonate 1 mM, Mn^{2+} 33 μM.; HRT 60 min; H_2O_2 addition rate 15 μmol/L min; pH 4.5

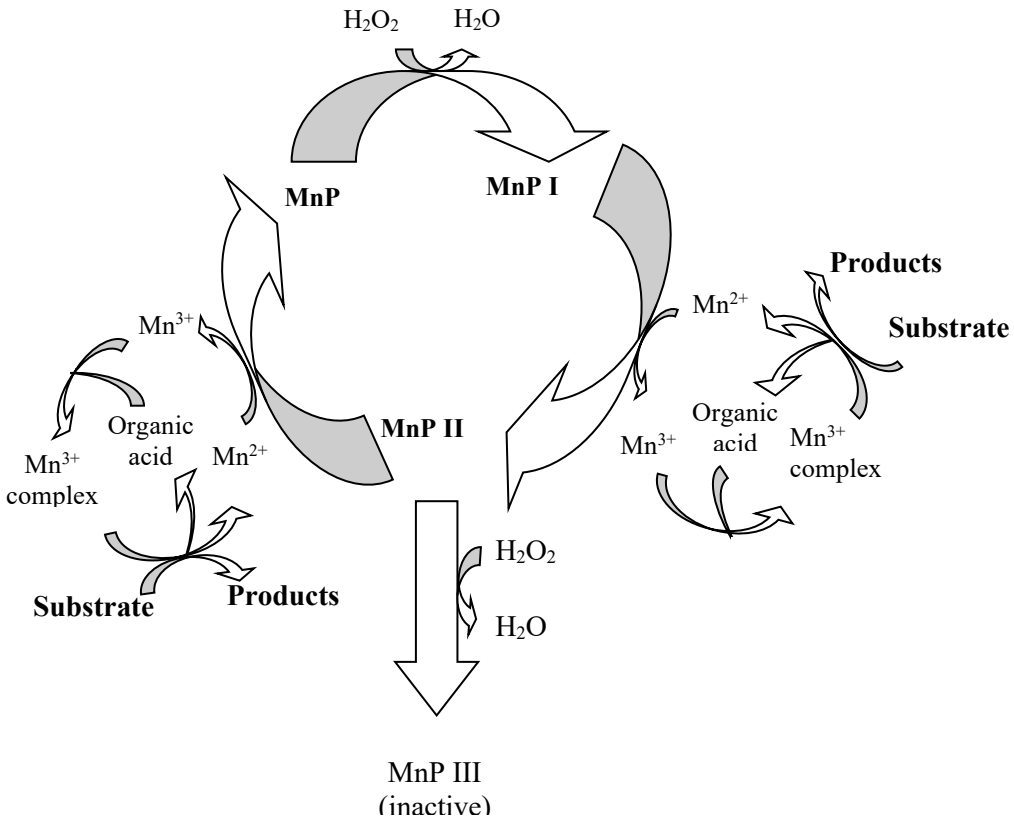
Fig. 6.6.9**.** Experimental (○) and simulated data (—) of Orange II concentration and dissolved oxygen (– · –) in a continuous process in unsteady state conditions. MnP activity 200 U/L; Concentrations in the influent: Orange II 100 mg/L, malonate 1 mM, Mn^{2+} 33 μM. Overload at 1 d: Orange II 200 mg/L; overload at 2 d: HRT 30 min; overload at 3 d: H_2O_2 7.5 μmol/L min

Fig. 6.6.10. Values of log K_{SW} obtained for 15 different solvents. Silicone oil and dodecane were selected as representative compounds for low and medium partition coefficients

Fig. 6.6.11. Degradation rate of anthracene (white bars) and efficiency (grey bars) at different hydrogen peroxide addition rate, malonate concentration and control of pH (*) by addition of malonic acid (250 mM)

Fig. 6.6.12. Values of k_La obtained for experiments at different agitation rates and volume of silicone oil

Fig. 6.6.13. Experimental data and fitted model (—) of anthracene degradation in batch experiments at different conditions of agitation speed and silicone oil volume: □ 200 rpm-10%; ■ 250 rpm-20%; ● 200 rpm-30%; ○ 300 rpm-30%



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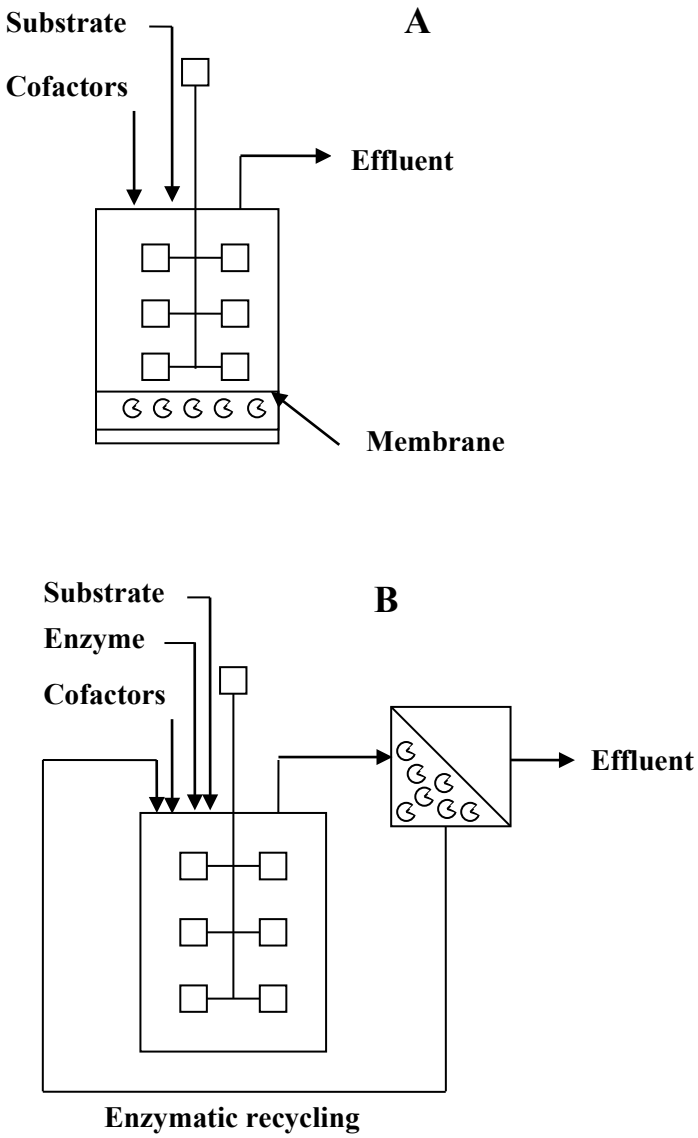

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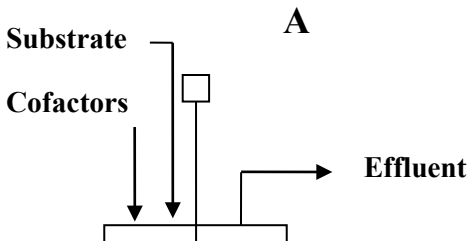

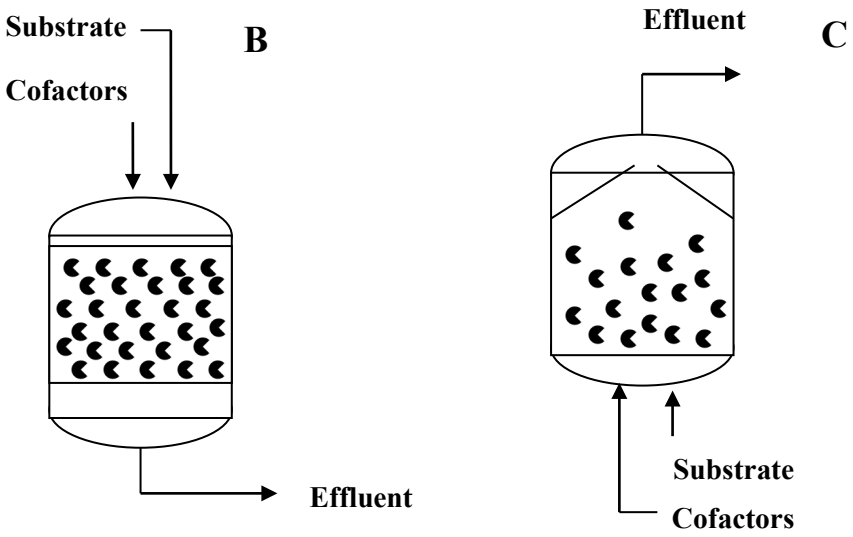

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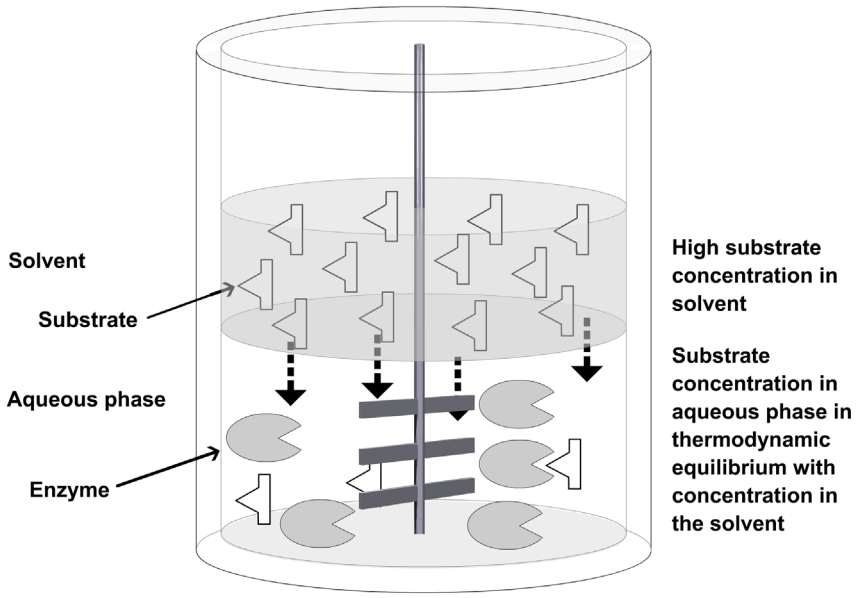

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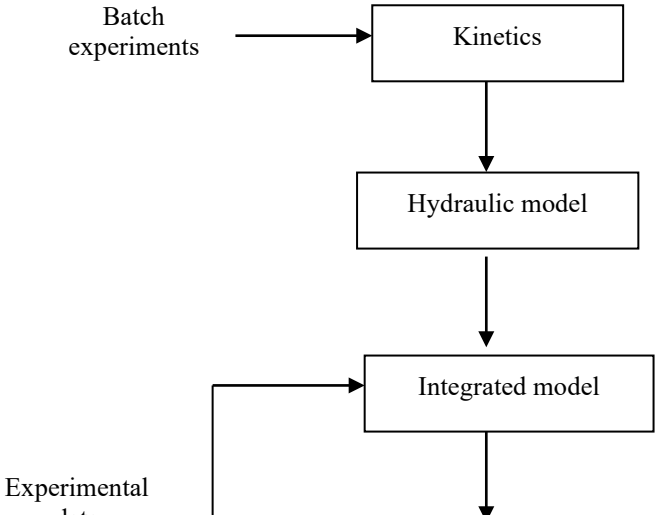

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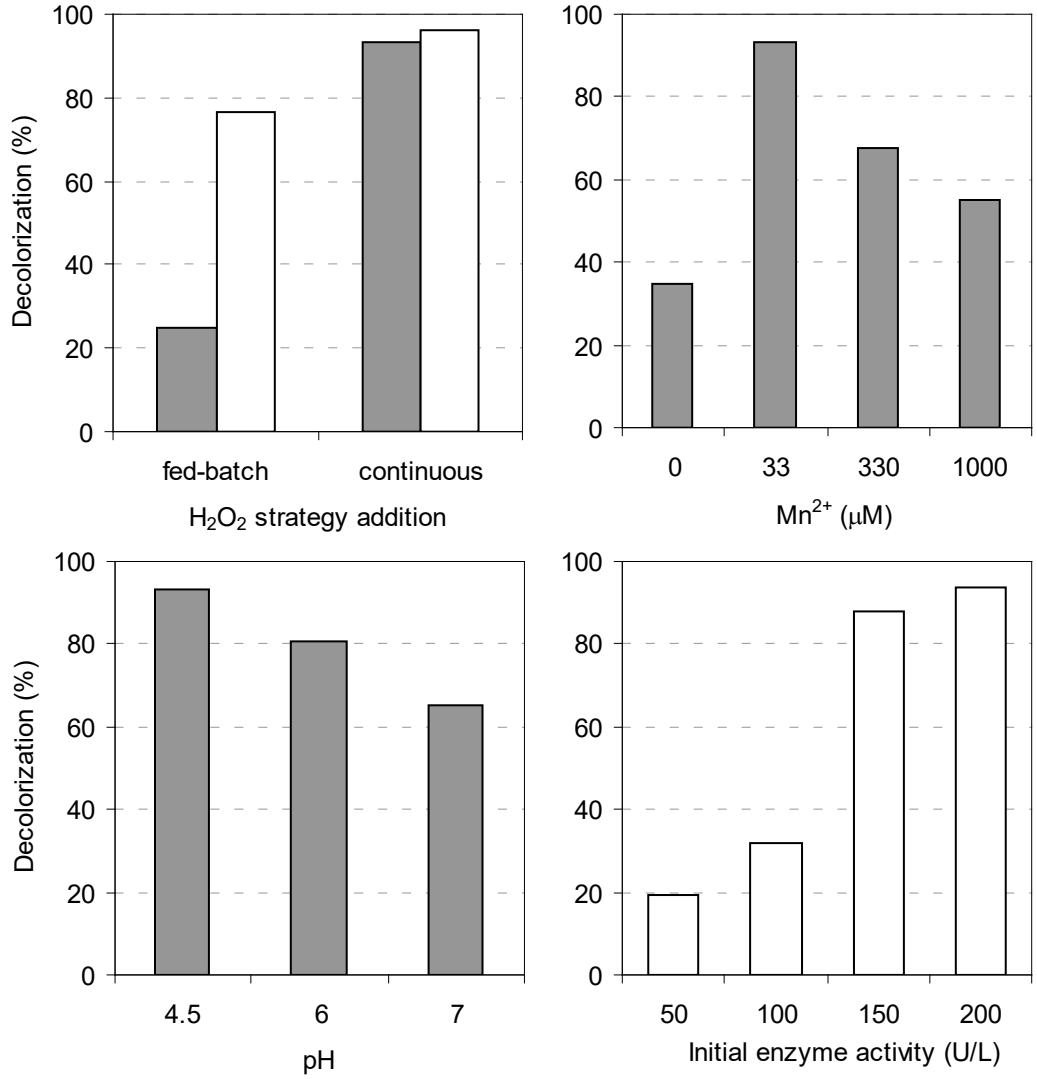

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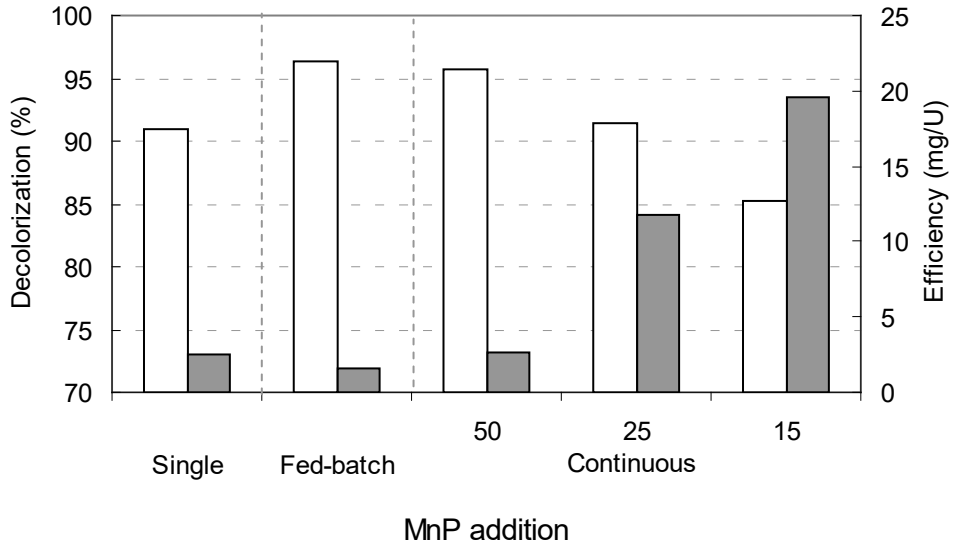

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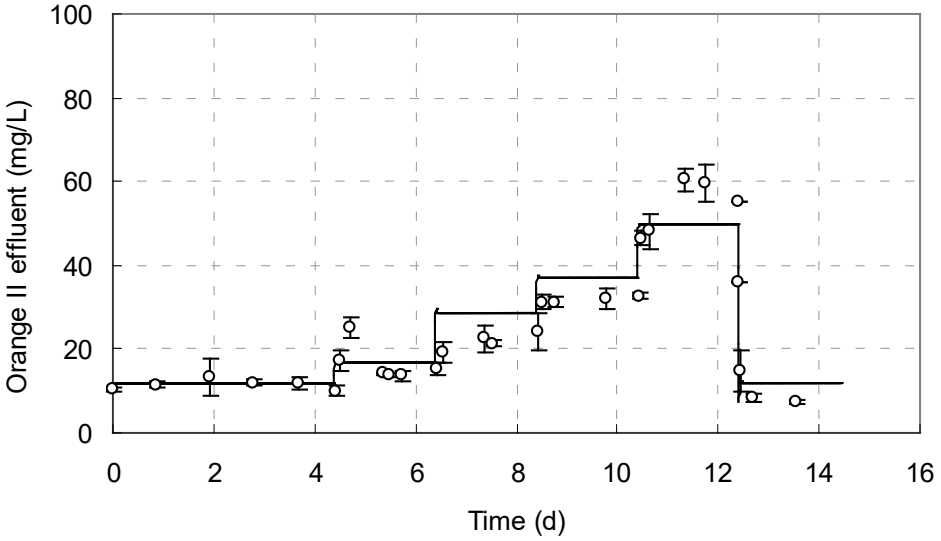

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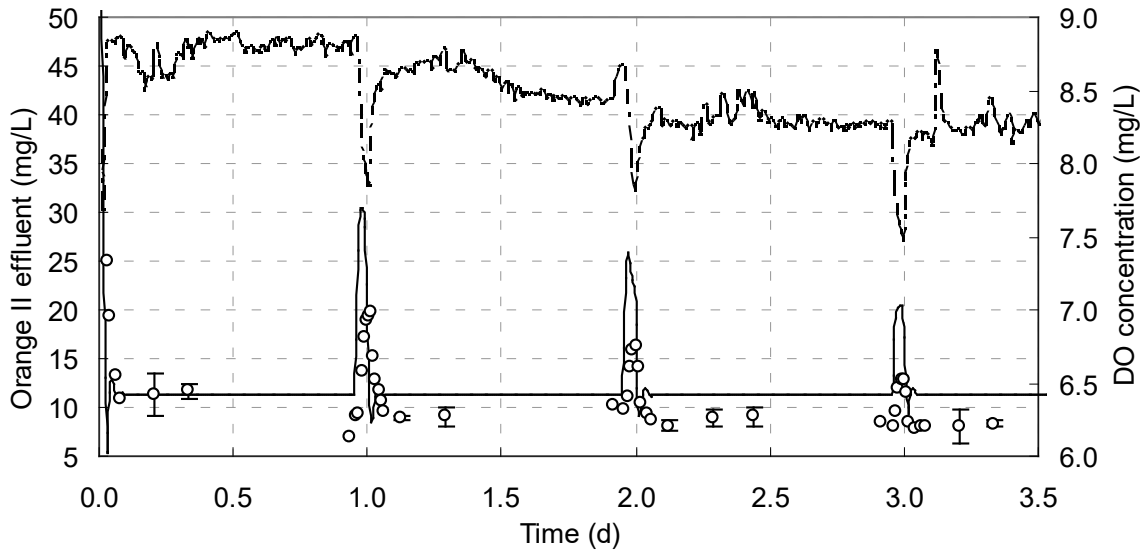

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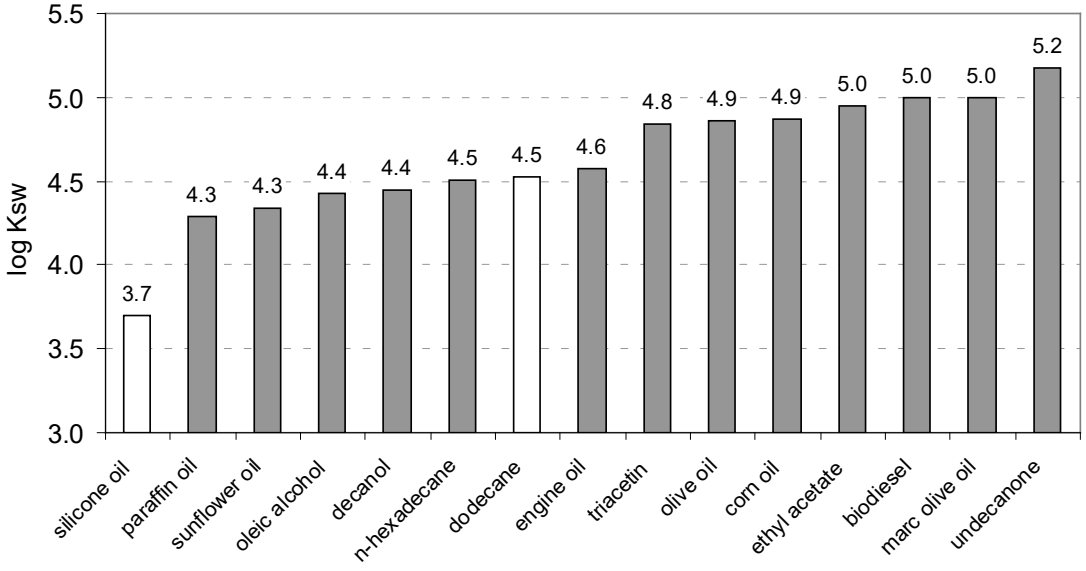

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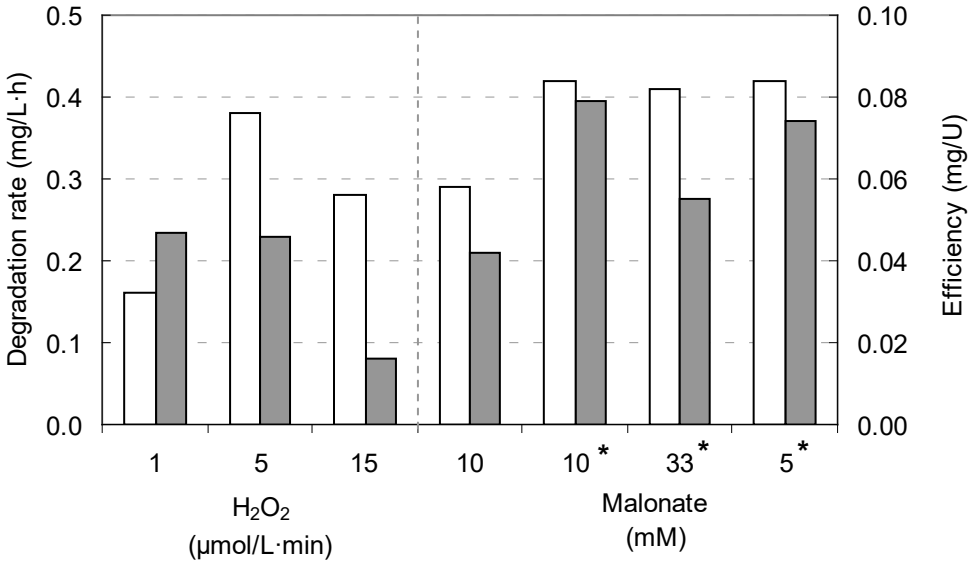

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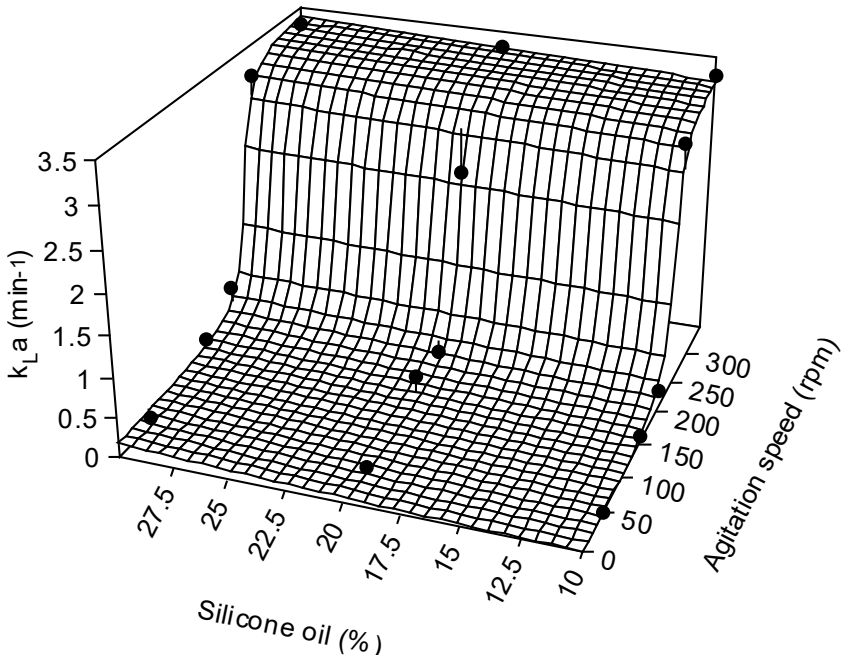

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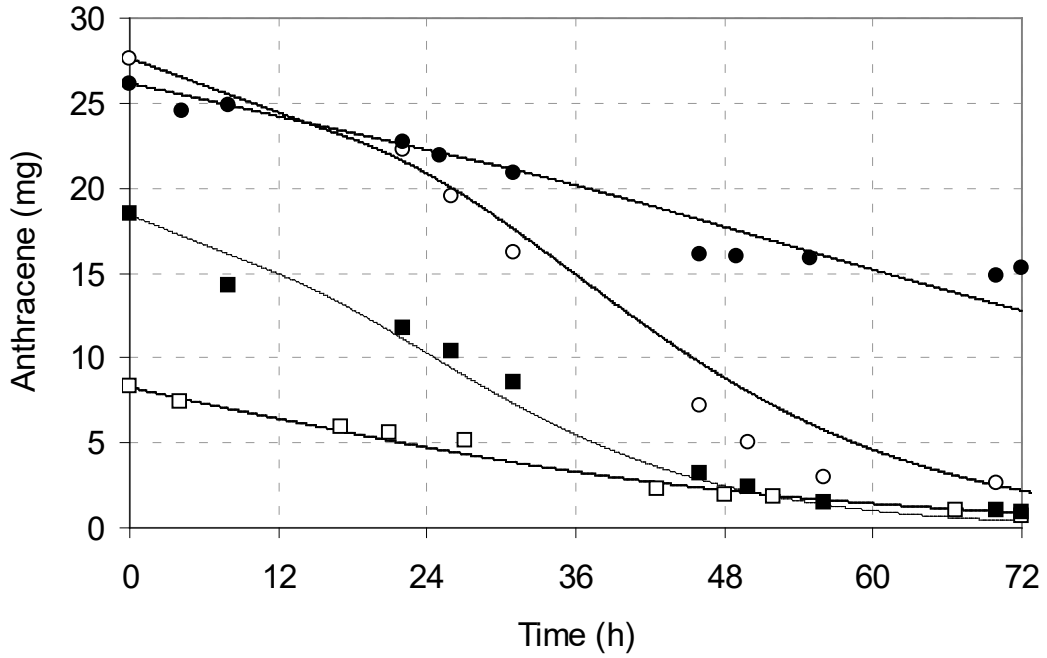

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Index Keywords