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## Removal of Endocrine Disruptors in Waste Waters by Means of Bioreactors

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### 1. Introduction

The presence of Endocrine Disrupting Chemicals (EDCs) represents an area of concern in the environmental field. An EDC is defined as “an exogenous substance that causes adverse health effects in an intact organism, or its progeny, in consequence to the induced changes in endocrine functions” (EU Commission, 1996). A large number of chemical compounds have been recognized as EDCs. Among these, natural and synthetic steroid hormones, phytoestrogens, alkylphenols, phthalates, pesticides, surfactants and polychlorinated biphenyls (Soto et al., 1995; Jobling et al., 1995; Routledge & Sumpter, 1997). EDCs are not defined on the basis of their chemical nature, but by their biological effects. They exhibit agonistic or antagonistic properties depending on the kind of interaction with the receptors. As estrogenic receptors have similar structure between different animals, including humans, EDCs can affect the endocrine functions of many living species. The main mechanisms through which they interfere with the endocrine system are: i) the simulation of the activities of physiological hormones, thereby participating in the same reactions and causing the same effects; ii) the inactivation, with competitive action, of hormone receptors and, consequently, the neutralisation of their activity; iii) the interference with the synthesis, transport, metabolism and secretion of natural hormones, altering their physiological concentrations and therefore their corresponding endocrine functions.

EDCs enter the environment from a variety of sources, such as effluent discharge pipes, agricultural runoff, landfills, atmospheric deposition and aerosols (Campbell et al., 2006). In particular aquatic ecosystems have been studied for the effect of wastewater treatment plant (WWTP) effluents, which are continuously discharged to the receiving water bodies (Jobling et al., 1998; Routledge et al., 1998; Tilton et al., 2002). Due to their incomplete removal during the waste treatment process, synthetic and natural estrogens are considered as the major responsible for the estrogenic activity associated with WWTP effluents (Gutendorf & Westendorf, 2001). So natural steroid hormones and the synthetic ethynylestradiol, alkylphenols, bisphenol A and phthalates are EDCs identified in sewage effluents (Desbrow

et al., 1998; Körner et al., 2000; Lye et al., 1999; Spengler et al., 2001). In consequence reproductive disorders and feminization of fish populations are alarming signs of endocrine disruption. Adverse effects have been also observed in humans, such as the increasing number of endocrine responsive cancers and the decreasing reproductive fitness of men (Daston et al., 1997).

Owing to these noxious effects remediation processes are requested in order to remove these pollutants. Conventional approaches (e.g. landfilling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the formation of toxic intermediates (Dua et al., 2002; Spain et al., 2000). Thus, biological decontamination methods are preferable because whole microorganisms or enzymes degrade numerous environmental pollutants without producing toxic intermediates (Furukawa, 2003; Pieper & Reineke, 2000).

To reduce the harmful effects due the EDCs presence in aqueous systems we will report here in the following some our results obtained with a biotechnological approach based on their enzymatic bioremediation as an alternative technology to the classical membrane processes.

In particular our attention will be focused on the bioremediation of Bisphenol A (BPA) and some of its congeners, such as Bisphenol B (BPB), Bisphenol F (BPF) and Tetrachlorobisphenol A (TCBPA), taken as model of EDCs of phenolic origin, and of Dimethylphthalate (DMP), taken as model of phthalates.

Bisphenol A (BPA) is an industrial raw material for polycarbonate and epoxy resins, unsaturated polyester-styrene resins and flame retardants. The final products are used as coatings on cans, powder paints, additives in thermal paper and in dental fillings, and as antioxidants in plastics. Several studies demonstrated that BPA is an EDC. It mimics or interferes with the action of endogenous hormones (Gaido et al. 1997; Kim et al. 2001; Krishnan et al. 1993; Matthews et al. 2001; Synder et al. 2003; Tinwell et al. 2000), causing adverse alterations in reproductive and developmental processes as well as metabolic disorders. Like BPA, also BPB, BPF and TCBPA are used as materials for epoxy resins and polycarbonates lining large food containers and water pipes. Coatings can also be made from mixtures of BPA congeners. All show estrogenic activity, but the activities varied markedly from compound to compound (Kitamura et al., 2005).

Phthalates are plasticizers used in polymer industry to improve their flexibility, workability and handling properties. They are used in films, in tubing, in liners of bulk liquid holding tanks or in conveyor belt material (Kirkpatrick et al., 1989). Phthalates, as bisphenols, are not bound chemically in the plastics and can consequently migrate into food that comes into contact. The presence of phthalates in packaging materials and their migration into packaged foods have been confirmed by a number of authors (Castle et al., 1988; Nerin et al., 1993; Page & Lacroix, 1992; Petersen, 1991).

This chapter has been written in order to promote the technology of waste bioremediation by means of bioreactors, in particular with our innovative process based on non-isothermal bioreactors. To this aim some of our published results have been selected and discussed. New perspectives will be also indicated.

## 2. Bioremediation versus remediation

For problems of water treatment in ecosystems the traditional membrane-based processes are not useful since they alter the life conditions. Ultrafiltration and reverse osmosis, for example, allow endocrine disruptors removal, but since the filtrate consists in pure water its

intake in the ecosystem alters the concentrations of salts and bioelements necessary for the life. On the contrary, the selective removal of endocrine disruptors by enzyme treatment (bioremediation) appears more suitable, since the treatment is effective only towards the target harmful chemical remaining unchanged the other components present in the water. For this reason to bioremediate polluted waters in small ecosystems we propose, in place of reactors, the use of bioreactors, i.e. reactors where a biological element is operating. In particular we have suggested the use of non-isothermal bioreactors (Attanasio et al., 2005; Diano et al., 2007; Durante et al., 2004; Georgieva et al., 2008; Georgieva et al., 2010; Ignatova et al., 2009; Mita et al., 2010). With these apparatuses we have found that 1°C of temperature difference across the catalytic membrane increases the enzyme reaction rate from 30% to 80% in comparison to the same reaction rate measured under comparable isothermal conditions. The increase of enzyme activity has been found to depend upon: i) the substrate concentration, ii) the average temperature in the bioreactor, and iii) the temperature difference across the catalytic membrane. The main advantage on using non-isothermal bioreactors is the reduction in the treatment times that is proportional to the size of the temperature difference applied across the catalytic membrane.

### 3. The catalytic systems

Laccase from *Trametes Versicolor* and tyrosinase from mushroom have been employed to biodegrade the phenol compounds, whereas Lipase from *Candida Rugosa* for removing the phthalates.

Laccase and tyrosinase were immobilized on polyacrylonitrile (PAN) beads employed into a fluidized bed bioreactor working under isothermal conditions.

Lipase was immobilized on a Polypropylene (PP) membrane from GE Osmonics (GE Labstore-Osmonics, Minnetonka, Minnesota), with a thickness of 150 µm and a nominal pore diameter of 0.22 µm. When made catalytic, the membrane was employed in a planar membrane reactor working under isothermal or non-isothermal conditions.

#### 3.1 Carrier functionalitaton

##### 3.1.1 PAN bead preparation and activation

PAN powder (18 g), LiNO<sub>3</sub> (1 g) and glycerin (3 g) were dissolved in 78 mL of dimethylformamide. The homogenized mixture was pipetted and precipitated in water. The beads obtained were water-washed and immersed for 24 hr in a 30% (v/v) glycerin aqueous solution. After this step the beads were dried in an oven at 70°C for a time sufficient to reach a constant weight.

20 cm<sup>3</sup> (12 g) of PAN beads were activated at 50°C for 60 min by treatment with 15% (w/v) NaOH aqueous solution. After washing in distilled water, the beads were treated with a 10% (v/v) aqueous solution of 1,2-diaminoethane (15 mL) at room temperature for 60 min. Then the beads were washed once more in distilled water.

##### 3.1.2 Polypropylene membrane activation

Polypropylene is a non-polar material that lacks reactive groups for enzyme immobilization. Consequently, functional groups have been created on the PP membrane by means of a plasma reactor. Plasma was powered by a mixture of acrylic acid (Sigma-Aldrich, 99%) and He according to the ratio of 3:20 sccm (standard cubic centimetres per minute). The experimental conditions (power = 80W, pressure = 400 mTorr, time = 10min) gave rise to a

very stable coating on the membrane, showing the following abundance of reactive groups:  $\text{COOH} < \text{CO} < \text{COH} < \text{CC}$ .

### **3.2 Immobilization techniques**

#### **3.2.1 Laccase immobilization**

Laccase immobilization was carried out through a diazotation process, involving the phenolic group of tyrosine residues far from the catalytic site. The PAN beads were treated at room temperature for 1 hr with a 2.5% (v/v) glutaraldehyde (GA) aqueous solution (15 mL). GA was used as the coupling agent. After washing at room temperature with double distilled water, the beads were treated, at room temperature for 90 min, with a 2% (w/v) Phenylendiamine (PDA) solution in 0.1 M sodium carbonate buffer, pH 9.0. PDA was used to obtain aminoaryl derivatives on the supports. Once water-washed, the beads were treated at 0°C for 40 min with an aqueous solution containing 2M HCl and 4%  $\text{NaNO}_2$ . At the end of this treatment, the beads were washed at room temperature in 0.1 M citrate buffer solution, pH 5.0, and then treated at 4 °C for 16 hr with the same buffer solution containing laccase at concentration of 3 mg/mL. At the end, in order to remove the unbound enzymes, the beads were washed in 0.1 M citrate buffer solution, pH 5.0.

The amount of immobilized enzyme was determined by measuring, through the Lowry protein assay method (Lowry et al. 1951), the initial and final concentrations of protein in the solution used for the immobilization and taking into account also the protein amount found in the washing solutions. Under the experimental conditions reported above, and using 12g (20 cm<sup>3</sup>) of activated PAN beads, the amount of immobilized laccase resulted to be  $3.56 \pm 0.40$  mg. When not used, the beads were stored at 4°C in 0.1 M citrate buffer pH 5.0.

#### **3.2.2 Tyrosinase immobilization**

Tyrosinase was immobilized by using glutaraldehyde in a condensation process involving its  $\text{NH}_2$ -groups. For this purpose, the PAN beads were treated at room temperature for 1 hr with a 2.5% (v/v) GA aqueous solution (15 mL). After washing at room temperature, the beads were incubated at 4°C for 16 hr in a 0.1M phosphate buffer solution, pH 6.5, containing tyrosinase at concentration of 3 mg/mL. At the end of this step, in order to remove the unbound enzyme, the beads were washed in the phosphate buffer solution. The amount of immobilized tyrosinase, measured by Lowry protein assay method, resulted to be  $3.21 \pm 0.60$  mg. When not used, the beads were stored at 4°C in 0.1M phosphate buffer pH 6.5.

#### **3.2.3 Lipase immobilization**

Lipase was immobilized on the activated PP membrane through a diazotation process involving the phenolic groups of tyrosine residues. This procedure was chosen because the tyrosine residues are far from the catalytic site. To generate aminoaryl derivatives on the plasma activated PP membranes, the membranes were treated for 90 min with a 2% (w/v) PDA aqueous solution of 0.1M sodium carbonate buffer, pH 9.0. Later, the membranes were washed with double distilled water. The obtained aminoaryl derivatives were treated for 40 min at 0°C with an aqueous solution containing 4% (w/v)  $\text{NaNO}_2$  and 2M HCl, in a ratio of 1:5. At the end of this treatment the membranes were washed at room temperature in a buffer solution (0.1M phosphate, pH 7.0), and then treated for 16 h at 4°C with 30mL of the same buffer solution containing 20mg/mL of enzyme power. After this step the membranes were washed with 0.1M phosphate buffer, pH 7.0, to remove the material not bound. Under



the experimental conditions above reported, the amount of immobilized protein on PP membranes, measured by Lowry protein assay method, was  $3.26 \pm 0.2$  mg. When not used, the membranes were stored at  $4^{\circ}\text{C}$  in 0.1M phosphate buffer pH 7.0.

## 4. The bioreactors

### 4.1 The fluidized bed bioreactor

A fluidized bed reactor (Figure 1) was used for the continuous removal of the single bisphenols from the buffered solution by laccase or tyrosinase immobilized on PAN beads. The bed reactor was constituted by a polystyrene pipe (1.7 cm inner diameter, 20 cm length) packed with 12 g ( $20\text{cm}^3$ ) of PAN catalytic beads. The bioreactor was fed with 40 mL of bisphenols substrate solution, at concentration 1mM and thermostated at  $25^{\circ}\text{C}$ , recirculating at a flow rate of 140 mL/min by means of a peristaltic pump.

The amount of enzymatic degradation was calculated after 90 min of enzyme treatment considering the initial and final bisphenols concentration in the reaction solution.

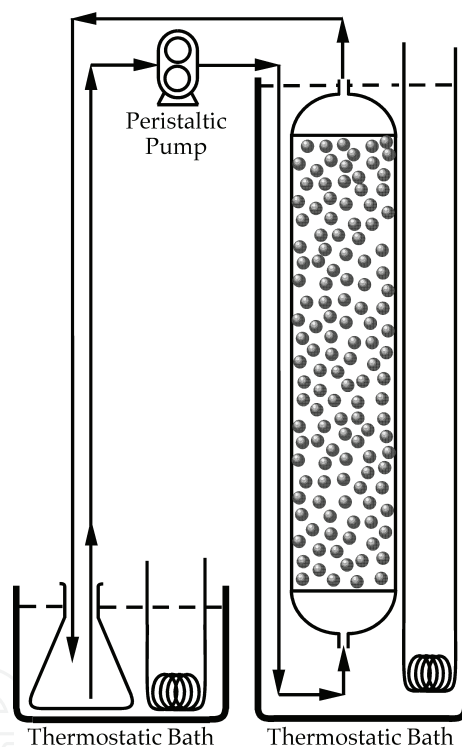


Fig. 1. Schematic (not to scale) representation of the fluidized bed bioreactor.

### 4.2 The planar membrane bioreactor

The bioreactor (Figure 2a) consists of two metallic flanges in each of which it is bored a shallow cylindrical cavity, 70 mm in diameter and 2.5 mm depth, constituting the working volume filled with the aqueous solutions containing BPA. The catalytic membrane is clamped between the two flanges so as to separate and, at the same time, to connect the solutions filling the half-cells. Solutions are circulated in each half-cell by means of two peristaltic pumps through hydraulic circuits starting and ending in a common glass container. By means of independent thermostats, the two half-cells are maintained at predetermined temperatures. Thermocouples, placed 1.5 mm away from the membrane

surfaces, measure the local temperature of the solutions in each half-cell. These measures allow the calculation of the temperature profile into the whole bioreactor and across the catalytic membrane.

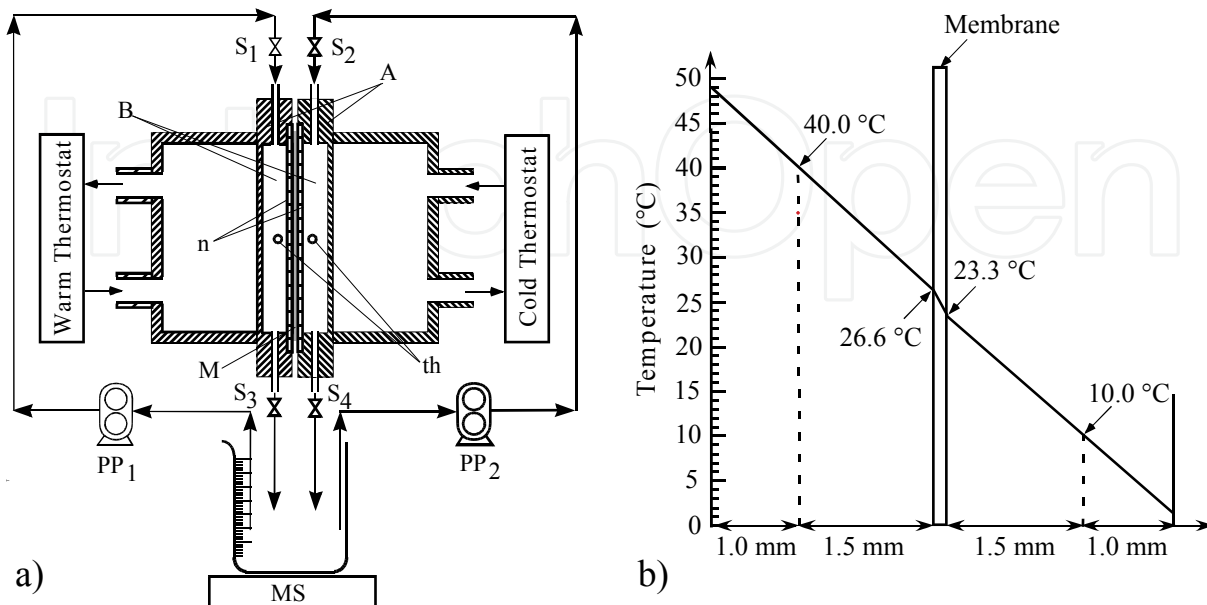


Fig. 2. a) Schematic representation of the planar bioreactor. Half-cells (A); internal working volumes (B); membrane (M); supporting nets (n); thermocouples (th); stopcocks (Si); thermostatic magnetic stirrer (MS); peristaltic pumps (PPi); b) Temperature profile in a non-isothermal bioreactor under the following experimental conditions:  $\Delta T = 30^\circ\text{C}$ ,  $T_{av} = 25^\circ\text{C}$ .

To estimate the real effects of temperature gradients on the activity of immobilized enzymes, the actual temperatures on the surfaces of the catalytic membrane ( $T_w^*$  and  $T_c^*$ ) must be known. The subscripts "w" and "c" stay for warm and cold side, respectively. Being impossible to measure the temperatures on each membrane face, these were calculated from those measured at the position of the thermocouples ( $T_w$  and  $T_c$ ), because the solution motion in the two half-cells was laminar. Indeed, in each half-cell the solution motion is constrained by two fins with rounded tips, at a flow rate of  $3.5 \text{ mL min}^{-1}$ . Under these conditions, the Reynolds number  $Re$  is lower than  $Re_{crit}$ , being  $Re$  lower than 10 (Diano et al., 2000). It follows that heat propagation through the bioreactor occurs by conduction between isothermal liquid planes perpendicular to the direction of the heat flow. By knowing the thermal conductivities and thickness of both filling solutions and membrane (Lide 1990; Touloukian 1970), it is possible to calculate the temperatures on the membrane surfaces by means of the heat flux continuity principle. It was found that the correlation between the temperatures read at the thermocouple positions,  $T$ , and the ones on the surfaces of the catalytic membranes,  $T^*$ , is given by:

$$\begin{cases} T_w^* = T_w - a\Delta T \\ T_c^* = T_c + b\Delta T \end{cases} \quad (1)$$

where  $a$  and  $b$  are numerical constants. Being our system symmetric,  $a=b$  and, hence

$$\Delta T^* = (1-2a) \Delta T = \text{const } \Delta T \quad (2)$$

$$T_{av} = \frac{T_w + T_c}{2} = T_{av}^* = \frac{T_w^* + T_c^*}{2} \quad (3)$$

$T_{av}$  and  $T_{av}^*$  being the average temperatures of the bioreactor and membrane, respectively. With the PP membrane, we have found  $a = b = 0.445$  and therefore  $\Delta T^* = 0.11 \Delta T$ . It follows that in non-isothermal experiments  $T_w^* < T_w$ ;  $T_c^* > T_c$ ; and  $\Delta T^* < \Delta T$ . Figure 2b shows the actual temperature profile in the bioreactor, when  $T_w = 40^\circ\text{C}$  and  $T_c = 10^\circ\text{C}$ , i.e. under the conditions  $\Delta T = 30^\circ\text{C}$  and  $T_{av} = 25^\circ\text{C}$ .

The functioning of this bioreactor is based on the application of the process of thermodialysis (Mita et al., 1992; Gaeta et al., 1992; Diano et al., 2000). Thermodialysis is the selective matter transport across a hydrophobic porous membrane separating two thermal solutions maintained at different temperatures. The driving force is the differential radiation pressure associated to the heat flux acting on the solvent and on the solute particles confined in the membrane pores. Each pore constitutes a microscopic Soret cell into which a modified thermal diffusion occurs, the modifications being on the water structure owing to its interaction with the pore walls.

When all fluxes (water and solutes) are allowed, as in Figure 3 where is illustrate the case of a two components solution, three matter fluxes are observed: a macroscopic volume flux,  $J_{vol}$ , from the warm to the cold side of the reactor; a drag solute flux,  $J_{S, drag}$ , associated to the volume flux, and a thermodiffusive solute flux, generally from the cold to the warm side.

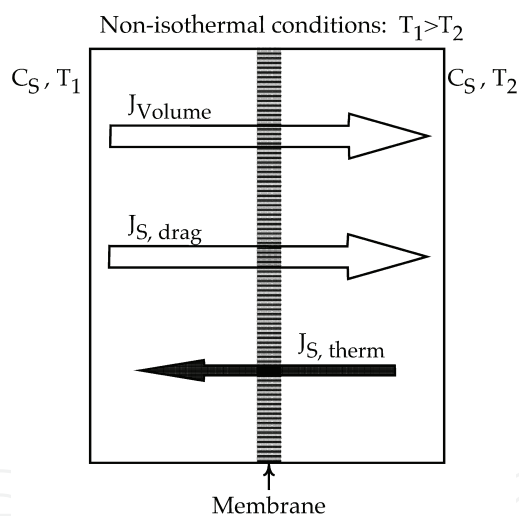


Fig. 3. Water and solutes fluxes

The expressions for each of the three fluxes are:

$$J_{vol} = \frac{\text{cm}^3}{\text{cm}^2 \text{s}} = D_{H_2O}^* \frac{\Delta T}{\Delta x} \quad (4)$$

$$J_{S, drag} = \frac{\text{mol}}{\text{cm}^2 \text{s}} = \sigma J_{vol} C_s \quad (5)$$

$$J_{S, th} = \frac{\text{mol}}{\text{cm}^2 \text{s}} = D_{th}^* C_s \frac{\Delta T}{\Delta x} \quad (6)$$



where,  $\Delta T$  is the temperature difference measured in the two half cell,  $\Delta x$  is the membrane thickness,  $C_s$  is the solute concentration expressed in moles  $\text{cm}^{-3}$ ,  $\sigma$  is a Staverman coefficient related to selectivity of the membrane,  $D_{H_2O}^*$  and  $D_{th}^*$  are the modified thermal diffusion coefficients, in  $\text{cm}^2 \text{s}^{-1} \text{K}^{-1}$ , for water and solute, respectively. If the solute is an appropriate pollutant, the enzymes immobilized on the membrane in the unit of time will encounter a number of substrate molecules higher than that encountered under the isothermal condition, where alone isothermal diffusion occurs, so that the enzyme reaction rate in the former case is increased in respect to the latter case in a manner proportional to the size of the temperature gradient.

## 5. Bioremediation quantification

The quantification of pollutant removal, and hence the enzyme reaction rate, is followed by measuring during the time by HPLC the changes in the substrate concentration in the common glass container. To show the followed methodology in Figure 4 two typical experimental models of pollutant degradation are reported.

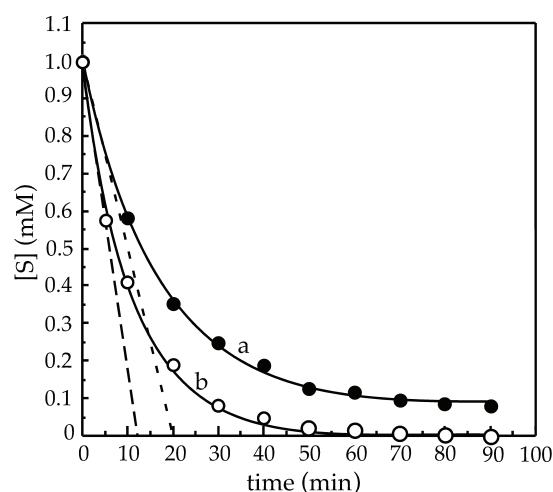


Fig. 4. Pollutant concentration decreases as function of time.

In particular curve “a” represents the case in which the enzyme activity, after a certain time, is inhibited by the substrate concentration or by a “suicide” effect. Curve “b”, instead, represents the case in which enzyme inhibition does not occur. In the figure the pollutant concentration, expressed in mM, is reported as a function of time. Curve “a” is represented by an analytical expression given by:

$$C(t) = C_{\infty} + (C_0 - C_{\infty}) e^{-kt} \quad (7)$$

where  $C_{\infty}$  and  $C_0$  are the BPA concentrations at  $t=\infty$  and  $t=0$ , respectively, and  $k$  is a time constant ( $\text{min}^{-1}$ ) related to the rate by which the enzyme reaction occurs. Curve “b” is expressed by the expression:

$$C(t) = C_0 e^{-kt} \quad (8)$$

In both cases, the initial reaction rate, measured as  $\mu\text{moles min}^{-1}$ , is obtained from the value of  $\left(\frac{dC}{dt}\right)_{t=0}$  multiplied by the solution volume in which the enzyme reaction is occurring.

When, instead, the pollutant decrease is linear, as it will be seen in the case of phthalates, the  $\left(\frac{dC}{dt}\right)_{t=0}$  is coincident with the slope of the line best fitting the experimental results.

The removal efficiency ( $RE_t$ ) at any time “t” is obtained in percentage by the expression:

$$RE_t(\%) = \frac{C(t) - C_0}{C_0} \times 100$$

(9)

6. Results

6.1 With the fluidized bed bioreactor

In order to determine the catalytic power of the catalytic PAN beads towards the single bisphenols, it has been investigated the rate of removal of each bisphenol at a concentration 1 mM in citrate buffer at pH 5.0 and at T=25°C. 1 mM was chosen considering that this concentration is higher than the effective concentrations in ecosystems (see log  $K_{ow}$  in the Table 1) and that the enzyme removal rate of a substrate decreases with increase of the substrate concentration. This means that the observed effects at 1 mM concentration are lower than those observable at smaller (and more natural) concentrations. Indeed, in the literature BPA measurements showed low concentrations: from 0.0005 to 0.41 mgL<sup>-1</sup> in surface water, from 0.018 to 0.702 mg L<sup>-1</sup> in sewage effluents, from 0.01 to 0.19mg kg<sup>-1</sup> in sediments and from 0.004 to 1.363 mgkg<sup>-1</sup>dw in sewage sludge. Measured concentrations of BPF result lower than those of BPA in all environmental media.

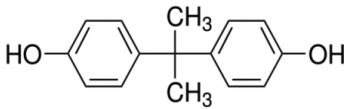
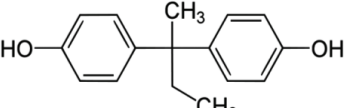
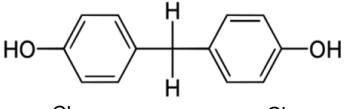
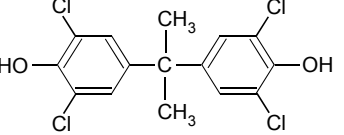
Substrate	Structural formula	Molecular weight	Water solubility	Log $K_{ow}$
BPA		228.29 g/mol	280 mg L <sup>-1</sup>	3.32
BPB		242.31 g/mol	220 mg L <sup>-1</sup>	3.90
BPF		200.23 g/mol	360 mg L <sup>-1</sup>	3.06
TCBPA		366.07 g/mol	200 mg L <sup>-1</sup>	4.02

Table 1. Schematic representation of the structure of studied bisphenols and some of their chemical and physical characteristics.

In Figure 5 the decreases of BPA, BPB, BPF and TCBPA concentrations are reported as function of time when immobilized laccase (●) or tyrosinase (○) are used. The substrate concentrations decrease with the enzyme treatment time following an exponential curve of

the type  $C(t) = C_0 e^{-kt}$ , where  $C(t)$  and  $C_0$  are the pollutant concentration at  $t$  and zero time, and  $k$  ( $\text{min}^{-1}$ ) is a rate constant which depends on the  $C_0$  value or, better, on the ratio between substrate molecules and available enzyme active sites.

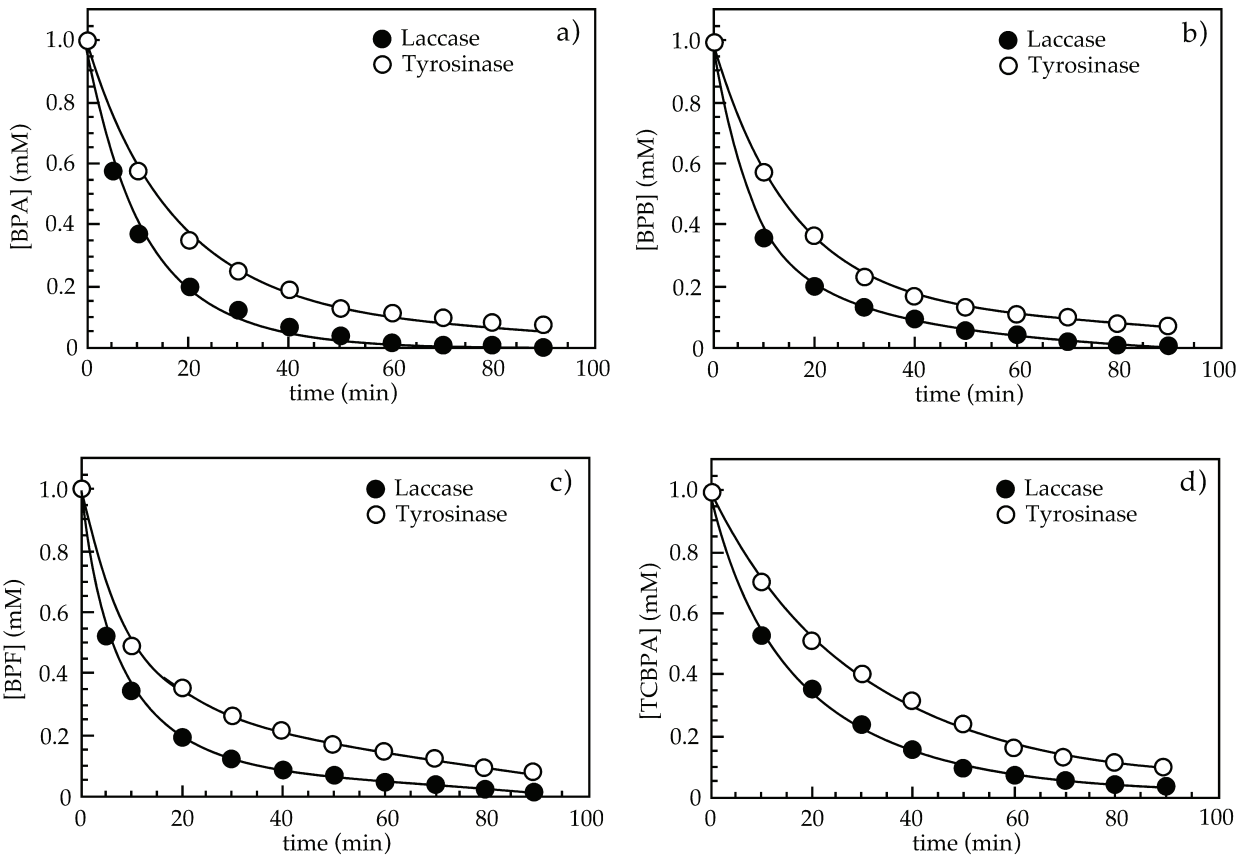


Fig. 5. Decreases of pollutant concentration as function of time. (●) laccase; (○) tyrosinase. The  $k$  values for each substrate are reported in Table 2.

Substrate	Laccase			Tyrosinase		
	$k$ ( $\text{min}^{-1}$ )	$\tau_{50}$ (min)	$\text{RE}_{90}$ (%)	$k$ ( $\text{min}^{-1}$ )	$\tau_{50}$ (min)	$\text{RE}_{90}$ (%)
BPA	0.087	8.0	100	0.059	13.0	92
BPB	0.083	7.5	100	0.054	13.4	93
BPF	0.117	5.0	100	0.070	10.0	94
TCBPA	0.057	11.5	96	0.033	21.0	91

Table 2. Rate constant ( $k$ ),  $\tau_{50}$  and removal efficiency ( $\text{RE}_{90}$ ) of laccase and tyrosinase immobilized on PAN beads towards the studied bisphenols.

The  $k$  values relative to BPF, BPA, BPB and TCBPA have been found to decrease in this order, from 0.117 to 0.057  $\text{min}^{-1}$  for laccase and from 0.070 to 0.033  $\text{min}^{-1}$  for tyrosinase. For each pollutant the  $k$  values of laccase are higher than the corresponding values of tyrosinase. The  $k$  values for tyrosinase follow the same order than those found for laccase. Moreover, looking at the results in Figure 5, two other efficiency factors can be calculated:  $\tau_{50}$  and  $\text{RE}_{90}$ .  $\tau_{50}$  is the time required to obtain, under our experimental conditions, the 50% of

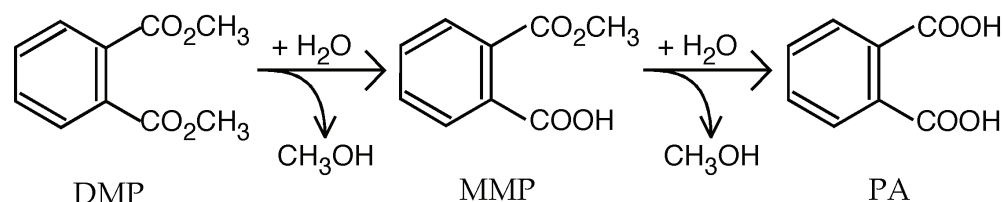
substrate biodegradation. The obtained  $\tau_{50}$  and  $RE_{90}$  values are also listed in Table 2. By comparing the time to obtain the 50% of initial concentration reduction, it is interesting to observe that by using the tyrosinase that time is quite doubled compared to that calculated for the laccase. Also interesting is the observation that, at least under our experimental conditions, 90 min of treatment with the enzyme laccase are sufficient to obtain the complete biodegradation of BPA, BPB and BPF. For TCBPA, a 96% reduction of its initial concentration has been calculated. Instead, 90 min of treatment with the enzyme tyrosinase are sufficient to obtain a biodegradation of all substrates near 90%.

Additionally, for both enzymes, the BPF is the substrate towards which the enzymes have the greatest biodegradation ability. In any case the biodegradation power of both enzymes is interesting for practical application.

## 6.2 With the planar membrane bioreactor

As reported in the introduction, lipase from *Candida rugosa* was used to biodegrade DMP. Lipase, like the other esterases, catalyses the hydrolysis and transesterification of ester groups. However, while esterases act on water soluble substrates, lipases catalyse reactions of water insoluble substrates. The presence of a water/lipid mixture is an essential prerequisite for an efficient catalysis reaction.

According to scheme 1, DMP hydrolysis by lipase may involve both methyl groups getting phthalic acid (PA) and two molecules of methanol, or may cause the rupture of a single bond thus producing monomethylphthalate (MMP) and methanol.



Scheme 1. Possible mechanism of DMP oxidation.

To ascertain the mechanism involving our enzyme, preliminary experiments have been carried out using MMP as substrate. It was found that our lipase did not catalyze this substrate, at least in any detectable amount after four hours of incubation. Incidentally it is important to stress the circumstance that MMP does not exhibit the same toxicological properties of DMP, as found by us with the MTT test, a rapid and sensitive method for screening the assessment of cytotoxicity of materials.

In each experiment, after the first ten minutes, subsequent points indicate that the sum of the moles of substrate and reaction product give a constant value equal to the initial value of DMP moles, as the stoichiometry of the reaction is 1:1 (Scheme 1 and Fig. 6). However, this sum is lower by about 10% with respect to the moles corresponding to the initial value of the substrate concentration. This difference is attributed to an initial substrate adsorption on either the membrane or the tubing of the hydraulic circuits. This percentage lost of DMP was constant in each experiment performed, regardless of the initial DMP concentration values. It must be noted that the bioreactor with the catalytic membrane was washed after each run with the 0.1 M phosphate buffer, pH 7.0.

The enzyme activity, expressed as  $\mu\text{moles min}^{-1}$ , is given by the slope of the lines that best fit the experimental points showing the decrease in DMP moles or the increase in MMP moles. No significant differences were found in the two calculations. Just to give an example for the

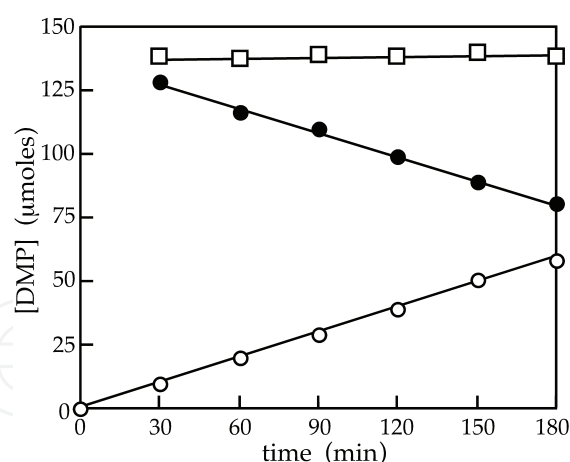


Fig. 6. Variation of DMP (O), MMP (●) and DMP + MMP (□) in function of time.

followed methodology in Figure 6 it has been reported the case of an experiment carried out with a 5 mM DMP initial solution. The DMP concentration has been converted in  $\mu\text{moles}$  by multiplying the measured concentration for the volume of the treated solution.

We now examine the behaviour of the catalytic membranes in the presence of temperature gradients. Figure 7 shows the results obtained under non-isothermal conditions by varying the DMP concentration from 1 to 15 mM. For comparison, the data obtained under isothermal conditions ( $\Delta T=0$ ) have also been added.

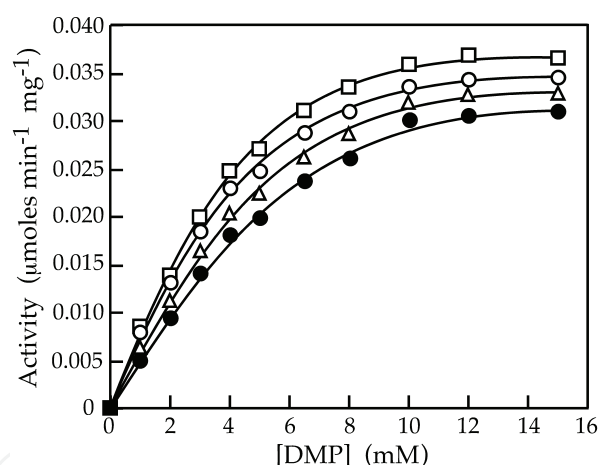


Fig. 7. Enzyme activity as function of DMP concentration.  $T_{av}=25^{\circ}\text{C}$ . ●:  $\Delta T=0^{\circ}\text{C}$ ; Δ:  $\Delta T=10^{\circ}\text{C}$ ; ○:  $\Delta T=20^{\circ}\text{C}$ ; □:  $\Delta T=30^{\circ}\text{C}$ .

Data in figure indicate that: i) the dependence of the reaction rate on the substrate concentration shows a behaviour described by a Michaelis-Menten equation either under isothermal or non-isothermal conditions; ii) for each DMP concentration the reaction rate under non-isothermal conditions is higher than the corresponding reaction rate under isothermal conditions; iii) at each substrate concentration, the reaction rate increases with the increase in the applied  $\Delta T$ . From the curves in Figure 7, the kinetic parameters reported in Table 3 have been calculated. These values show that: i) the  $K_m$  values obtained under non-isothermal conditions are lower than those obtained under the corresponding isothermal condition, thus demonstrating that the non-isothermal conditions increase the affinity of immobilized lipase for DMP; ii) under non-isothermal conditions the  $V_{max}$  values

for the immobilized lipase increase with the increase in the value of  $\Delta T$ , compared to those obtained under isothermal conditions, and approach the value ( $0.063 \mu\text{moles min}^{-1}\text{mg}^{-1}$  of enzyme) obtained for the free enzyme in the course of another experimentation (private communication), thus proving the effectiveness of non-isothermal bioreactors.

$T_{av}$ (°C)	$\Delta T$ (°C)	$K_M$ (mM)	$V_{max}$ ( $\mu\text{moles min}^{-1} \text{mg}^{-1}$ of enzyme)
25	0	5.5	0.031
	10	5.1	0.033
	20	4.7	0.035
	30	4.2	0.037

Table 3. Kinetic parameters calculated at  $T_{av}$ =25°C and  $\Delta T$ =0, 10, 20, 30°C.

As reported in the section 4.2, the obtained results are explained by considering that, in the presence of a temperature gradient, the immobilized enzymes in the unit of time “encounter” more substrate molecules, since additional thermodiffusive fluxes add to the diffusive ones (Diano et al., 2000). This means that under non-isothermal conditions the immobilized enzymes “see” in the microenvironment around the catalytic site a substrate concentration that is higher than in the bulk solution. This is a new kind of “partitioning effect” that is related to the presence of the temperature gradient.

Let us consider again Figure 7 where it is possible to observe that at each DMP concentration the enzyme reaction rate increases with the increase of the size of the applied temperature gradient. To verify the type of dependence, it is appropriate to plot, at each substrate concentration, the enzyme reaction rate as a function of the applied  $\Delta T$ . This has been done, as one example, in Figure 8 for the case of 5 mM DMP. From this figure, it is possible to observe that the lipase catalytic activity linearly increases with the applied  $\Delta T$ . The best line interpolating the experimental points is described by the equation:

$$y_{\Delta T \neq 0}(T_{av}) = y_{\Delta T = 0}(T_{av}) \left( 1 + \frac{\alpha}{100} \Delta T \right)$$

(10)

where  $y_{\Delta T \neq 0}(T_{av})$  and  $y_{\Delta T = 0}(T_{av})$  are the catalytic activity values measured under non-isothermal ( $\Delta T \neq 0$ ) or isothermal ( $\Delta T = 0$ ) conditions, respectively, at a fixed value of  $T_{av}$ . From the above equation one obtains:

$$\alpha = \frac{y_{\Delta T \neq 0}(T_{av}) - y_{\Delta T = 0}(T_{av})}{y_{\Delta T = 0}(T_{av})} \frac{100}{\Delta T} = \frac{\text{P.A.I.}}{\Delta T}$$

(11)

where the  $\alpha$  coefficient ( $\%, ^\circ\text{C}^{-1}$ ) represents the Percentage Activity Increase (P.A.I.), when a macroscopic temperature difference  $\Delta T=1^\circ\text{C}$  is read at the position of the thermocouple.

Following the procedure described above, the values of the P.A.I., calculated for each substrate concentration, have been reported in Figure 9a as a function of DMP concentration. The curve parameter is the applied  $\Delta T$ . Figure 9a shows that the P.A.I. values decrease with the increase in substrate concentration. This is explained by considering that when the immobilized enzyme works at substrate concentrations close to saturation, the addition of further substrate fluxes driven by the temperature gradients is less effective in increasing the enzyme activity. Alternately, when the enzymes work at low concentrations, far from the saturation value, any additional substrate flux effectively increases the enzyme activity.



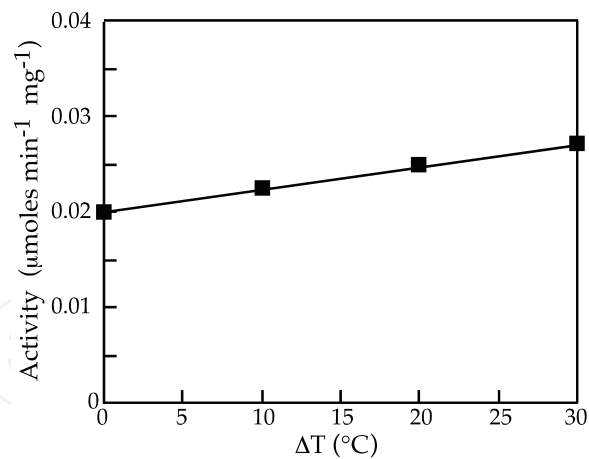


Fig. 8. Activity in function of temperature gradient  $\Delta T$ .

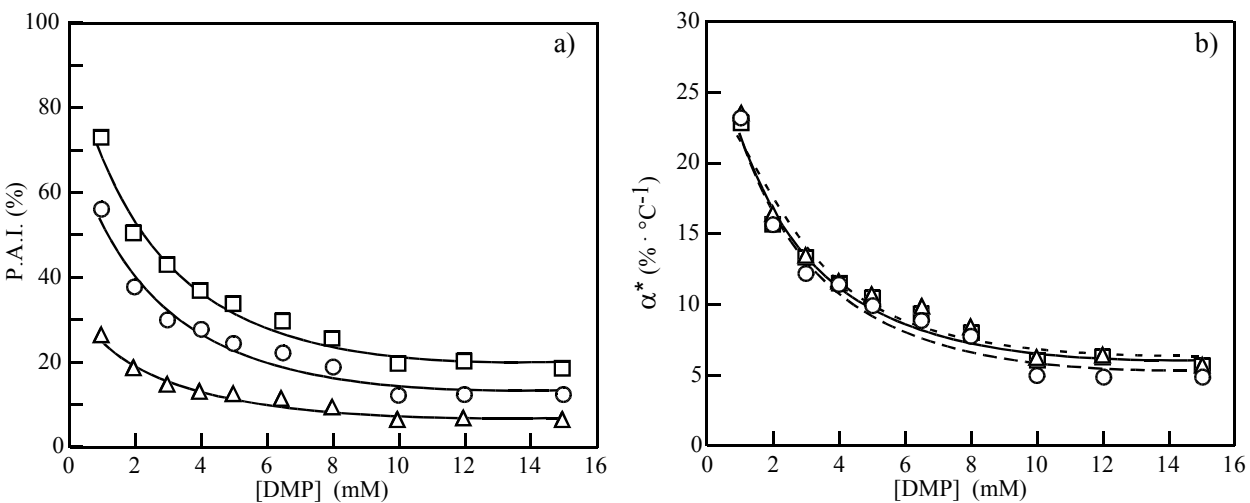


Fig. 9. (a): Percentage Activity Increase in function of DMP concentration. (b):  $\alpha^*$  coefficient in function of DMP Concentration. Symbols:  $\Delta$ :  $\Delta T=10^\circ\text{C}$ ;  $\circ$ :  $\Delta T=20^\circ\text{C}$ ;  $\square$ :  $\Delta T=30^\circ\text{C}$ .

Another interesting parameter for the process is  $\alpha^*$  (%  $\cdot$  °C $^{-1}$ ), the percentage activity increase of the enzyme reaction rate for  $\Delta T^*=1^\circ\text{C}$ , i.e., when an actual temperature difference of  $1^\circ\text{C}$  is applied across the catalytic membrane. The expression for  $\alpha^*$  is:

$$\alpha^* = \frac{\text{P.A.I.}}{\Delta T^*} \tag{12}$$

Figure 9b shows, as a function of DMP concentration, the  $\alpha^*$  values obtained from the results in Figure 9a. Because this process is similar to a normalization process, overlapping curves are present. It is interesting to observe that at low concentrations of DMP (1 mM) the  $\alpha^*$  value amounts to 24%, while at high DMP concentrations (10–15 mM) the  $\alpha^*$  values amount to 6%. From the applied point of view, these results indicate that at concentrations of DMP lower than those used by us, such as those actually existing in the environment, a temperature difference of  $1^\circ\text{C}$  across the catalytic membrane is enough to obtain increases of over 20% in the removal of DMP concentration. Looking at our similar publications, it is possible to see that the  $\alpha^*$  values for lipase are smaller than those found for other catalytic systems, may be because the aqueous system is not completely suitable for lipase activity.

From the industrial point of view, the results discussed above indicate a substantial reduction in the processing times to bioremediate water polluted by DMP and thus a reduction in the process's costs. In fact, it is possible to correlate the parameters  $\alpha$  or P.A.I., which are functions of  $\Delta T$ , with the reduction in the bioremediation time,  $\tau_r$ , defined as

$$\tau_r (\%) = \frac{\tau_{iso} - \tau_{non-iso}}{\tau_{iso}} \times 100 \quad (13)$$

where  $\tau_{iso}$  and  $\tau_{non-iso}$  are the time required to obtain the same percentage of DMP biodegradation under isothermal and non-isothermal conditions, respectively. To correlate  $\tau_r$  with the applied  $\Delta T^*$  it is necessary to calculate the time required to obtain the same amount of DMP removal under isothermal and non-isothermal conditions. This calculation can be done graphically or analytically.

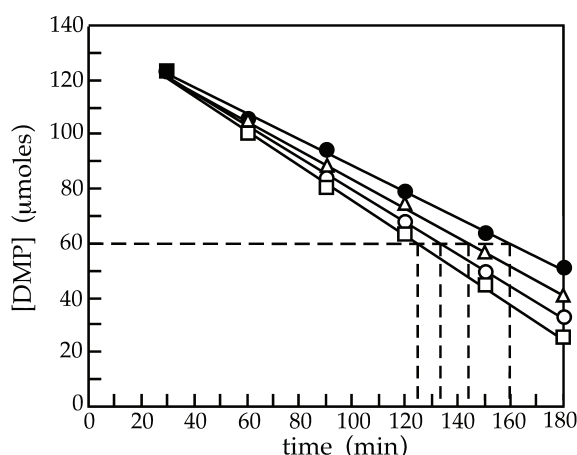


Fig. 10. DMP in function of reaction time. ●:  $\Delta T=0^\circ\text{C}$ ;  $\Delta$ :  $\Delta T=10^\circ\text{C}$ ; ○:  $\Delta T=20^\circ\text{C}$ ; □:  $\Delta T=30^\circ\text{C}$ .

As one example of the graphical calculation let us see Figure 10, where we have been reported as a function of the time of enzyme treatment, the DMP decrease in the case of  $T_{\text{average}}=25^\circ\text{C}$ , with  $\Delta T=0$  or 10 or 20 or  $30^\circ\text{C}$ . The initial DMP concentration was 5 mM. To obtain the same biodegradation of DMP, for example a 50% reduction, 161 minutes are needed for the isothermal condition, while 145 or 134 or 125 minutes are required for  $\Delta T=10^\circ\text{C}$ ,  $\Delta T=20^\circ\text{C}$ ,  $\Delta T=30^\circ\text{C}$ , respectively. It follows that a value of  $\tau_r=9.9\%$  is obtained with a  $\Delta T=10^\circ\text{C}$ , a  $\tau_r=16.8\%$  with a  $\Delta T=20^\circ\text{C}$ , and a  $\tau_r=25\%$  with a  $\Delta T=30^\circ\text{C}$ . The  $\tau_r$  values increase with an increase in the applied  $\Delta T$  and therefore with the P.A.I.

The analytical approach is based on the consideration that the same DMP degradation is obtained when  $RR_{C,\Delta T=0} \cdot \tau_{iso} = RR_{C,\Delta T \neq 0} \cdot \tau_{non-iso}$ , where RR stands for the reaction rate. By recalling that

$$RR_{C,\Delta T=0} \tau_{iso} = RR_{C,\Delta T \neq 0} \left( 1 + \frac{\alpha}{100} \Delta T \right) \tau_{non-iso} \quad (14)$$

after a series of mathematical steps one obtains

$$\tau_r (\%) = \left( \frac{\alpha \Delta T}{\alpha \Delta T + 100} \right) 100 = \left( \frac{\alpha^* \Delta T^*}{\alpha^* \Delta T^* + 100} \right) 100 = \left( \frac{P.A.I.}{100 + P.A.I.} \right) 100 \quad (15)$$

In Figure 11a, the  $\tau_r$  values obtained at different DMP concentrations with  $\Delta T=30^{\circ}\text{C}$  have been reported as a function of the P.A.I. calculated for each DMP concentration. As expected, the reduction in bioremediation time is an increasing function of the percentage increase in the enzyme activity (P.A.I.) and, consequently, in the temperature difference applied across the membrane. Highlighted in black is the case for a DMP concentration equal to 5 mM, for which the P.A.I. is 33.4% and the  $\tau_r$  is 25%.

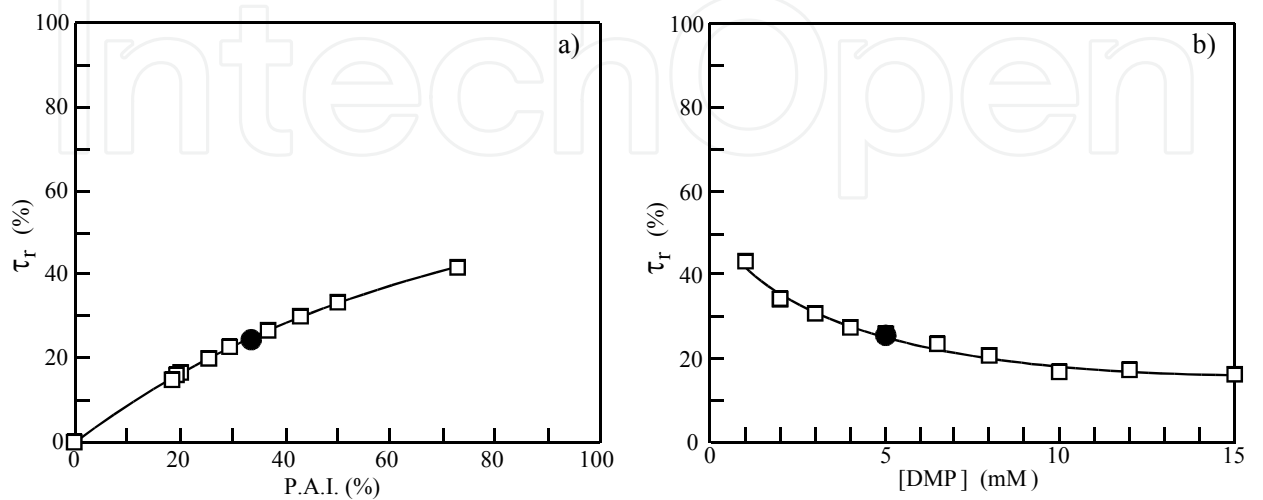


Fig. 11. (a): Percentage reduction of the productions time ( $\tau_r$ ) in function of Percentage Activity Increase. (b):  $\tau_r$  in function of DMP concentration.

Because the P.A.I. is related to the substrate concentration, we have reported the reduction in biodegradation time as a function of the DMP concentration in Figure 11b. Again, highlighted in black is the result relative to the DMP concentration of 5 mM. As is evident from Figure 11b, the reduction in the biodegradation time decreases with the increase in the DMP concentration. Also, this result is interesting for practical applications, because the concentrations used by us are higher than those actually found in polluted water, owing to DMP's small solubility in water.

From the above results, it follows that the decrease in DMP concentration is a linear function of the applied temperature difference and is inversely proportional to the initial DMP concentration. To quantify this observation, in Table 4 we have reported the percentage decreases of DMP concentration after 180 minutes of enzyme treatment.

$T_{av}$ ( $^{\circ}\text{C}$ )	$\Delta T$ ( $^{\circ}\text{C}$ )	[DMP]			
		1 mM	3 mM	5 mM	8 mM
25	0	68.5 %	63.0 %	58.1 %	43.4 %
	10	84.0 %	72.5 %	66.1 %	47.7 %
	20	100.0 %	82.7 %	72.9 %	51.9 %
	30	100.0 %	89.5 %	79.3 %	55.1 %

Table 4. Percentage decreases of DMP concentration after 180 minutes of enzyme treatment.

7. Conclusions

The obtained results have shown that the laccase from *Trametes versicolor* and the tyrosinase from mushrooms immobilized on PAN beads filling a fluidized bed bioreactor are able to

oxidize different bisphenols. In particular, the BPF is the substrate towards which the immobilized enzymes have the highest bioremediation power. Moreover the higher removal efficiencies ( $\approx 100\%$ ) for all bisphenols were obtained with immobilized laccase. The immobilized tyrosinase, under the same experimental conditions, showed smaller removal efficiency ( $\sim 90\%$ ), notwithstanding the specific activity of this enzyme results to be 1500U/mg, about 75 time that of laccase.

Coming to the experiments carried out in planar membrane bioreactors working under non-isothermal conditions, the results have shown the possibility of using the enzyme lipase from *Candida rugosa* in the pathway for the biodegradation of phthalates to bioremediate water polluted by these compounds. The use of non-isothermal bioreactors proved the utility of this technology in solving some of the pollution problems affecting human life and wildlife. Moreover, our studies may increase the limited knowledge regarding the direct exploitation of purified enzymes in the hydrolysis of phthalates, since the literature exhibits very few papers in this field.

## 8. Perspectives

Our results encourage new studies in order to bioremediate waters polluted by EDCs. By considering that in real samples EDCs are present in mixture, it will be interesting for the future to coimmobilize different enzymes able to hydrolyze different pollutants. But a more intriguing observation can be advanced by considering the meaning of the word “cleaning up”. From the analytic point of view “cleaning up” means to “reduce or eliminate” the pollutant concentration. From the biological point of view, indeed, and particularly in the case of EDCs, besides “the reduction or elimination” of the pollutant concentration, “cleaning up” means the removal of the endocrine effects observed before the enzyme treatment. So, tests on cell line and on living organisms are request to assess the toxicity or the endocrine power of the reaction products. If these tests will result negative, only in this case we can speak of occurred “cleaning up”.

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The steady increase in industrialization, urbanization and enormous population growth are leading to production of huge quantities of wastewaters that may frequently cause environmental hazards. This makes waste water treatment and waste water reduction very important issues. The book offers a collection of studies and findings concerning waste water treatment, minimization and reuse.

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