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Employment of immobilised lipase from *Candida rugosa* for the bioremediation of waters polluted by dimethylphthalate, as a model of endocrine disruptors

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ABSTRACT

A planar bioreactor, equipped with a polypropylene membrane on which a lipase was immobilised, has been employed in a bioremediation process involving water polluted by dimethylphthalate (DMP), a model for a class of endocrine disruptors. The dependence of enzyme activity on pH, temperature and DMP concentration has been characterised under isothermal conditions, whereas the kinetics parameters have been studied under non-isothermal conditions. The following sequence was found for the values of lipase affinity, K_m, towards the DMP: $K_m^{free} < K_{m,non\text{-}isoth}^{imm} < K_{m,isoth}^{imm}$. A comparison of the results obtained under isothermal and non-isothermal conditions indicated that there was an advantage in using non-isothermal bioreactors in the environmental field. These advantages in particular resulted in: (i) an increase in the enzyme activity proportional to the applied transmembrane temperature difference and (ii) a reduction in the bioremediation times and, consequently, the process costs. The advantages in using bioremediation processes in the place of classical membrane processes, such as ultrafiltration or reverse osmosis, are also discussed.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a heterogeneous group of chemicals that interfere with the endocrine system and cause adverse alterations in reproductive and development processes while also causing metabolic diseases. Depending on their action on female or male hormones, they are defined as anti-estrogens or anti-androgens. Endocrine disruptors include polychlorinated biphenyls (PCBs), dioxins, some pesticides, alkylphenols, phthalates, polybrominated flame retardants, phytoestrogens, and some heavy metals. The main mechanisms through which these substances interfere with the endocrine system are: (i) simulation of the activities of physiological hormones, thereby participating in the same reactions and causing the same effects; (ii) inactivation, with competitive action, of hormone receptors and consequently the neutralisation of their activity; and (iii) interference with the synthesis, transport, metabolism and secretion of natural hormones, altering their physiological concentrations and therefore their corresponding endocrine functions.

There are two main sources of evidence favouring these hypotheses. The first is the extensive experimental evidence showing that many xenobiotic compounds have intrinsic hormonal activity. Laboratory animal studies indicate that a wide variety of hormone-dependent physiological effects are associated with exposure to these environmental pollutants. Furthermore, in vitro molecular studies have demonstrated that these pollutants not only have affinity for hormone receptors but also are able to induce, via the receptor, agonistic or antagonistic effects. The second piece of evidence derives from incidents of human and wildlife acute exposure to hormonally active xenobiotics, which result in impaired reproductive capability in the exposed individuals and in their offspring. Cancer has also been implicated.

Because of the broad spectrum of diseases correlated with exposure to EDCs, affecting not only the quality of wild and human life but also that of their offspring, many research activities have tried

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to address these problems in the last years. The main research fields included: (a) bioremediation of polluted soils and waters; (b) biodetermination using biosensors; (c) study of the molecular mechanisms by which these harmful substances interact with the cellular machinery; and (d) establishment of epidemiological correlations between exposure and the diseases.

Recently we have studied the bioremediation of waters polluted by phenol compounds. In particular, we have studied the endocrine disruptor Bisphenol A [1], its biodetermination in aqueous solution by means of tyrosinase-based carbon paste electrodes [2], and the changes in the proliferation and viability indices of MCF-7 cancer cells exposed to BPA, untreated or enzyme treated [3,4].

In this paper, we have focused our attention to phthalates. Phthalates are used in many consumer products including toys, baby products, lotions, cosmetics, personal care products, fragrances, air fresheners, medical tubing components and devices, blood bags, PVC pipes and flooring, and pharmaceuticals [5,6]. They are ubiquitous in the environment. Several studies have shown that although phthalate exposure levels in humans are generally low, close to the detection limit, a small percentage of people are exposed to higher levels of phthalates. This information is based on the level of phthalate metabolites identified in the urine of some pregnant women and in human amniotic fluid [7–13]. In rats, at certain levels of exposure, phthalates cause liver cancer, spontaneous abortions, and reproductive tract malformations in male and female offspring [14–19]. The adverse reproductive effects seen in the male offspring, described as the "Phthalate Syndrome", are currently the focus of regulatory agencies because this syndrome occurs at lower dosage levels than other toxicities.

Taking account of these concerns, we decided to study the bioremediation of water polluted by phthalates. While numerous studies have demonstrated that microorganisms play a major role in the degradation of phthalates in the environment [20–26], only a few papers [27,28] reported the use of purified enzymes in these biodegradation processes. This paper describes the degradation of dimethylphthalate by means of lipase from *Candida rugosa*. The lipase has been immobilised on a planar polypropylene membrane in a bioreactor operating under isothermal or non-isothermal conditions. The advantages in using a bioremediation process in place of classical membrane processes, such as reverse osmosis or ultrafiltration, are discussed. Moreover the advantages of using non-isothermal bioreactors, compared to isothermal bioreactors, are analysed in terms of: (i) the percentage increase in catalytic activity per 1 °C of temperature difference across the catalytic membrane and (ii) the consequent reduction of bioremediation times.

2. Experimental

2.1. Chemicals

To carry out our experiments dimethylphthalate (DMP) was chosen as a model for the phthalate class. DMP is an oily liquid that is slightly sweet. The chemical formula is $C_{10}H_{10}O_4$ and the molecular weight is 194.19 g/mole.

For the enzyme, we used lipase (EC 3.1.1.3) from *C. rugosa* (1170 U/mg). Lipases, like esterases, catalyse the hydrolysis and transesterification of ester groups. However, while esterases act on water soluble substrates, lipases catalyse reactions of insoluble water substrates. The presence of a water/lipid mixture is an essential prerequisite for an efficient catalysis reaction. The active site of lipase from *C. rugosa* consists of three amino acid residues (Ser209, His449 and Glu341) that form a "catalytic triad" (as that of serine proteases), which is responsible for the nucleophilic attack that promotes ester bond cleavage.

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. To ascertain the relevant mechanism for our enzyme, we have carried out experiments using free lipase from *C. rugosa* and MMP. It was found that our lipase did not catalyse this substrate, at least in any detectable amount after 4 h of incubation. Incidentally it is important to stress the circumstance that the MMP does no 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.

Polypropylene (PP) membranes were purchased from GE Osmonics (GE Labstore-Osmonics, Minnetonka, MN) and used as supports for enzyme immobilisation. Their thickness was 150 μm and the nominal pore diameter was 0.22 μm.

All the chemicals, including the enzyme, were purchased from Sigma (Sigma Italia, Milan, Italy) and used without further purification.

2.2. Apparatus

The apparatus employed is represented in Fig. 1. Fig. 1a shows the modus operandi and Fig. 1b shows an exploded view. The bioreactor consists of two metallic flanges, in each of which is bored a shallow cylindrical cavity, 70 mm in diameter and 2.5 mm in depth, constituting the working volume that is filled with the aqueous buffer solutions containing DMP. The catalytic membrane is clamped between the two flanges so as to separate and, at the same time, 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 tube. Using independent thermostats, the two half-cells are maintained at predetermined temperatures. Thermocouples, placed 1.5 mm away from the membrane surfaces, measure the temperatures of the solutions at that point in each half-cell. This setup allows the calculation of the temperature profile across the catalytic membrane, as reported in Section 3.3.1.

2.3. Methods

2.3.1. Catalytic membrane preparation

The catalytic membranes were prepared in two successive steps: (a) activation by means of a plasmo-chemical reactor and (b) enzyme immobilisation by means of a diazotation process.

2.3.1.1. Membrane activation. Polypropylene is a non-polar material that lacks reactive groups for enzyme immobilisation. Consequently, functional groups have been created on the polypropylene 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 = 80 W, pressure = 400 mTorr, time = 10 min) gave rise to a very stable coating on the membrane, showing the following abundance of reactive groups: COOH < C=O < COH < CC.

2.3.1.2. Enzyme immobilisation. The lipase was immobilised on the activated 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) p-phenylenediamine (PDA) aqueous solution of 0.1 M 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%

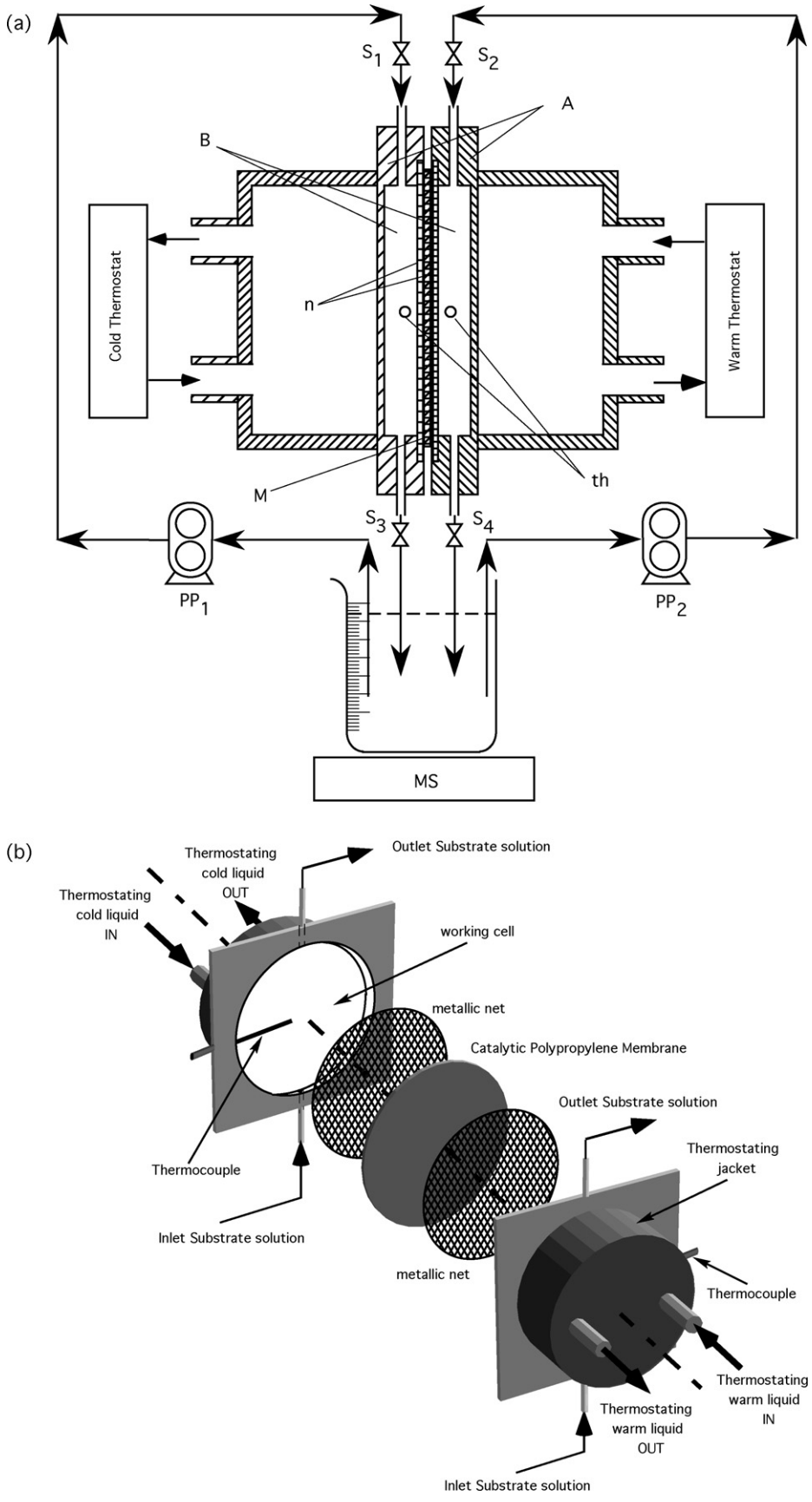


Fig. 1. The bioreactor: (a) modus operandi of the bioreactor and (b) exploded view of the bioreactor.

(w/v) $NaNO_2$ and 2 M HCl, in a ratio of 1:5. At the end of this treatment the membranes were washed at room temperature in a buffer solution (0.1 M phosphate, pH 7.0), and then treated for 16 h at 4 °C with 30 mL of the same buffer solution containing 20 mg/mL of extract from the container. At the end of this last step the membranes were further washed with 0.1 M phosphate buffer, pH 7.0 to remove material not bound.

The amount of immobilised lipase was calculated by subtracting the amount of protein recovered in the solution at the end of the immobilisation process (and in the washing solutions) from the amount of protein initially used for the immobilisation. The protein concentration was measured using the method of Lowry et al. [29]. Under the experimental conditions reported above, the amount of immobilised protein on PP membranes was 3.26 ± 0.2 mg, i.e., 0.041 ± 0.002 mg cm^{-2}.

2.3.2. Catalytic activity determination

Lipase catalytic activity was determined by monitoring, over time by means of a HPLC (LC-20 AT, Shimadzu, Kyoto), the disappearance of DMP or the appearance of MMP. In particular, at regular time intervals, 200 μL of solution were collected from the reaction volume and processed by chromatographic analysis. Each experiment lasted 3 h.

HPLC analyses were carried out with a Discovery HS C18 column (15 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, USA). The samples were eluted at a flow rate of 1.0 mL/min with 30% acetonitrile in water (0–2 min), followed by a linear gradient from 30% to 50% acetonitrile (2–4 min), and finally with 50% acetonitrile (4–10 min). At the end of the chromatogram, the mobile phase returned to its initial composition in 2 min and the column was equilibrated for 10 min. The wavelength used for detection was 275 nm. The chromatograms were processed with the software LC Solution (Shimadzu, Kyoto). The retention time for DMP occurred at 5.5 min, while that for MMP occurred at 1.3 min. A decrease in the peak of DMP and an increase in the peak for MMP were observed during the enzyme reaction.

Interestingly, after the first 10 min of the experiment, subsequent time points indicate that the sum of the moles of substrate and reaction product give a constant value equal to the initial value of DMP, as the stoichiometry of the reaction is 1:1 (Scheme 1). This sum is lower by about 10% with respect 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 circuit. This lost percentage 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 μmoles 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 followed methodology in Fig. 2 we report the case of an experiment carried out with a 5 mM DMP initial solution. The DMP concentration is converted in μmoles by multiplying the actual concentration for the treated solution volume.

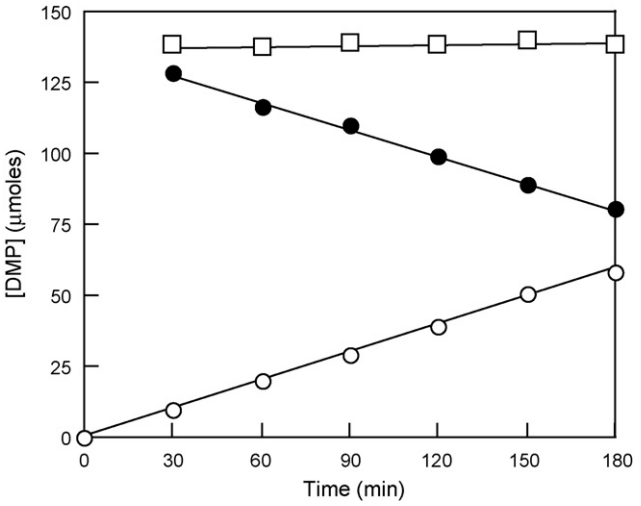


Fig. 2. Concentration of MMP (○), DMP (●) and DMP + MMP (□) as a function of time.

When not used, the membranes were stored at 4 °C in 0.1 M phosphate buffer, pH 7.0.

Membranes were stable for about two months. Membrane stability was assayed by measuring (every day) the membrane activity under standard conditions, i.e., 5 mM in 0.1 M phosphate buffer, pH 7.0 and $T = 25$ °C. When the membrane activity exhibited a value that was 8% lower than that of the initial value (i.e., a value twice the experimental error) the membrane was discharged.

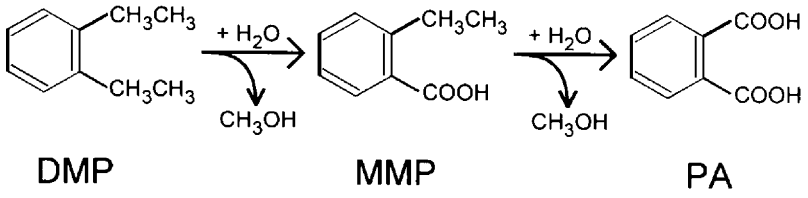
3. Results and discussion

The experimental results are grouped into three sections. The first, addressed to highlight the activity changes induced by the immobilisation process, will concern the study (under isothermal conditions) of the dependence of lipase activity, soluble or insoluble, on pH and temperature, and the determination of the kinetics parameters. The second section will be focused on the effects of non-isothermal conditions on the activity of immobilised lipase. The third section will deal with the evaluation of some quantitative parameters that are significant for industrial processes. These parameters are: (a) the actual temperature difference across the catalytic membrane and (b) the coefficients α, P.A.I. and τ_r that will be defined in the following.

All experiments were repeated five times, and the experimental points in the figures represent the average value. The experimental error never exceeded 4%.

3.1. Isothermal characterisation of soluble and insoluble lipase

In Fig. 3a, the relative activities of soluble or insoluble lipase are reported as a function of pH. In the case of soluble lipase we carried out the experiments holding the enzyme concentration (2 mg/mL), the temperature (25 °C) and the concentration of substrate (5 mM)



Scheme 1.

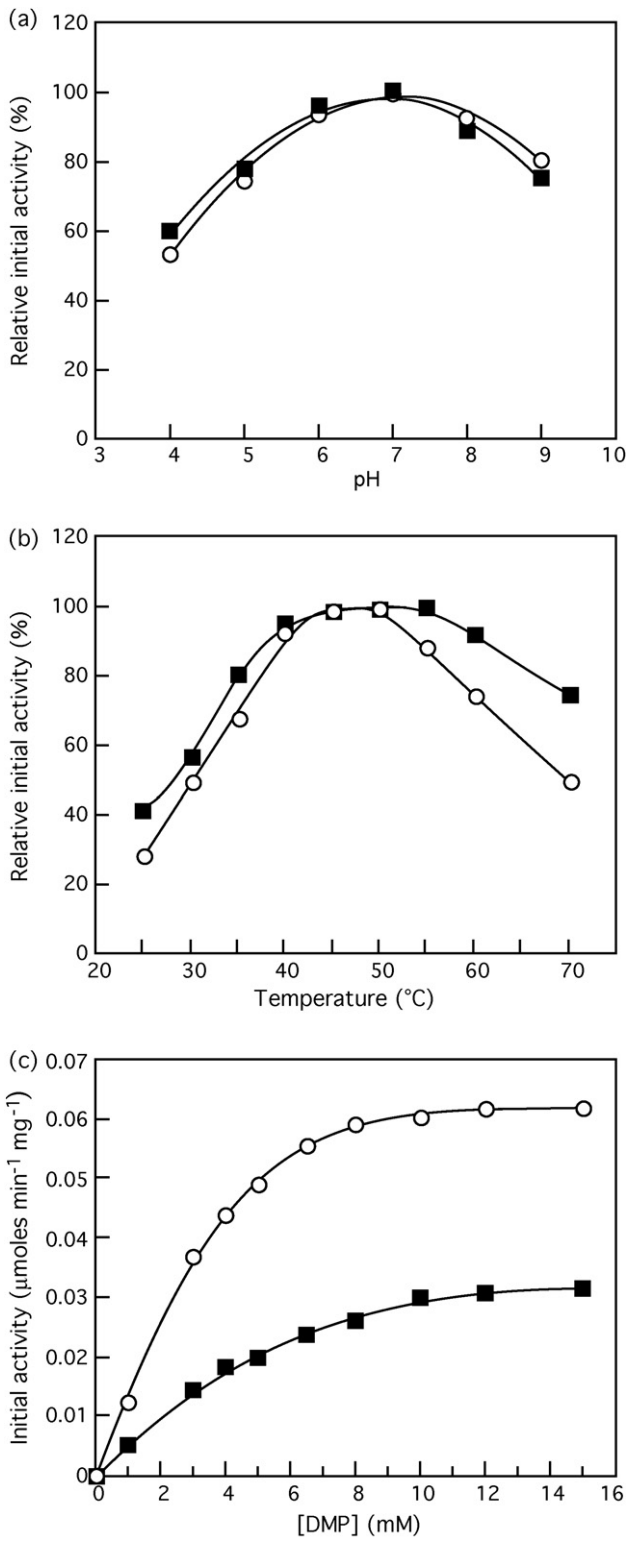


Fig. 3. Dependence of lipase activity on pH, temperature and DMP concentration: Relative initial enzyme activity as a function of pH (a) and temperature (b). Initial enzyme activity as a function of DMP concentration (c). Symbols: (○) free enzyme; (■) immobilised enzyme.

all constant while varying the pH in a range from 4.0 to 9.0. The volume of substrate solution was 10 mL.

For the immobilised lipase, the experimental conditions were the same as that for the free enzyme, with the exception of the enzyme amount (3.26 ± 0.35 mg of enzyme) and the reaction volume (30 mL).

As seen in Fig. 3a, both enzyme forms have an optimum pH value at 7.0. The only difference appears in the range in which the relative catalytic activity is higher than the 90% value defined by us as the "optimum pH range." This range is from 5.6 to 8.0 for the immobilised enzyme, while the free enzyme has a range from 5.8 to 8.3.

The data in Fig. 3b show the dependence of the relative activity of the free or immobilised lipase as a function of temperature. The experimental conditions, either for the free lipase or for the insoluble lipase, were the same as those used for the pH dependence studies, with the exception that this time the pH was kept constant at 7.0, whereas the temperature was varied in the range from 25 to 70 °C. Fig. 3b shows that the optimum temperature for soluble lipase occurs at about 45 °C, whereas the optimum temperature for immobilised lipase is shifted to about 55 °C. Moreover, the immobilised enzyme presents an optimum temperature range between 35 and 60 °C, which is more extensive than that of the free counterpart, which spans between 40 and 55 °C. Also in this case the "optimum temperature range" is the range in which the relative activity is higher than 90%. It follows that the immobilisation procedure imparts more thermal stability to the enzyme, protecting it from the environmental conditions.

In Fig. 3c, the activities of the soluble and insoluble lipase are reported as a function of DMP concentration. The experimental conditions (pH 7.0 and temperature 25 °C) were the same for the free and immobilised lipase, with only the DMP concentration being varied. The choice of the DMP range was determined by the limit of DMP solubility in water and by the HPLC detection limit. Both enzyme forms exhibit a Michaelis–Menten behaviour. From the data in Fig. 3c, through a Lineweaver–Burk plot, one obtains the kinetics parameters K_m and V_{max}. For the free lipase, the K_m value is 3.5 mM and the V_{max} value is 0.063 μmoles min^{-1} per mg of enzyme. For the immobilised lipase, the values were 5.5 mM for the K_m and 0.031 μmoles min^{-1} per mg of immobilised enzyme for the V_{max}, respectively.

When an enzyme is immobilised, there is a change in the kinetic parameters. This is due to the changes in protein conformation induced by the enzyme–support interactions and by the method of immobilisation, i.e., whether the amino acid residues involved in the immobilisation process are near or far from the catalytic site. Other causes are the differential distribution of substrate in the microenvironment around the catalytic site and in the bulk solution (partitioning effect), and the restrictions to substrate diffusion introduced by the chemicals involved in the immobilisation process. Specifically, if we compare the value of K_m obtained for the immobilised enzyme (5.5 mM) with that of the free lipase (3.5 mM), it is clear that the increase in K_m value appears as a loss of affinity for the substrate. Moreover, a comparison of the V_{max} values shows that the immobilised enzyme loses about 50% of its activity in the free form.

3.2. *Effects of non-isothermal conditions on the activity of immobilised lipase*

We now examine the behaviour of our catalytic membranes in the presence of temperature gradients. The presence of two independent thermostats, connected to the bioreactor, can keep the two half-cells at the same or at different temperatures. The subscripts "w" and "c" indicate the values of the warm and cold half-cells, respectively. When $T_w = T_c$, the temperature difference ΔT is equal to zero and the experiments are carried out under isothermal conditions ($\Delta T = 0$). Alternatively, when $T_w > T_c$, $\Delta T = T_w - T_c$ is different from zero and the experiments are carried out under non-isothermal conditions ($\Delta T \neq 0$). It must be remembered that the temperatures are read at the positions of the thermocouples, which are 1.5 mm away from the membrane surface. So, for exam-

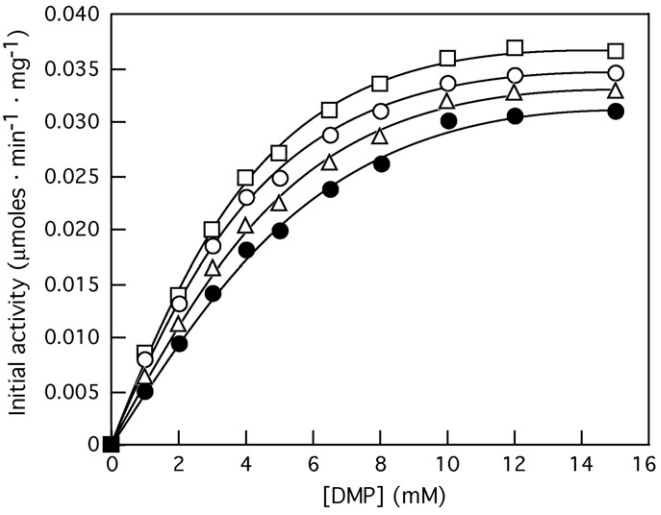


Fig. 4. Effect of ΔT on the enzyme kinetics: initial enzyme activity as a function of DMP concentration. Symbols: (●) $\Delta T = 0\,^{\circ}C$; (△) $\Delta T = 10\,^{\circ}C$; (○) $\Delta T = 20\,^{\circ}C$; (□) $\Delta T = 30\,^{\circ}C$.

ple, when $T_w = 40\,^{\circ}C$ and $T_c = 10\,^{\circ}C$, the macroscopic experimental conditions are identified as $\Delta T = 30\,^{\circ}C$ and $T_{average} = 25\,^{\circ}C$. To assess the effects of the temperature gradients on the activity of the immobilised lipase and, consequently, to assess the convenience in using the technology of non-isothermal bioreactors, we have carried out the experiments while maintaining a constant average temperature ($T_{average} = 25\,^{\circ}C$), and by applying three temperature differences: $\Delta T = 10$ or 20 or $30\,^{\circ}C$.

Fig. 4 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. Data in this 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 enzyme reaction rate under non-isothermal conditions is higher than the corresponding reaction rate under isothermal conditions; and (iii) at each substrate concentration, the enzyme reaction rate increases with the increase in the applied ΔT. From the curves in Fig. 4, we calculated the kinetic parameters reported in Table 1. These values show that: (i) the K_m values obtained when working under non-isothermal conditions are lower than those obtained in the corresponding isothermal condition, thus demonstrating that the non-isothermal conditions increase the apparent affinity of immobilised lipase for DMP; and (ii) under non-isothermal conditions the V_{max} values for the immobilised lipase increase with an increase in the macroscopic value of ΔT, compared to those obtained under isothermal conditions, and approach the value (0.063 μmoles $min^{-1}\,mg^{-1}$ of enzyme) obtained for the free enzyme, thus proving the effectiveness of non-isothermal reactors.

The above behaviour is explained by considering that, in the presence of a temperature gradient, the immobilised enzymes in the unit of time "encounter" more substrate molecules, since additional thermodiffusive fluxes add to the diffusive ones [30]. This means that under non-isothermal conditions the immobilised 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.

Table 1
Kinetic parameters of immobilised lipase.

T_{av} (°C)	ΔT (°C)	K_m (mM)	V_{max} (μmoles $min^{-1}\,mg^{-1}$ of enzyme)
25	0	5.5	0.031
	10	5.1	0.033
	20	4.7	0.035
	30	4.2	0.037

3.3. Evaluation of some quantitative parameters that are significant for processes of industrial interest

3.3.1. Temperature profile in the bioreactor

To quantify the advantages of using non-isothermal bioreactors, it is necessary to clarify that the catalytic membrane actually is not subjected to the macroscopic temperature difference ΔT detected by the thermocouples, but instead is subjected to a lower temperature difference ΔT^*. Indeed, we have demonstrated [30] that the solution motion in the bioreactor is laminar and that, utilising the principle of heat continuity, it is possible to write the following relationship: $\Delta T^* = k\Delta T$, where "k" is a numerical constant depending on the thermal conductivities of both the solutions filling the bioreactor and the membrane. In the present case, k is equal to 0.11. To obtain the k value we used the thermal conductivity of water [31], for the solution filling the bioreactor, and the value taken from Touloukian et al. [32] for the polypropylene membrane. We are conscious of the fact that our calculation is approximate. To give an example of the real situation, in Fig. 5 the temperature profile in the bioreactor is depicted for the experimental conditions $\Delta T = 30\,^{\circ}C$ and $T_{average} = 25\,^{\circ}C$. From this figure it is possible to appreciate how a macroscopic $\Delta T = 30\,^{\circ}C$ across the bioreactor is reduced to an effective $\Delta T^* = 3.2\,^{\circ}C$ across the catalytic membrane.

3.3.2. Evaluation of P.A.I. and α coefficients

Let us consider again Fig. 4 where it is possible to observe that at each DMP concentration the enzyme reaction rate increases with an increase in 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 ΔT. This has been done, as one example, in Fig. 6 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 ΔT. The best line

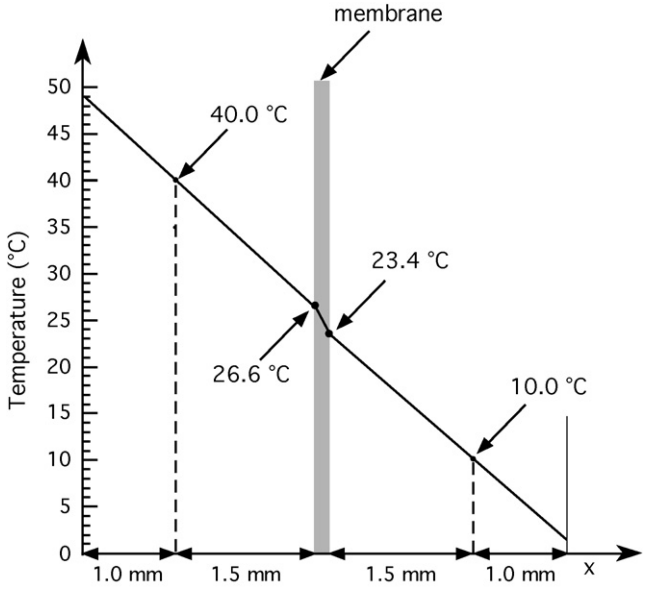


Fig. 5. Actual temperature profile into the bioreactor: temperature profile in a non-isothermal bioreactor under the following experimental conditions: $\Delta T = 30\,^{\circ}C$, $T_{av} = 25\,^{\circ}C$.

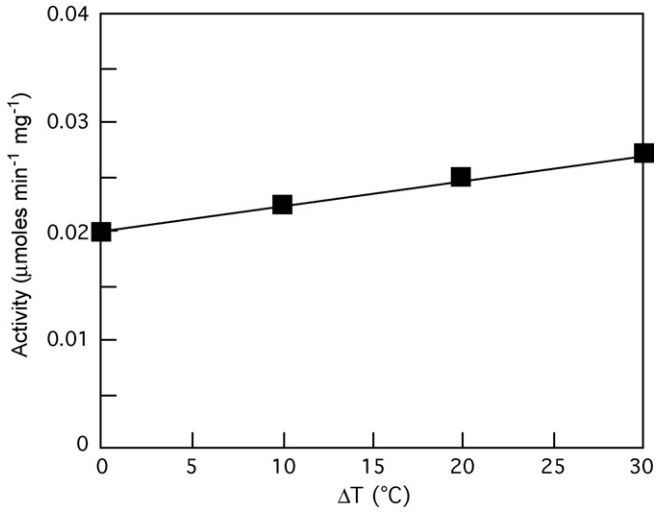


Fig. 6. Dependence of the enzyme activity on the applied ΔT: enzyme activity as a function of the temperature gradient ΔT. DMP concentration = 5 mM.

interpolating the experimental points is described by an equation of the type:

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

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 $T_{average}$.

From the above equation one obtains

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

where the α coefficient ($\%\,^{\circ}C^{-1}$) represents the percentage activity increase (P.A.I.), when a macroscopic temperature difference $\Delta T = 1\,^{\circ}C$ is read at the thermocouple positions.

Following the procedure described above, the values of P.A.I., calculated for each substrate concentration of Fig. 4, have been reported in Fig. 7a as a function of DMP concentration. Fig. 7a shows that the P.A.I. values decrease with the increase in substrate concentration. This is explained by considering that when the immobilised 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 activity.

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

The expression for α^* is:

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

Fig. 7b shows, as a function of DMP concentration, the α^* values obtained from the results in Fig. 7a. Because this process is similar to a normalisation process, overlapping curves are present. It is interesting to observe that at low concentrations of DMP (1 mM) the α^* value amounts to 24%, while at high DMP concentrations (10–15 mM) the α^* value amounts 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 $1\,^{\circ}C$ across the catalytic membrane is enough to obtain increases of over 20% in the removal of DMP concentration.

3.3.3. Evaluation of the reduction in bioremediation times under non-isothermal conditions

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 α^*or P.A.I., which are functions of ΔT^*, with the reduction in the bioremediation time, τ_r, defined as

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

where τ_{iso} and $\tau_{non\text{-}iso}$ are the time required to obtain the same percentage of DMP biodegradation under isothermal and non-isothermal conditions, respectively. To correlate τ_r with the applied ΔT^* 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.

As one example of the graphical calculation let us see Fig. 8, where we have reported, as a function of the time of enzyme treatment, the DMP decrease in the case of $T_{average} = 25\,^{\circ}C$, with $\Delta T = 0$

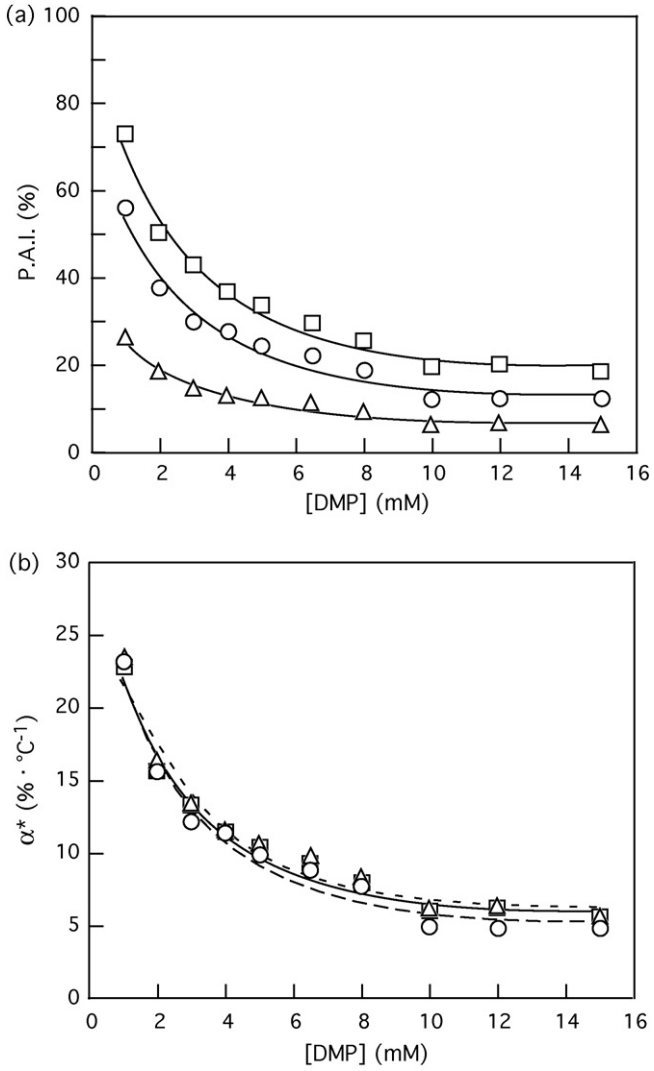


Fig. 7. Significant parameters of industrial interest: (a) percentage activity increase as a function of DMP concentration. (b) α^* coefficient as a function of DMP concentration. Symbols: (△) $\Delta T = 10\,^{\circ}C$; (○) $\Delta T = 20\,^{\circ}C$; (□) $\Delta T = 30\,^{\circ}C$.

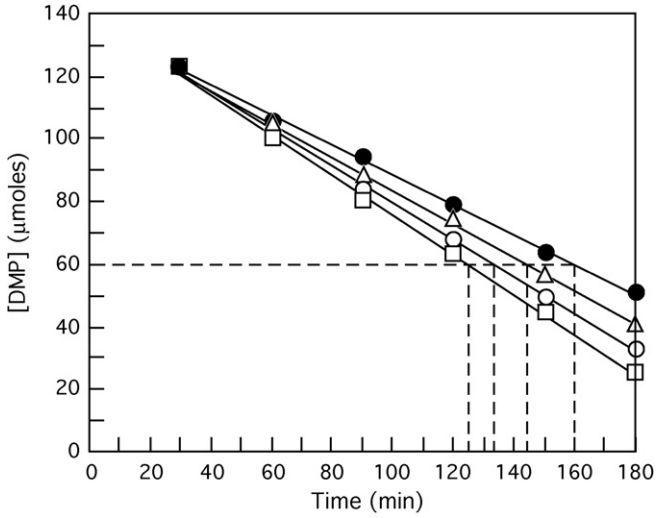


Fig. 8. Methodology for the calculation of the reduction times: DMP concentration as a function of the enzyme reaction time. Symbols: (●) $\Delta T = 0\,^{\circ}C$; (△) $\Delta T = 10\,^{\circ}C$; (○) $\Delta T = 20\,^{\circ}C$; (□) $\Delta T = 30\,^{\circ}C$.

or 10 or 20 or 30 °C. The initial DMP concentration was 5 mM. To obtain the same biodegradation of DMP, for example, a 50% reduction, 161 min are needed for the isothermal condition, while 145 or 134 or 125 min are required for $\Delta T = 10\,^{\circ}C$, $\Delta T = 20\,^{\circ}C$, $\Delta T = 30\,^{\circ}C$, respectively. It follows that a value of $\tau_r = 9.9\%$ is obtained with a $\Delta T = 10\,^{\circ}C$, a $\tau_r = 16.8\%$ with a $\Delta T = 20\,^{\circ}C$, and a $\tau_r = 25\%$ with a $\Delta T = 30\,^{\circ}C$. The τ_r values increase with an increase in the applied ΔT and therefore with the P.A.I.

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

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

one obtains after a series of mathematical steps

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

In Fig. 9a, the τ_r values obtained at different DMP concentrations with $\Delta T = 30\,^{\circ}C$ 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, of 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 τ_r is 25%.

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 Fig. 9b. Again, highlighted in black is the result relative to the DMP concentration of 5 mM. As is evident from Fig. 9b, the reduction in the biodegradation time decreases with an 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 quantise this observation, in Table 2 we have reported the percentage decreases of DMP concentration after 180 min of enzyme treatment, calculated as $R = (C_{t=0} - C_{t=180})/C_{t=0}$.

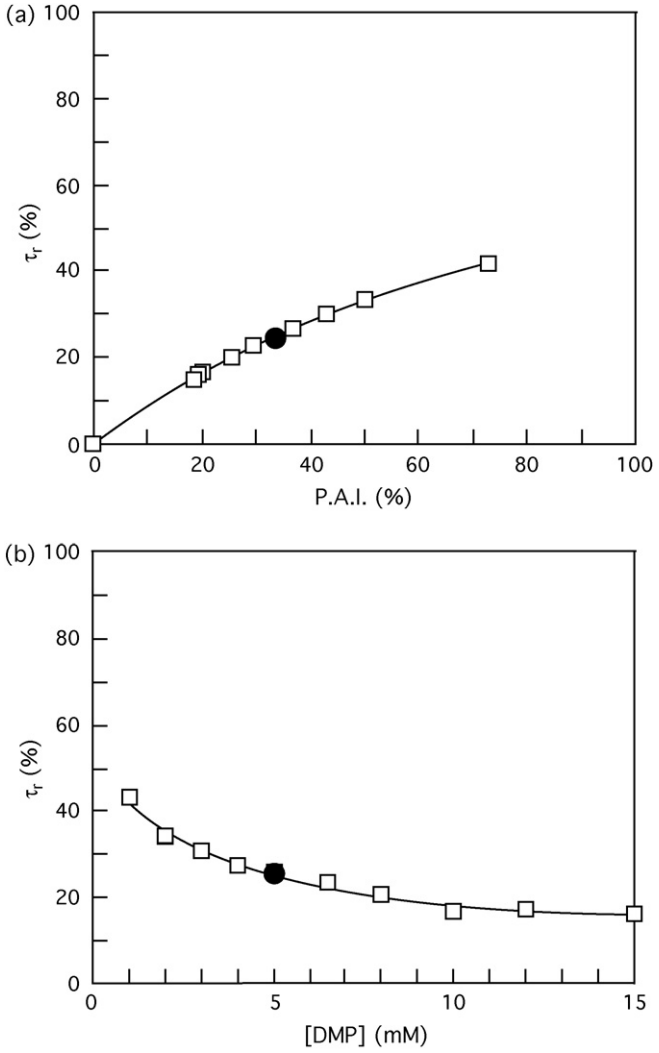


Fig. 9. Values of the bioremediation reduction times: percentage reduction of the bioremediation times (τ_r) as a function of percentage activity increase (a) and as a function of DMP concentration (b).

$C_{t=0}$ and $C_{t=180}$ are the DMP concentrations at the beginning of the experiment and after 180 min of lipase treatment, respectively.

There are other considerations with respect to the usefulness of employing a bioremediation process in place of a classical membrane remediation process. For water treatment problems in small ecosystems, the traditional membrane-based processes are not useful because they alter the local life conditions. Ultrafiltration or reverse osmosis, for example, can remove phthalates but, because the filtrate is pure water, their intake in the ecosystem alters the concentrations of salts and bioelements necessary for life. As a consequence, the ecosystem is destroyed. In contrast, the selective

Table 2
The percentage decreases of DMP concentration after 180 min of enzyme treatment under different experimental conditions.

T_{av} (°C)	ΔT (°C)	[DMP] = 1 mM	[DMP] = 3 mM	[DMP] = 5 mM	[DMP] = 8 mM
25	0	68.5%	63%	58.1%	43.4%
	10	84%	72.5%	66.1%	47.7%
	20	100%	82.7%	72.9%	51.9%
	30	100%	89.5%	79.3%	55.1%

removal of phthalates by means of enzyme treatment (bioremediation) appears suitable, because the treatment is effective only towards the harmful molecule target, leaving unaltered the concentrations of other components.

4. Conclusion

The experimental results reported above have shown the possibility of using the enzyme lipase from *C. 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.

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