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Bioremediation of waters polluted by Endocrine Disruptors by means of the process of thermodialysis.

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1. Introduction

Endocrine Disruptors Chemicals (EDCs) are compounds, synthetic or natural, so termed for their ability of interacting with the endocrine system thus inducing severe pathologies not only in exposed wildlife and humans, but also in their progeny. EDCs have been proved to induce cancer [1], to damage the male and female reproductive system [2-4], to induce obesity [5, 6] and cardiovascular diseases [7, 8]. A great number of compounds have been identified as belonging to this group of chemicals: natural and synthetic steroid hormones, phytoestrogens, alkylphenols, phthalates, pesticides, surfactants and polychlorinated biphenyls. Among the EDCs Bisphenol A (BPA) is one of the most abundant in the environment and the most studied.

Endocrine Disruptors reach the humans mainly through the food, but their presence is widespread in aqueous systems, particular in superficial waters, where they put severe risks for the preservation of the biodiversity of the species there living. Concerning BPA it enters into the environment mainly from the discharges of industrial wastewater treatment plants (WWTPs), leachates of waste plastic in landfills, processing of BPA in manufacture, and spray paints. Due to their retention and harmful effects, the fate of EDCs in the environment has become a social issue by the public community and the need of removing them from the aquatic ecosystems is an imperative.

Many physico-chemical and biotechnological methods have been used to this aim.

Physico-chemical methods include photo degradation [9], oxidation [10, 11], absorption on active carbon [12, 13] and classical membrane processes such as ultrafiltration and nanofiltration [14, 15].

Biotechnological methods are pursued by means of enzymes [16, 17], by whole microbial cells in suspension or grown as biofilm [18-20] or by

phytoremediation by means of plants [21, 22]. All these biotechnological processes are recognized as bioremediation.

Enzyme bioremediation has been proved to be the most appropriate since it selectively reduce the concentration of the harmful compound, remaining unchanged that of the other compounds.

In bioremediation processes with enzymes usually the biocatalyst is used immobilized. Immobilized enzymes offer many advantages in respect to their free counterpart. Among the advantages we recall higher resistance to extreme values of temperature and pH, major time stability and, more interesting from the industrial point of view, the possibility of reuse. The best way to immobilize the enzyme is its covalent bond to a solid carrier, for example beads, jell or membrane. Enzyme immobilization on a membrane made us to think on the possibility of applying to the catalytic composite system the process of Thermodialysis, discovered and patented by us many years ago [23] as "Process of thermal diffusion across porous partitions and relative apparatuses"

Under the name of Thermodialysis is known the process by which the component of a solution are selectively fractionated across a hydrophobic, porous and uncharged solid membrane separating two solutions, of equal or different composition, kept at different temperatures. Under these conditions the membrane per se "unselective" becomes "selective". The driving force of the process, according to the classical approach of the Thermodynamics of Irreversible Processes [24,25], is the temperature gradient which, in Gaeta's theory [26] produces differential forces on the solute and solvent molecules. From this point of view the solution in the membrane pores can be considered a microscopic Soret cell [27] where a modified thermal diffusion occurs.

The employment of catalytic hydrophobic porous membranes in bioreactors operating in the presence of a temperature gradient has been patented by us since 1992 [28]. The rationale for this process is illustrated in section 2.

2. Rationale

In Figures 1 and 2a schematic pictures of the non-isothermal bioreactor are represented. The bioreactor consists of two metallic flanges in each of which a shallow cylindrical cavity is bored, 70 mm in diameter and 2.5 mm in depth. Each half-cell, so built, constitutes the working volume filled with the aqueous solution to be treated. The catalytic membrane is clamped between the two flanges in order to separate and, simultaneously, connect the solution filling the half-cells. Solutions are circulated in each half-cell by means of two peristaltic pumps through hydraulic circuits starting and ending in the same reservoir. In this way the only changes in the solution are those relative to the substrate

interacting with the immobilized enzyme. Using independent thermostats, the half-cells are maintained at predetermined temperatures. Thermocouples placed 1.5 mm away from the membrane surfaces measure the local temperature of the solution in each half-cell, i.e. T_w (warm temperature) and T_c. (cold temperature). These measurements allow calculating the real temperature profile across the catalytic membrane. Being laminar the solution motion in each half-cell and being the Reynolds number lower than Re critical [29],the heat propagation in the bioreactor occurs by conduction between isothermal liquid planes perpendicular to the direction of the heat flow. In this case, if the thermal conductivity and thickness of both filling solutions and membrane are known, it is possible to calculate the temperatures on the membrane surfaces by applying the heat flux continuity principle. In Figure 2b the situation corresponding to T_w= 40 °C and T_c = 20°C is represented, while with T^*_w and T^*_c indicate the effective temperature at the two sides of the catalytic membrane. It has been found that $\Delta T^* = (T^*_w - T^*_c) = \beta \Delta T = \beta (T_w - T_c)$, where β with the membranes employed by us (Teflon or Nylon) has been calculated to be about 0.1.

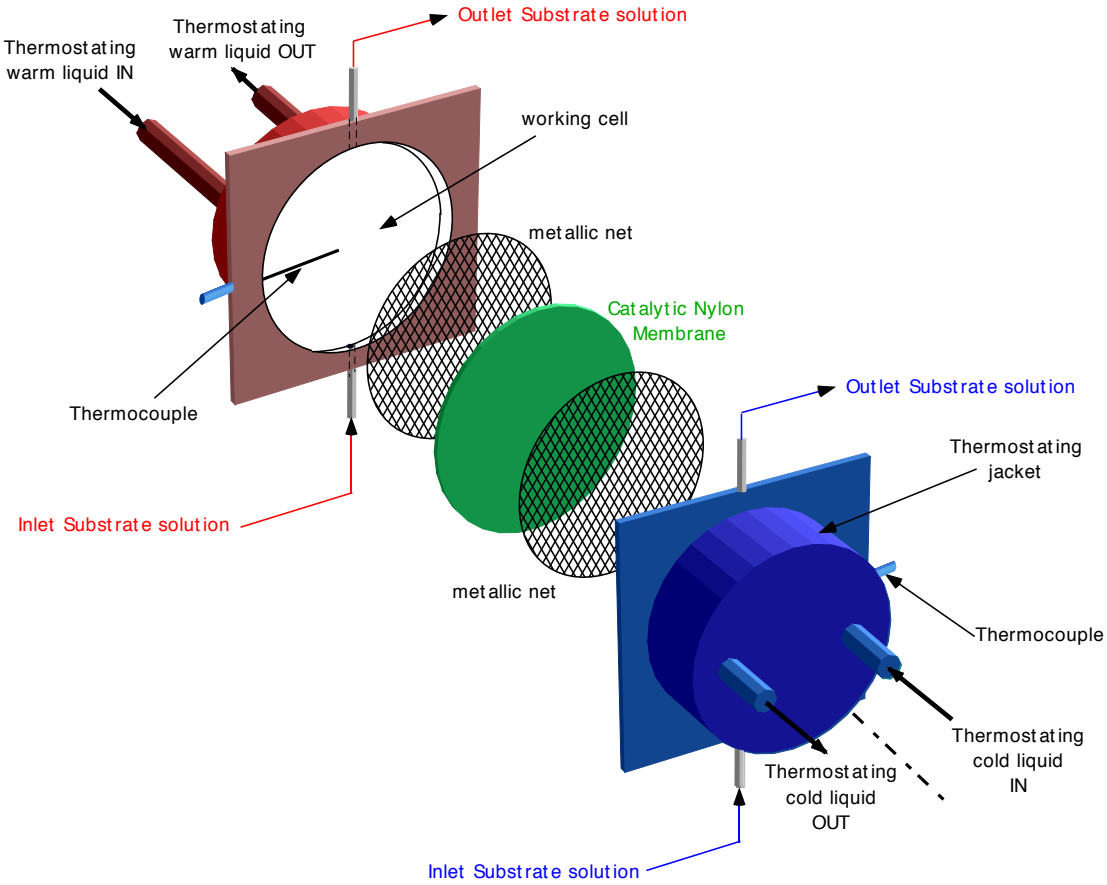

Figure 1 : Schematic representation, not to scale, of the non-isothermal membrane bioreactor

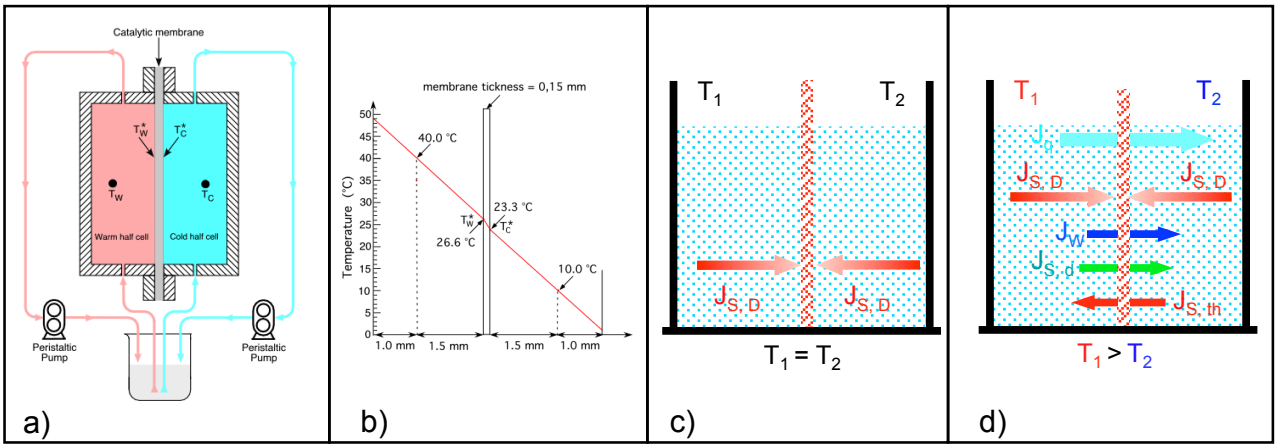

Figure 2: a) Modus operandi of the non-isothermal membrane bioreactor; b) Temperature profile across the non-isothermal membrane bioreactor; c) Diffusive substrate fluxes when the membrane bioreactor operates under isothermal conditions; Heat and matter fluxes associated to the non-isothermal membrane bioreactor.

In Figure 2 the modus operandi of an enzyme immobilized on a hydrophobic and porous membrane employed in an isothermal (Figure 2c) or non-isothermal (Figure 2d) bioreactor is also illustrated. Under isothermal conditions the enzyme interacts with the substrate molecule only by diffusion according to the Fick law. Under non-isothermal conditions the catalytic membrane, in addition to the heath flow, is crossed by the matter fluxes indicated in Figure 1d, i.e. by: i) a massive water flux, J_w expressed in cm^3/c^2 s, driven by the thermodialysis process and proceeding from the warm to the cold half-cell; ii) a substrate flux, $J_{s,d}$ expressed in moles/c^2 s, known as solute drag and associated to the water flux; and iii) a further substrate flux, $J_{s,th}$ expressed in moles/cm^2 s, driven by the temperature gradient. The analytical expressions for each of these fluxes are reported in box 1 were it is possible to see how they are proportional to the applied temperature gradient $\Delta T^*/\Delta x$ (or $\Delta T/\Delta x$). Of course the diffusive substrate fluxes, J_D, still remain. Consequently in presence of a temperature gradient the enzymes immobilized encounter, in the unit of time, more substrate molecules that in absence of a temperature gradient. Since the reaction rate is determined by the number of substrate molecules interacting in the unit of time with the catalytic site it follows that under non-isothermal conditions the reaction rate increases proportionally to the size of the temperature gradient applied across the membrane. In addition the massive water flow, J_w, produced by the process of thermodialysis plays a synergetic role in favor of the reaction rate by removing from the catalytic site the reaction product/s, so allowing the enzyme to perform more reactions in the unit of time. In other words the non-isothermal conditions increase the turnover number of the reaction proportionally to the applied transmembrane temperature gradients.

$$J_{S,D} = D_s \frac{\Delta C}{\Delta x} = \frac{cm^2}{s} \frac{moles}{cm^3} \frac{1}{cm} = \frac{moles}{cm^2 s}$$

$$J_{W,th} = \frac{1}{A} \frac{\Delta V}{\Delta t} = D^*_{W,th} \frac{\Delta T^*}{\Delta x} = \frac{cm^2}{s\ K} \frac{K}{cm} = \frac{cm}{s}$$

$$J_{S,drag} = \sigma J_{W,th} C_s = \frac{cm}{s} \frac{moles}{cm^3} = \frac{moles}{cm^2\ s}$$

$$J_{S,th} = D^*_S C_S \frac{\Delta T^*}{\Delta x} = \frac{cm^2}{s\ K} \frac{moles}{cm^3} \frac{K}{cm} = \frac{moles}{cm^2\ s}$$

Legend

D_S is the substrate diffusion coefficient $\left(\frac{cm^2}{s} \right)$; ΔV is the water volume (cm^3) crossing the membrane surface A (cm^2) in the time Δt (s); $\frac{\Delta C}{\Delta x}$ and are the concentration and temperature gradient, respectively; $D^*_{W,th}$ and D^*_S are thermal diffusion coefficients of water and substrate induced by the temperature gradients; σ is the Staverman reflection coefficient of substrate.

Box 1

3. Phenomenology of a catalytic process mediated by the process of thermodialysis.

To introduce the experimental part we firstly report the typical phenomenology of how the experimental results are handled.

In Figure 3a the typical time dependence of an enzymatic removal of a pollutant under isothermal and non-isothermal conditions is shown. Each curve is fitted by a general equation of the type:

$$C(t) = C_1 + C_2 \exp(-kt) \qquad (1)$$

were C(t) is the pollutant concentration existing at time t, $C_1 + C_2 = C_0$, i.e. the initial pollutant concentration, C_1 is the asymptotic steady state pollutant concentration in the case of enzyme inhibition by the reaction product. In absence of product inhibition $C_1 = 0$ and $C_2 = C_0$ and equation 1 becomes:

$$C(t) = C_0 \exp(-kt) \qquad (2)$$

In both equations k, measured in $time^{-1}$, is a constant indicative of the rate of substrate removal. k depends from the nature and concentration of the substrate, from the isothermal or non-isothermal conditions. Interesting enough the plot of the k values as a function of ΔT (or ΔT^*), at fixed pollutant concentration, exhibits (Figure 3b) a linear dependence increasing with the increase of ΔT. Other interesting parameters that can be derived by the results in Figure 3a and from equations 1 or 2 are the times requested to halve ($\tau_{c=0,5\ co}$) or to eliminate ($\tau_{c=0}$) the pollutant initial concentration. Both parameters (Figures 3c and 3d) decrease with the increase of the applied ΔT, confirming that under non-isothermal conditions the enzyme reaction rate is greater in respect to that of the isothermal conditions. These results can be attributed exclusively to the process of thermodialysis. Indeed in the absence of any flux driven by the temperature gradient and considering only the temperature dependence of the enzyme activity, the small temperature difference across the membrane faces suggests that what is gained on the warm surface of the catalytic membrane it's exactly lost on its cold face.

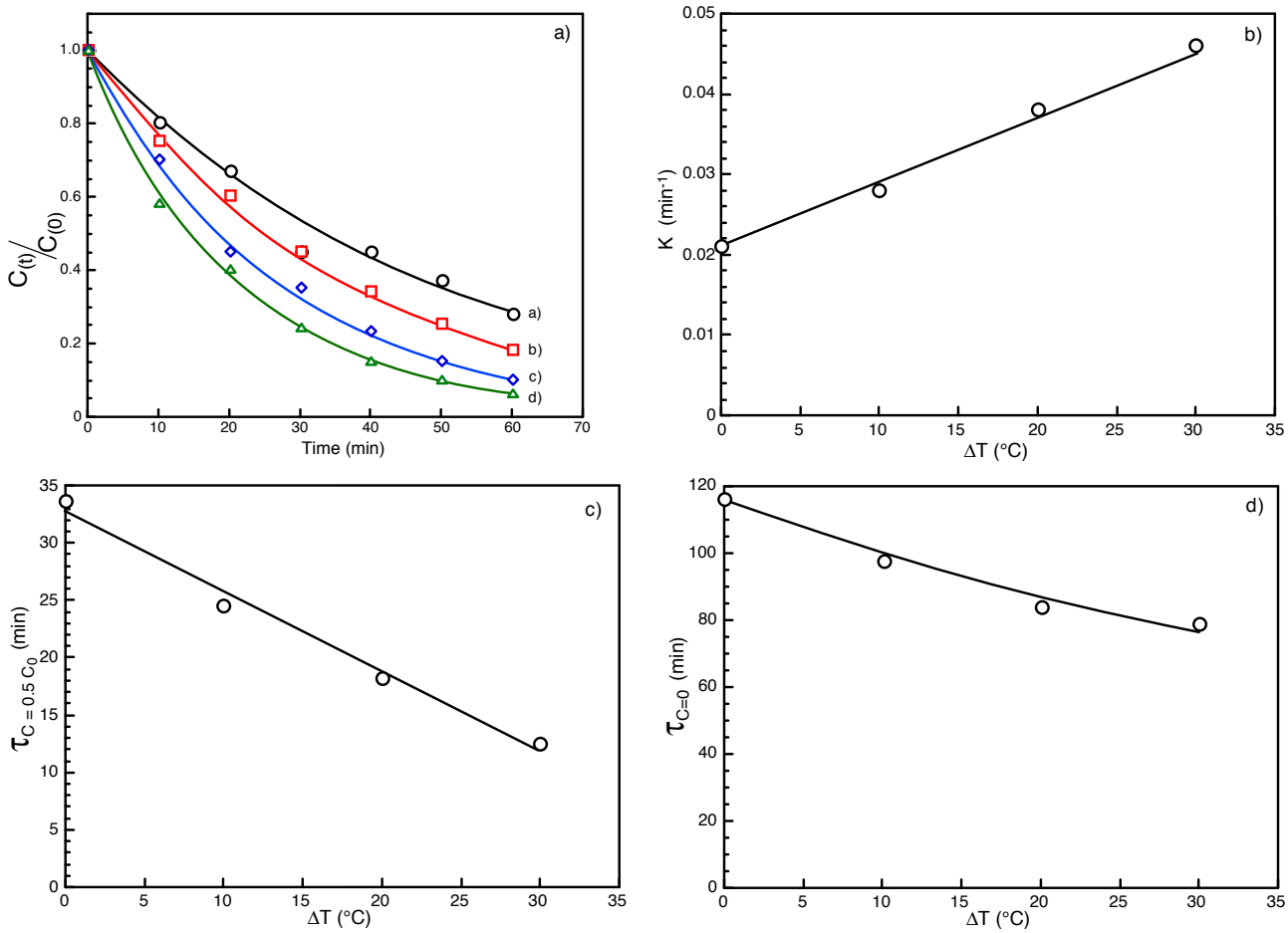

Figure 3: a) Normalized substrate concentration as a function of time under ΔT = 0°C (○○), ΔT = 10 °C (●●), ΔT = 20 °C = (✱✱), ΔT = 30 °C (□□); b) k values as a function of the applied ΔT; c) $\tau_{c=0.50\ c0}$ as a function of the applied ΔT; d) $\tau_{c=0}$ as a function of the applied ΔT.

4) Actual results

From the curves similar to those reported in Figure 3a it is possible to derive the initial enzyme reaction rate, expressed in moles/cm^2 s, by multiplying the value of the tangent to the curve in the initial part, i.e. the value of Δc/Δt for t near to zero, for the volume of treated solution. Following this approach one obtains the actual results reported in Figure 4, shoving, as a function of BPA concentration, the initial reaction rates under isothermal and non-isothermal conditions when laccase (Figure 4a) or tyrosinase (Figure 4b) were covalently immobilized on a hydrophobic nylon membrane (Hydrolon from Pall), 150 µm thick and 0.2 µm nominal pores, chemically grafted with glycidyl methacrylate. Detailed description of technologies used for membrane activation and enzyme immobilization can be found in reference [30].

Results in Figures 4a and 4b show: i) a Michaelis-Menten behavior either under isothermal or under non-isothermal conditions; ii) at each BPA concentration the initial enzyme reaction rate under non-isothermal conditions is greater than the one under isothermal conditions.

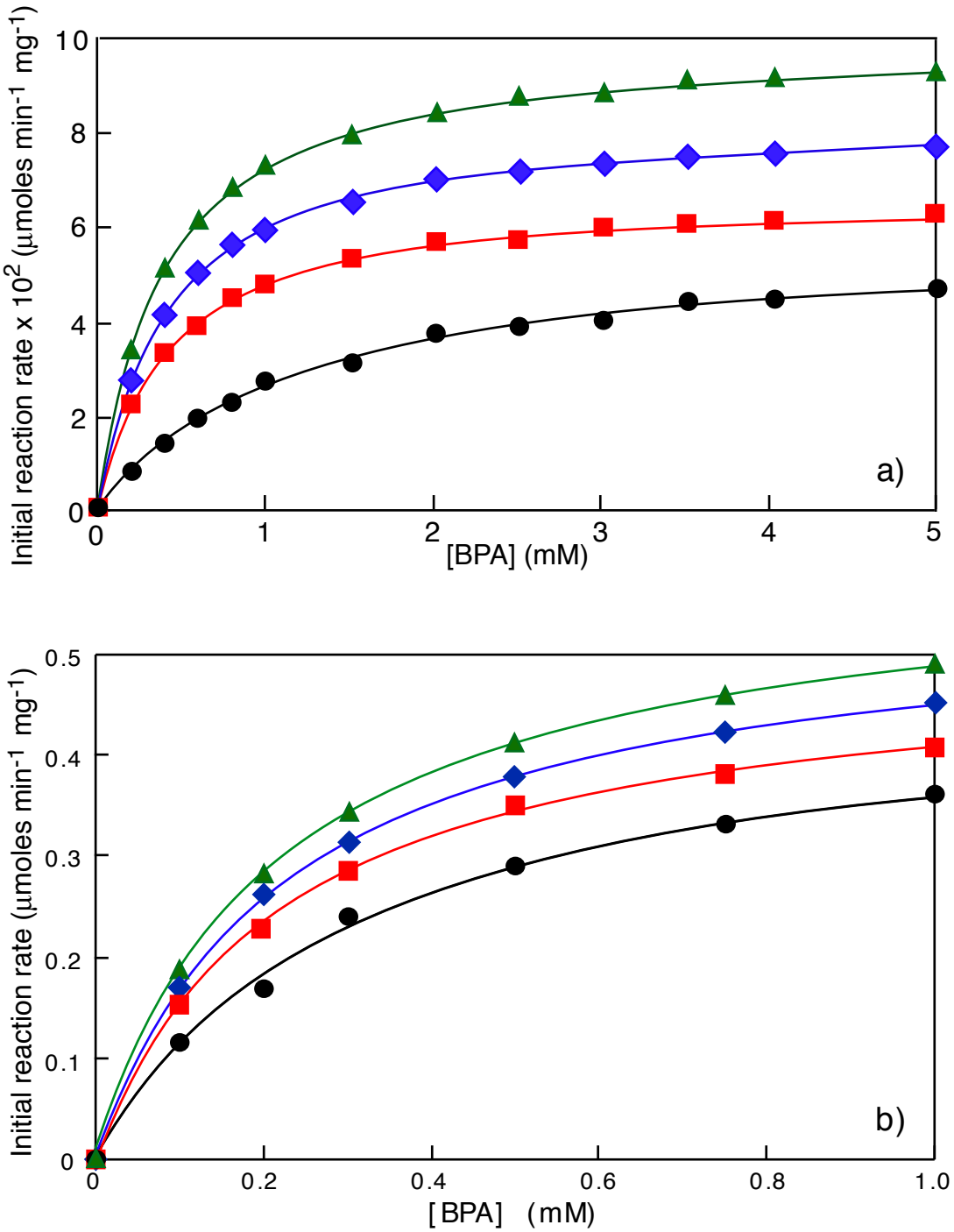

Figure 4: Initial reaction rate as a function of BPA concentration in the case of immobilized laccase (a) or immobilized tyrosinase. Symbols ΔT = 0°C (), ΔT = 10 °C (), ΔT = 20 °C = (), ΔT = 30 °C (). Adapted from reference [30].

By plotting as a function of the applied ΔT the values of the initial reaction rate at each concentration one obtains the results of Figure 5 where the cases of BPA concentrations equal to 0.4 mM and to 4 mM are reported. The results refer to the treatment with laccase. The same occurs for all the concentrations explored and also when the results relative to the tyrosinase are considered.

Being linear the dependence between the initial reaction rate and the ΔT one can write:

$$y_{non\text{-}iso}(c) = y_{iso}(c)\left[1 + \alpha\Delta T\right] \tag{3}$$

where $y_{non\text{-}iso}(c)$ and $y_{non\text{-}iso}(c)$ are the values of the initial reaction rate under non-isothermal and isothermal conditions at the pollutant concentration (c) and α represents the Percentage Activity Increase (P.A.I.) of the initial enzyme activity when the temperature difference between the thermocouples position is 1°C. The expression for α is:

$$\alpha\left(\%, {}^{o}C^{-1}\right) = \frac{y_{non\text{-}iso}(c) - y_{iso}(c)}{y_{iso}(c)}\frac{1}{\Delta T} = \frac{P.A.I(c)}{\Delta T} \tag{4}$$

When the actual temperature difference ΔT* across the two membrane surfaces is considered, one obtains:

$$\alpha^{*}\left(\%, {}^{o}C^{-1}\right) = \frac{y_{non\text{-}iso}(c) - y_{iso}(c)}{y_{iso}(c)}\frac{1}{\Delta T^{*}} = \frac{P.A.I(c)}{\Delta T^{*}} \tag{5}$$

where α* is the percentage activity increase when the transmembrane ΔT* is equal to 1. Considering the expressions (4) and (5) one obtains:

$$\alpha^{*} = \alpha\frac{\Delta T}{\Delta T^{*}} = \frac{P.A.I(c)}{\Delta T^{*}} \tag{6}$$

In Figure 6 the α* values obtained by elaborating the results in Figures 4a and 4b have been reported as a function of BPA concentration. It is interesting to observe that the percentage activity increases (the α* are proportional to P.A.I.) induced by the temperature difference on laccase activity are about five times higher than those induced on tyrosinase. Calculations have been reported only for the common BPA concentration range explored with laccase and tyrosinase.

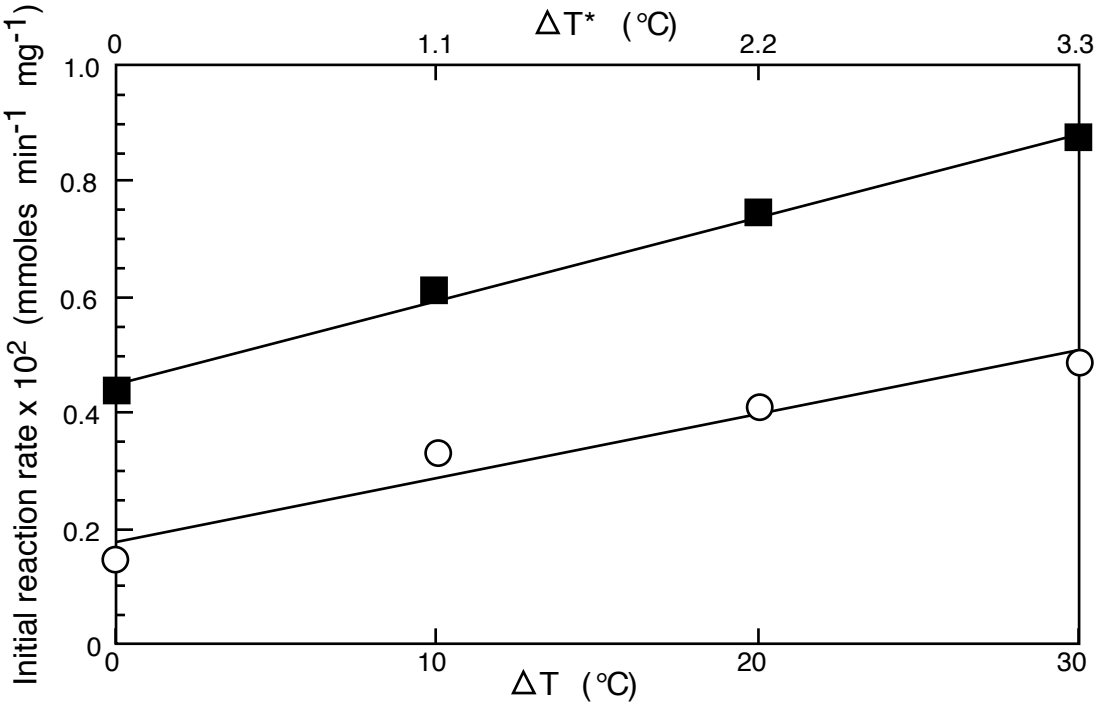

Figure 5: Initial reaction rate of immobilized laccase as a function of ΔT (or ΔT*) as a function of BPA concentration equal to 0.4 (○○) or 4.0 mM (●●). Adapted from reference [30].

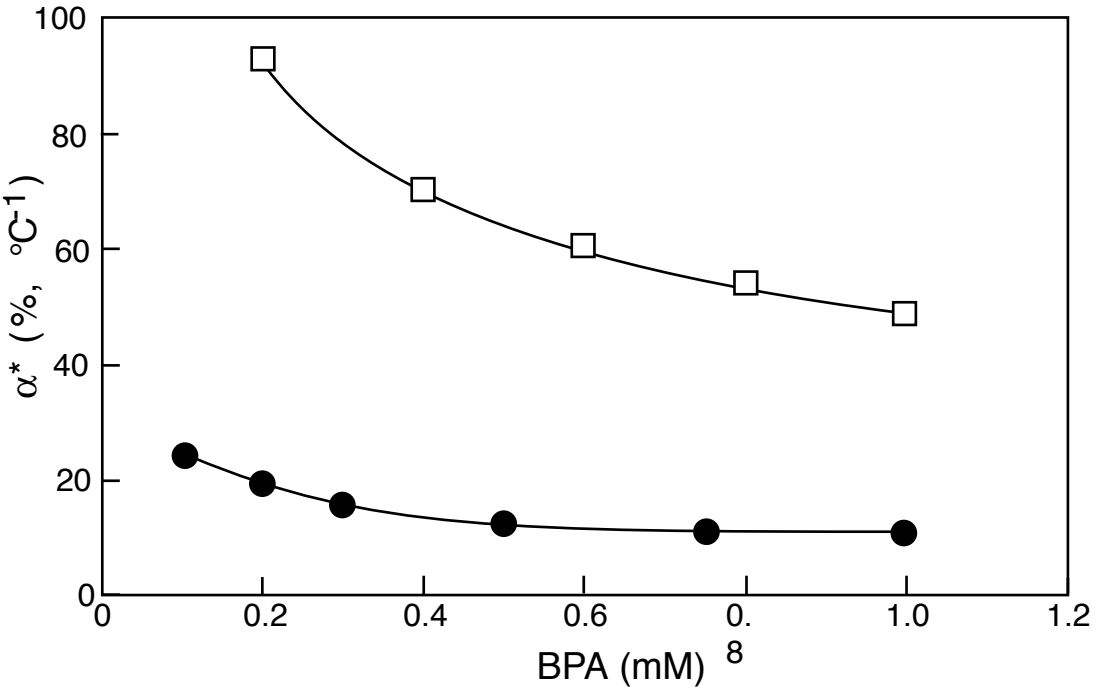

Figure 6: α*values for immobilized laccase (●●) or tyrosinase (○○) as a function of BPA concentration. Adapted from reference [30].

5) Practical considerations

All the results above reported clearly indicate that: 1) it is possible to bioremediate aqueous systems polluted by BPA by using immobilized laccase or tyrosinase; ii) the bioremediation efficiency is increased when the immobilized catalyst operates under non-isothermal conditions; iii) under non-isothermal conditions the laccase results more efficient than the tyrosinase.

To better quantify the effect of the transmembrane temperature gradient it is interesting to correlate, at each BPA concentration, the α*value with the reduction of the bioremediation time, τ_r, defined as

$$\tau_r(\%) = \frac{\tau_{iso} - \tau_{non\text{-}iso}}{\tau_{iso}} \times 100 \qquad (7)$$

where $\tau_{non\text{-}iso}$ and τ_{iso} are the times required to obtain the same percentage of pollutant removal under non isothermal and isothermal conditions, respectively. The same value of BPA removal, i.e. the same value of BPA concentration under non-isothermal ($c_{non\text{-}iso}$) and isothermal (c_{iso}) conditions is reached, i.e. when

$$y_{iso}(c)\tau_{iso} = y_{non\text{-}iso}(c)\tau_{non\text{-}iso} \qquad (8)$$

Remembering equation 2, one can write:

$$y_{iso}(c)\tau_{iso} = y_{iso}(c)[1+\alpha\Delta T]\tau_{non\text{-}iso} \qquad (9)$$

by which, taking in account equation (1) and equation (2) one obtains:

$$\tau_r(\%) = \frac{PAI}{1+PAI} \times 100 \qquad (10)$$

Equation 10 is an equation similar to that of an equilateral iperbola whose generic plot of τ_r as a function of P.A.I. is represented by the continuous line reported in Figures 7a and 7b. Looking at these figures it is also possible to appreciate how the experimental points obtained with the laccase (Figure 7a) or with tyrosinase (Figure 7b) immobilized exactly fit with the theoretical curve. The indication coming out from figures 7 is that the reduction of the bioremediation times is function of the percentage increase of the enzyme activity (P.A.I.) and therefore of the temperature difference applied across the catalytic membrane. This is a clear demonstration of the effectiveness of the thermodialysis process on the activity of an enzyme immobilized on a hydrophobic porous membrane. A confirmation of this conclusion comes out from the results of Figure 8 where the reduction of bioremediation times is

reported as a function of the BPA concentration. Interesting enough the reduction of the bioremediation times decrease with the increase of BPA concentration and this result is expected by considering that when an enzyme works at high substrate concentrations the additional substrate fluxes driven by the thermodialysis process are less effective than those occurring at low concentrations.

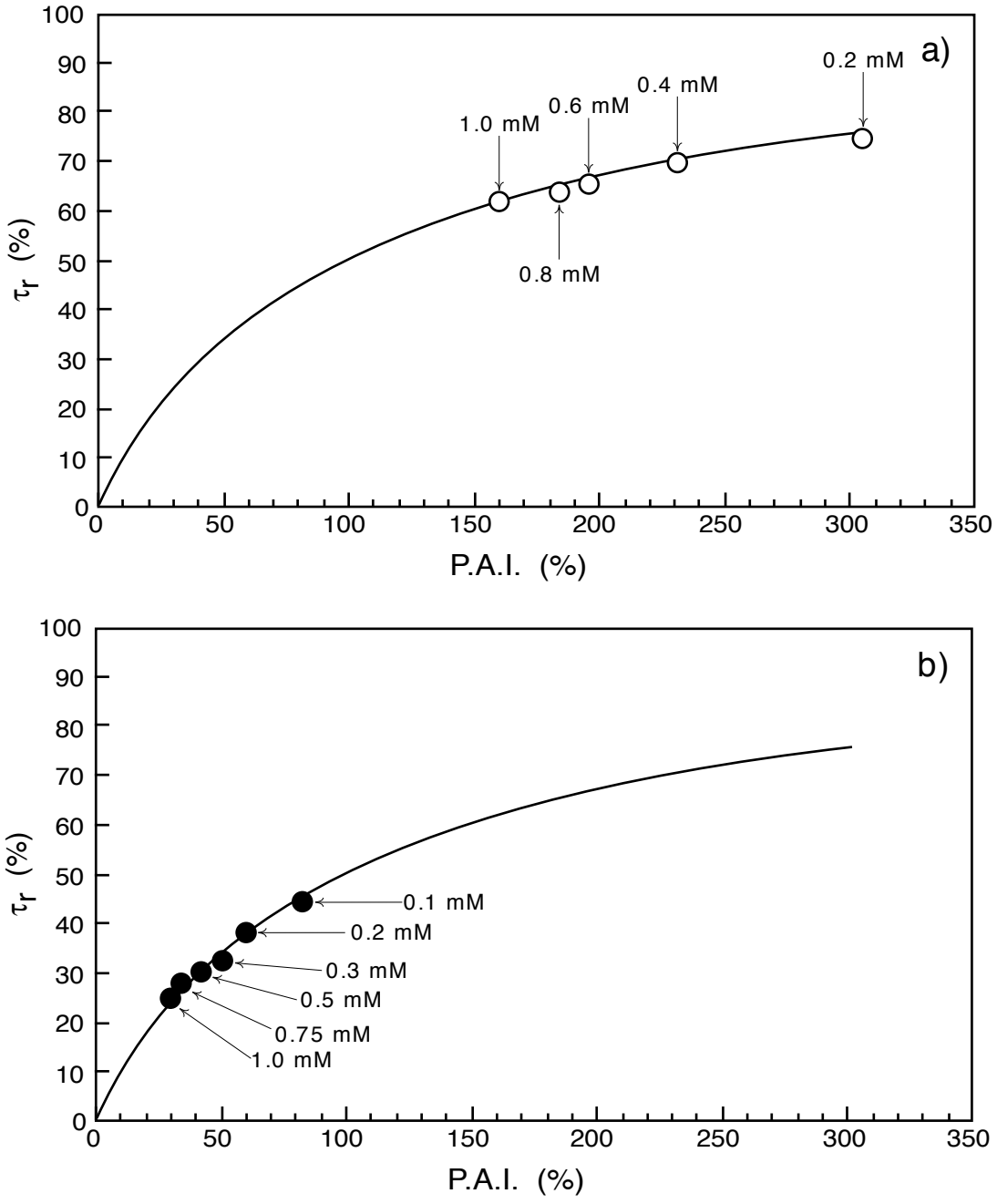

Figure 7: Bioremediation reduction times as a function of Percentage Activity Increase (P.A,I.) for immobilized laccase (◔◔) or immobilized tyrosinase (◕◕). Adapted from reference [30].

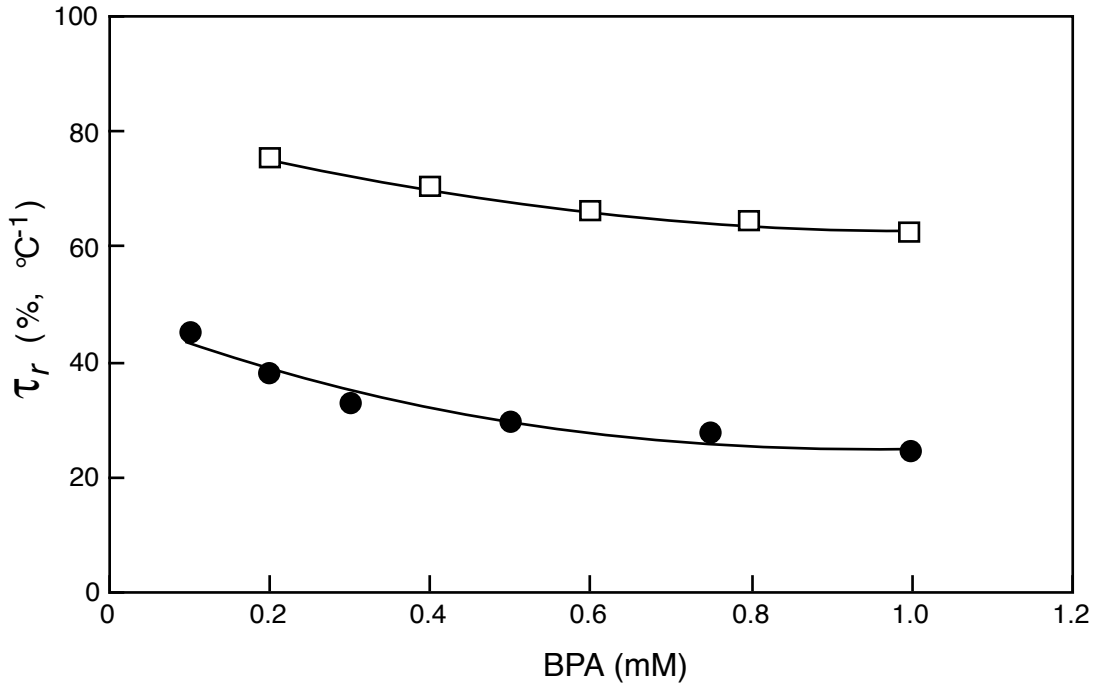

Figure 8: Bioremediation reduction times as a function of BPA concentration for immobilized laccase (**00**) or immobilized tyrosinase (ஜஜ). Experimental conditions: T_{av} = 20 °C and DT 0 30°C. Adapted from reference [30].

All together these results are important from the ecological point of view since in the present case mM concentrations have been used, while those existing in nature (in superficial and deep waters as well as in biological fluids) are of the order of nM.

At this point the biologist has a question: the clean-up is real? From the chemistry point of view the bioremediation is complete when the pollutant concentration becomes zero, but this is not completely true for a biologist particularly when the pollutant is an endocrine disruptor insomuch the reaction products can exhibit estrogenic activity more powerful than its parent. To solve this question we have measured the proliferation of MCF-7 cell in presence of the same BPA solution enzyme treated or untreated. The initial BPA concentration was 0.2 mM and the enzyme treatment lasted 36 h. Of course negative and positive controls were used. The crystal violet assay was employed. In figure 9 the results of this investigation are reported. Cells were grown in a multiwell for 48 h. Panel A is a picture of the natural cell growth, panel B represents the cell growth with the buffer employed in the bioreactor during the enzyme reaction, panel C represents the cell grown in presence of untreated BPA solution, Panel D indicates the cell grown in presence of the initial BPA solution enzyme treated for 36 h. A simple inspection to the figure

shows that the cell growth under condition C is strongly inhibited in respect to panel A and B, while panel D shows the same cell growth than in panel A and B.

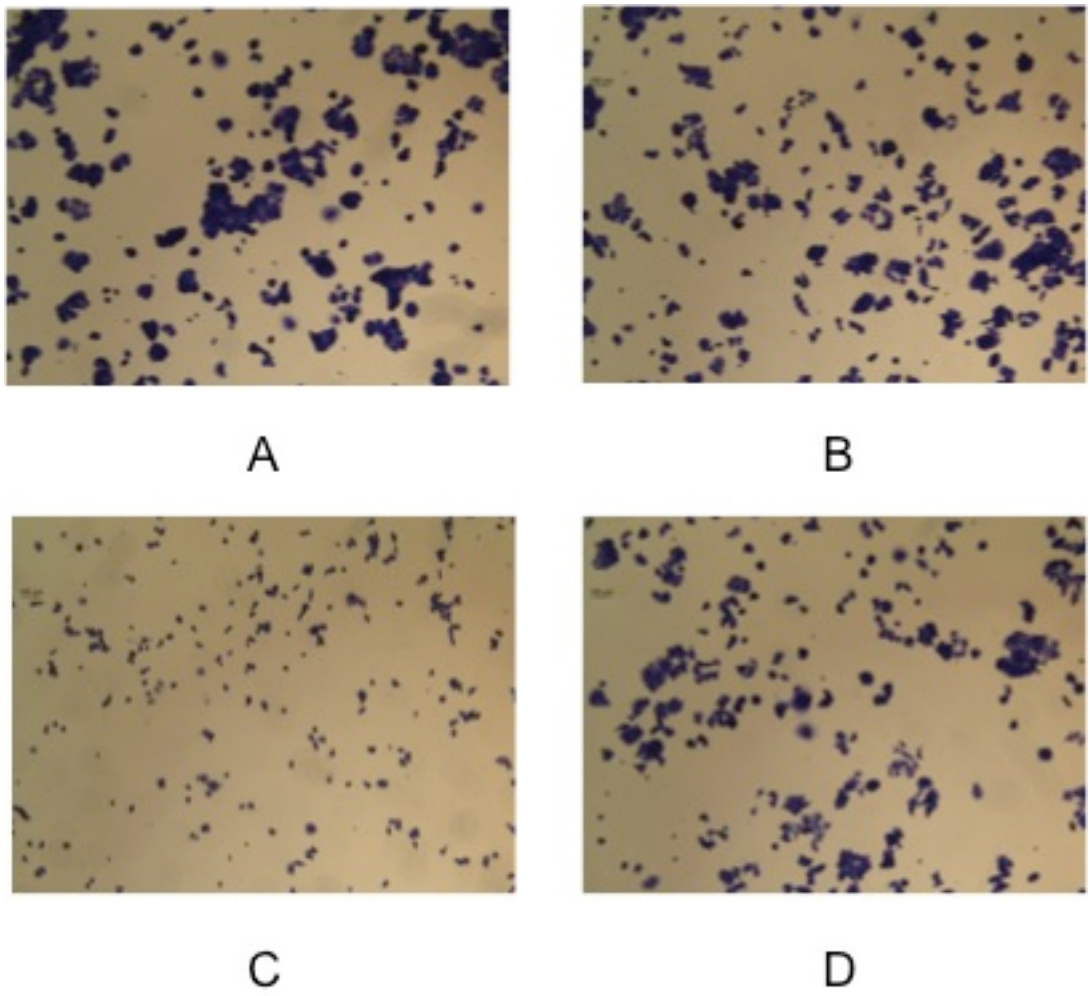

Figure 9: Inverted microscope images showing the effect of bioremediation on MCF-7 cells growth after 48 hour of incubation: (A) control, (B) veicle, (C) pre-bioremediation, (D) post-bioremediation.

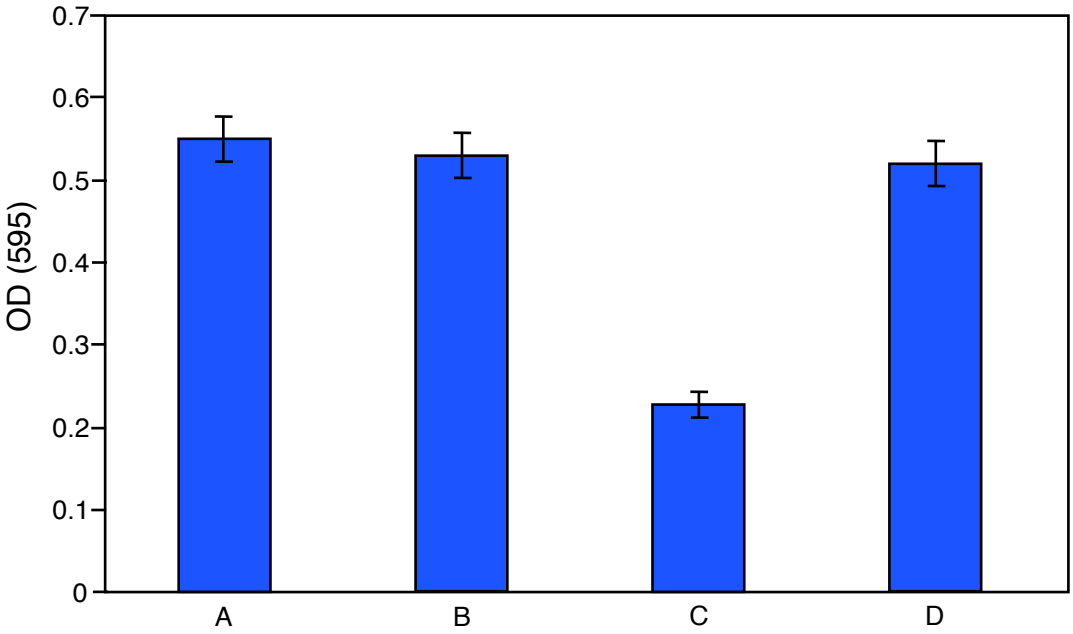

Figure 10: Crystal Violet assay: Measure, as optical density at 595 nm, of MCF-7 cells proliferation after 48 hour of incubation: (A) control, (B) veicle, (C) pre-bioremediation, (D) post-bioremediation.

In Figure 10 the results of Figure 9 are reported as optical densities after the assay with the crystal violet method.

At this point to give an idea of all the work done by us using our patented process in Table 1 a list of the different catalytic systems used in the last years is reported.

Table 1:

Some catalityc systems employed in non-isothermal membrane bioreactors after 2000.

Enzyme	Substrate	Membrane	Membrane activation	Material grafted	Spacers and coupling agent	Reference
β-galactosidase from *Aspergillus Oryzae*	Lactose	teflon	γ-radiation	MAA and HEMA	HMDA and GA	31
Penicillin G acylase from *E. Coli*	Cephalexin	nylon	γ-radiation	MMA	HMDA and GA	32
β-galactosidase from *Aspergillus Oryzae*	Lactose	nylon	Chemical	MAA	HMDA and GA	33

β-galactosidase from *Aspergillus Oryzae*	Lactose	nylon	Chemical	Sty-MAA	HMDA and GA	33
β-galactosidase from *Aspergillus Oryzae*	Lactose	nylon	Chemical	BMA	HMDA and GA	34
β-galactosidase from *Aspergillus Oryzae*	Lactose	nylon	Chemical	BMA	HMDA and GA	35
Urease	Urea	nylon	Ghemical	BMA	HMDA and GA	36
Penicillin G acylase from *E. Coli*	Cephalexin	nylon	Chemical + γ radiation	MMA	HMDA and GA	37
β-galactosidase from *Aspergillus Oryzae*	Glucose	nylon	Chemical	BMA at different concentrations	HMDA and GA	38
β-galactosidase from *Aspergillus Oryzae*	Glucose	nylon	Chemical	GMA	PDA and diazotization	39
β-galactosidase from *Aspergillus Oryzae*	Glucose	nylon	Chemical	GMA	PDA and condensation	39
Penicillin G acylase from *E. Coli*	PGME and 7-ADCA	nylon	Chemical	BMA	HMDA and GA	40
Urease from Jack beans	urea	nylon	Chemical	MMA	HMDA or HZ with GA	41
β-galactosidase from *Aspergillus Oryzae*	lactose	Nylon	Chemical	GMA	[HMDA or HTDA or HZ] + GA	42
Urease from Jack beans	urea	nylon	Chemical	CHMA	HDMA and GA	33
Laccase from *Rhus Vernicifera*	Quinol	nylon	Chemical	GMA	HMDA	44
β-galactosidase from *Aspergillus Oryzae*	Milk	Polypropylene hollow fibers	Chemical	HMDA	HMDA and GA	45
Laccase from *Trametes Versicolor*	Syringic acid	nylon	Chemical	GMA	PDA	46
Laccase from *Trametes Versicolor*	BPA	nylon	Chemical	GMA	PDA	47
Laccase from *Rhus Vernicifera*	Phenol	Polypropylene	Chemical with cromic acid	none	EDA and GA	48
Acetylcholinesterase	Acetylcholine iodide	PAN membranes	Chemical	EDA	With or without chitosan	49
Lipase from *Candida Rugosa*	Dimethylphthalate	Polypropylene	Plasma reactor	Acrylic Acid and He	PDA	50

Symbols:MAA: Methacrylic Acid – HEMA: Hyddroxyethyl Methacrylate - BMA = Butyl Methacrylate - HMDA: Hexamethylenediamina – GA: Glutharaldeyde – MMA: Methyl Metacrylate - Sty = Styrene - HZ= Hydrazine – EDA: Ethylenediamine - CHMA = Cyclohexyl methacrylate - GMA = Glycidil Methacrylate, PDA = Phenylenediamine

Summing up it is possible to conclude that the bioremediation process carried out by means of enzymes immobilized on hydrophobic porous membranes
operating in non-isothermal bioreactors is more advantageous in respect to the same catalytic membrane operating in isothermal bioreactors.

6)
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