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Non-isothermal cephalexin hydrolysis by penicillin G acylase immobilized on grafted nylon membranes

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Abstract

A new catalytic membrane has been prepared using a nylon membrane grafted by γ-radiation with methylmethacrylate (MMA) and using hexamethylenediamine (HMDA) as spacer. Penicillin G acylase (PGA) and cephalexin were employed as catalyst and substrate, respectively. Cephalexin hydrolysis was studied in bioreactors operated under isothermal and non-isothermal conditions. A hydrolysis increase was found when the temperature of the warm membrane surface was kept constant and the temperature of the other membrane surface was kept at a lower value. The hydrolysis increase was linearly proportional to the applied temperature difference. Cephalexin hydrolysis increased to about 10% when a temperature difference of 1°C was applied across the catalytic membrane. These results have been attributed to the non-isothermal cephalexin transport across the membrane, i.e., to the process of thermodialysis. In this way, the enzyme immobilized on and into the membrane reacts with a substrate concentration higher than that produced by simple diffusion under isothermal conditions.

Keywords: Immobilized enzymes; Biocatalytic membranes; Penicillin G acylase; γ-Radiation; Non-isothermal bioreactors; Cephalexin

1. Introduction

The design and construction of new catalytic membranes represent one of the growing areas in the study of applications of immobilized enzymes to biotechnological processes. Transfer of the results from the research laboratories to industry gives, sometimes, origin to negative surprises [1]. The immobilization process is a critical fundamental step since allowing easy separation of the enzyme from the reaction mixtures often improves catalyst stability at elevated temperatures and at extreme pH values.

One of the most interesting enzymes used in biotechnological processes is penicillin G acylase (PGA). This enzyme plays a relevant role

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in pharmaceutical industries, since it catalyzes the synthesis of important intermediates in the production of semi-synthetic penicillins and cephalosphorines [2,3], as well as in the resolution of racemic mixtures [4].

Many papers have been recently published [5–9] on the immobilization of penicillin acylase using different polyacrylic carriers and different immobilization methods. Recently, we have also gained interest in the immobilization of this enzyme [10]. The biochemical and biophysical characterization of the immobilized enzyme derivatives, prepared until now, has so far only been done under isothermal conditions.

Recently, it has been found [11–17] that it is possible to increase the activity of membrane-bound enzyme by operating under non-isothermal conditions. The activity of the catalytic membrane increases from 20 to 40% when a temperature difference of 1°C is applied across it. The activity increase depends on the enzyme and immobilization methods used. The results here referred to were obtained with purified enzymes as well as with immobilized cells. In the latter case, the activity of both internal and cell wall-bound enzymes was studied.

A prerequisite for obtaining these results was the presence of a hydrophobic teflon membrane coupled to a hydrophilic catalytic membrane. The hydrophobic Teflon membrane in the presence of a temperature gradient induces transmembrane mass transport of substrate and products by the process of thermodialysis [18–22]. This process consists in the selective mass transport across a hydrophobic membrane separating two identical or different aqueous solutions, maintained at different temperatures. Thermodialysis is considered as one of the physical causes inducing the observed increase of the enzyme reaction rate when catalytic membranes are employed in non-isothermal bioreactors.

With the aim of obtaining membranes, both catalytic and hydrophobic, we have recently modified commercial Teflon or nylon membranes by means of a double grafting technique and by using γ-radiation [23–27]. One of these membranes, loaded with β-galactosidase, has been successfully employed in non-isothermal bioreactors [27].

The present work aims to study under isothermal and non-isothermal conditions the behaviour of PGA immobilized onto a nylon membrane grafted with methylmethacrylate (MMA) and using hexamethylenediamine (HMDA) as spacer. From this new membrane, we expect two advantages. The first concerns the replacement of the double membrane system, one hydrophobic and the other catalytic, employed until now in non-isothermal bioreactors with a single membrane carrying out, at the same time, catalysis and thermodialysis. The other concerns the realization of a process by which it is possible to increase the hydrolysis of cephalexin in industrial bioreactors, thus reducing the production time with all the consequent economical implications. Here, the hydrolysis of cephalexin is used as model reaction.

2. Experimental

2.1. The bioreactor

The apparatus employed consisted of two cylindrical half-cells (Fig. 1), filled with the working solution and separated by a planar membrane. Solutions containing the substrate were recirculated in each half-cell by means of a peristaltic pump through hydraulic circuits starting and ending in the common cylinder C. Each half-cell was thermostatted at a temperature T_i $(i = 1, 2)$. When the apparatus worked under isothermal conditions, T_1 was equal to T_2. Thermocouples, placed at 1.5 mm from each of the membrane surfaces, measured the temperatures inside each half-cell and allowed the calculation of the temperature profile across the catalytic membrane when the apparatus was kept under non-isothermal conditions. The temperatures measured by the thermocouples are indicated by the symbol T, the ones calculated

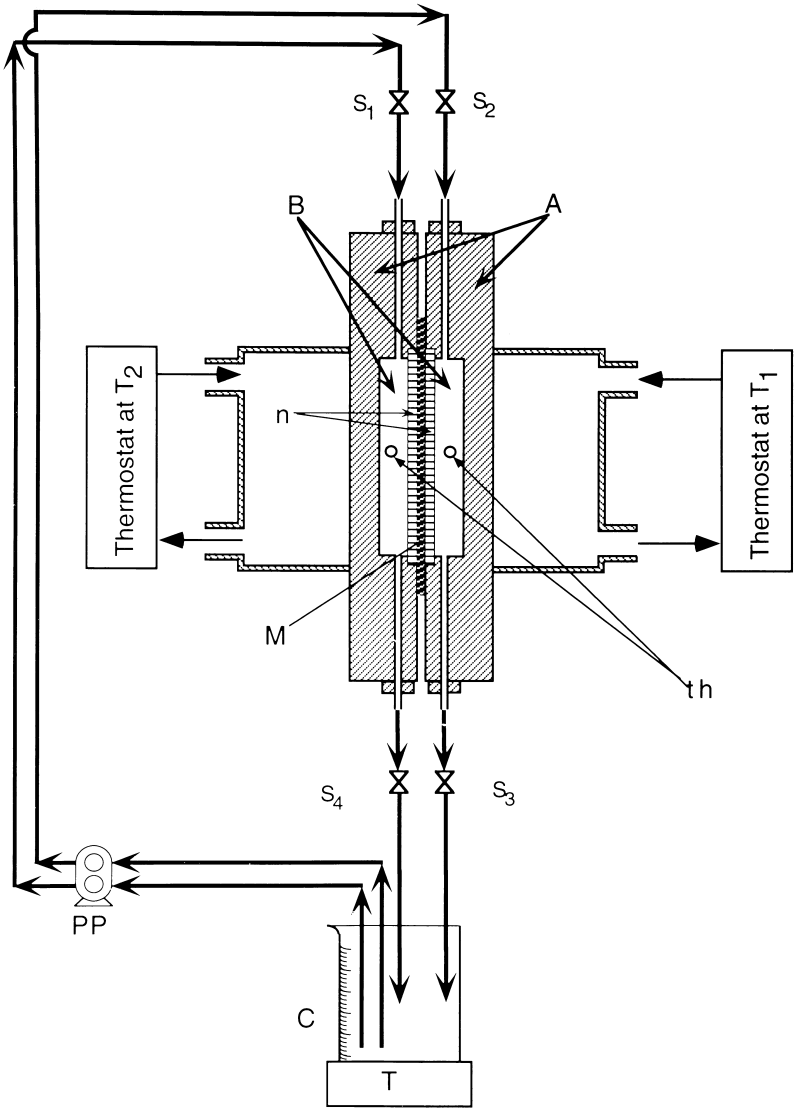

Fig. 1. Schematic (not to scale) representation of the bioreactor. (A) Half-cells; (B) internal working volumes; (C) external working volume; (M) membrane; (n) supporting nets; (th) thermocouples; (S_i) stopcocks; (T) thermostatic magnetic stirrer; (PP) peristaltic pump.

at the membrane surfaces are indicated by the symbol T^*. The values related to the warm and cold side, respectively, will be indicated by the subscripts w and c. Under these assumptions, $\Delta T = T_w - T_c$ and $\Delta T^* = T_w^* - T_c^*$, as well as $T_{av} = (T_w + T_c)/2$ and $T_{av}^* = (T_w^* + T_c^*)/2$. In non-isothermal experiments $T_w^* < T_w$, $T_c^* > T_c$, and $\Delta T^* < \Delta T$.

The correlation between the temperatures read by the thermocouples and the actual temperatures on the catalytic membrane surfaces will be given in the experimental part.

2.2. Materials

As solid support to be grafted, we used nylon Hydrolon membranes, a precious gift from the Italian Division of Pall (Pall Italia, Milano, Italy). These membranes are hydrophobic and have a nominal pore size of 0.2 μmol. The pore

size is related to the size of the minimum value of the diameter of the smallest particles that the membrane retains, since in the membrane there are no ''classical'' pores but irregular cavities crossing the membrane thickness.

All the chemicals, excluding the enzyme and its substrate, were purchased from Sigma (Sigma-Aldrich, Milano, Italy) and used without further purification. As monomer to be grafted, MMA was used. HMDA (70% aqueous solution) was used as spacer between the grafted membrane and the enzyme. A 2.5% glutaraldehyde (GA) aqueous solution was employed as bi-functional agent for covalently coupling the enzyme to the activated membrane.

The PGA and its substrate, cephalexin, were a gift from Gist-brocades, Delft, and DSM, Geleen, The Netherlands. The enzyme specific activity, with cephalexin, was 250–300 μmol min^{-1} ml^{-1} for the original enzyme solution. The cephalexin has a purity of 92.5% w/w, 6% (w/w) being water and the remaining substances being impurities.

The PGA hydrolyses the cephalexin to phenyl glycine (PG) and 7-aminodesacetoxy cephalosporanic acid (7-ADCA).

2.3. Methods

2.3.1. Membrane grafting

Membrane grafting was done by irradiation with γ-rays. The irradiation source was Caesium 137 in a gammacell 1000 Elite by Nordion International, Canada. The average dose rate in the core of the radiation chamber (central dose rate) was 2.35×10^4 rad h^{-1}.

The nylon membranes to be grafted were immersed in a solution of ethanol:water (1:1 v/v) containing 10% (v/v) of MMA and irradiated for 8 h. After this treatment, the membranes were washed with water to remove the homopolymers adhered to its surface and after that soaked in acetone for about 1 h to swell the membranes and allowing the release of the included homopolymer. After a further washing with water, the membrane was dried to estimate the value of the grafting percentage obtained. To evaluate the percentage of grafting, we adopted the classical definition used for this parameter. The degree of grafting (X, %) was determined by the difference between membrane mass before, G_B, and after, G_A, the grafting according to the expression:

$$X(\%) = \frac{G_A - G_B}{G_B} \times 100.$$

The result of all these steps is a nylon-poly-MMA grafted membrane ready to be activated for enzyme binding.

2.3.2. Membrane activation

To activate the grafted membranes, they were soaked in a 10% HMDA (v/v) aqueous solution for 1 h at room temperature. After a further washing with water, the aminoalkylated membranes were immersed, for 1 h at room temperature, in a GA 2.5% (v/v) aqueous solution, thus obtaining membranes activated and ready to bind the enzyme.

2.3.3. Enzyme immobilization

To immobilize the enzyme, the activated membranes were immersed for 20 h at 4°C in a 0.1 M phosphate buffer solution, pH 7.0, containing 15% (v/v) of the original enzyme solution.

After washing with water, the membranes were ready for use. When not used, the catalytic membranes were stored at 4°C in 0.1 M phosphate buffer solution, pH 7.0.

2.3.4. Determination of the time stability of the catalytic membrane

Time stability of the biocatalytic membranes was assessed by analyzing every day their activity under the same experimental conditions. After 2 days, during which the membranes lost some activity, a stable condition was reached,

remaining unchanged for more than 2 months. Only these stabilized membranes have been used in the comparative experiments reported herein.

Under standard conditions, i.e., 25 ml of 0.1 M phosphate-buffer solution, pH 7.0 and $T = 30°C$, containing a 20-mM cephalexin concentration, the absolute membrane activity was 1 μmol min^{-1}, corresponding to an activity of 285 μmol min^{-1} per m^2 of membrane surface.

2.3.5. Treatment of the experimental data

All the experimental results reported below have been done recirculating in the two half-cells of the bioreactor 25 ml of a 20 mM cephalexin solution at pH 7.0. The catalytic membrane activity was assessed through the amount of alkaline solution (0.5 N NaOH) needed to keep the treated cephalexin solution at the initial pH value. Membrane activity is expressed as μmol min^{-1}. One experiment lasted for 30 min.

Every experimental point reported in the figures represents the average of three experiments done under the same conditions. The experimental errors never exceeded 5%.

3. Results and discussion

3.1. Isothermal characterization of the catalytic membrane

All the membrane used in the experimentation had, before the immobilization phase, an MAA grafting percentage value of 43 ± 3%.

In Fig. 2, the catalytic membrane activity as a function of the temperature is reported. The activity of the free enzyme is also reported. The figure shows that the optimum temperature of the enzyme reaction is practically coincident for the free and the immobilized form of the biocatalyst. Our results can be attributed to the presence of the spacer, HMDA, which binds the enzyme far enough away from the electric field generated by the amide groups constituting the backbone of the nylon membrane. In this way, the enzyme structure undergoes small modifications.

3.2. Non-isothermal characterization of the catalytic membrane

Having characterized the catalytic membrane behaviour under isothermal conditions, the ef-

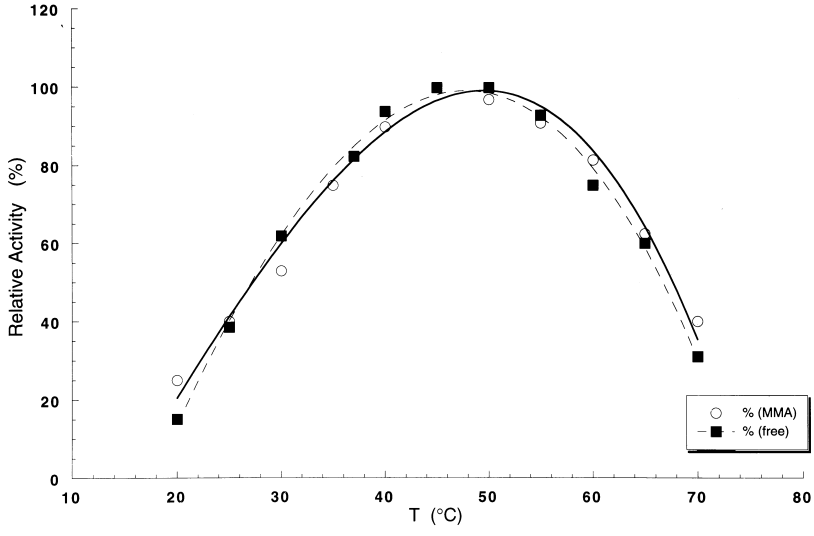

Fig. 2. Relative activity for the free and immobilized PGA as a function of temperature.

fect of temperature gradients on the activity of the immobilized PGA was further studied.

In Fig. 3, the catalytic membrane activity as a function of the temperature difference read by the thermocouples is reported. Each curve is referred to a specific average temperature. The results indicate a linear increase of the enzyme reaction rate with the applied temperature difference, at each of the average temperatures used.

A parameter giving information on the usefulness of non-isothermal bioreactors in industrial processes is the percentage activity increase (P.A.I.) defined, at every average temperature, as:

$$\text{P.A.I.} = \frac{[\text{Activity}]_{T_{av}}^{\Delta T \neq 0} - [\text{Activity}]_{T=T_{av}}^{\Delta T = 0}}{[\text{Activity}]_{T_{av}}^{\Delta T = 0}} \times 100 \quad (1)$$

where $[\text{Activity}]_{T_{av}}^{\Delta T \neq 0}$ and $[\text{Activity}]_{T=T_{av}}^{\Delta T = 0}$ are the catalytic membrane activity found, at determined $T = T_{av}$, under non-isothermal and isothermal conditions, respectively. The P.A.I. values, calculated from the results of Fig. 3, are reported in Fig. 4 as a function of ΔT. Inspection of Fig. 4 shows that the P.A.I. is a linear function of the applied ΔT. At the same ΔT, P.A.I. decreases with increasing average temperature. This means that for obtaining a significant increase of cephalexin hydrolysis, it is sufficient to operate at low average temperatures and temperature gradients instead of higher average temperatures and gradients. Looking at Fig. 4, for example, it is evident that a 50% activity increase is obtained under the conditions $T_{av} = 25°C$ and $\Delta T \cong 15°C$ while at $T_{av} = 30°C$ a ΔT of 22°C is needed. It is unquestionable that the former conditions are more interesting for practical application of the technology of non-isothermal bioreactors in industrial processes.

In Fig. 5, the catalytic membrane activity is reported as a function of the system average temperature. The temperature difference read by the thermocouples is the parameter of each represented curve. Also, in this case, it is interesting to observe how, keeping the average temperature constant, the catalytic membrane activity increases with the increase of the applied temperature difference.

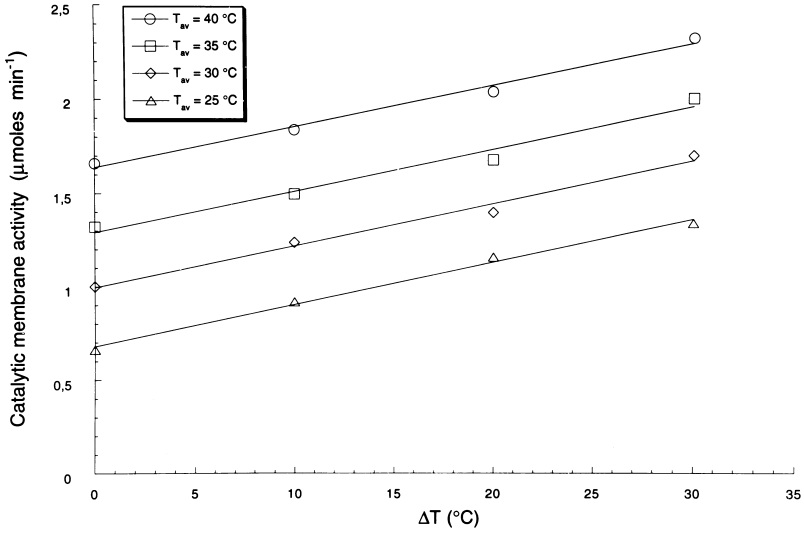

Fig. 3. Catalytic membrane activity as a function of temperature difference read by the thermocouples. Curve parameter is the average temperature $T_{av} = (T_w + T_c)/2$.

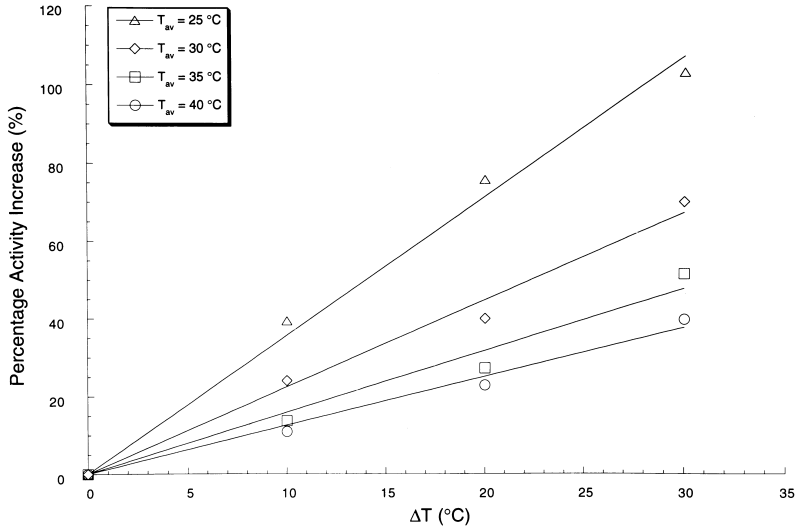

Fig. 4. Percentage activity increase, calculated according to Eq. (1), as a function of temperature difference read by the thermocouples. Curve parameter is the average temperature.

In Fig. 6, the P.A.I. of the catalytic membrane has been reported as a function of the average temperature at which the bioreactor is operated. Conclusions similar to the ones deduced from the results reported in Fig. 4 are obtained observing the results of Fig. 6. The results in Fig. 6 show that at the same average temperature, the percentage activity increase is a function of the applied ΔT. The figure also shows how the same value of the P.A.I. can be obtained by operating the bioreactor at a low average temperature and gradients or under

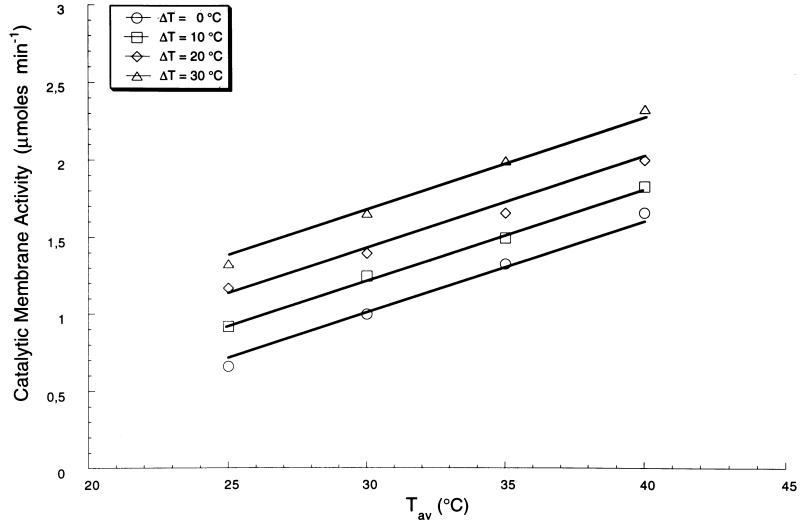

Fig. 5. Catalytic membrane activity as a function of T_{av}. Curve parameter is ΔT, i.e., the temperature difference read by the thermocouples.

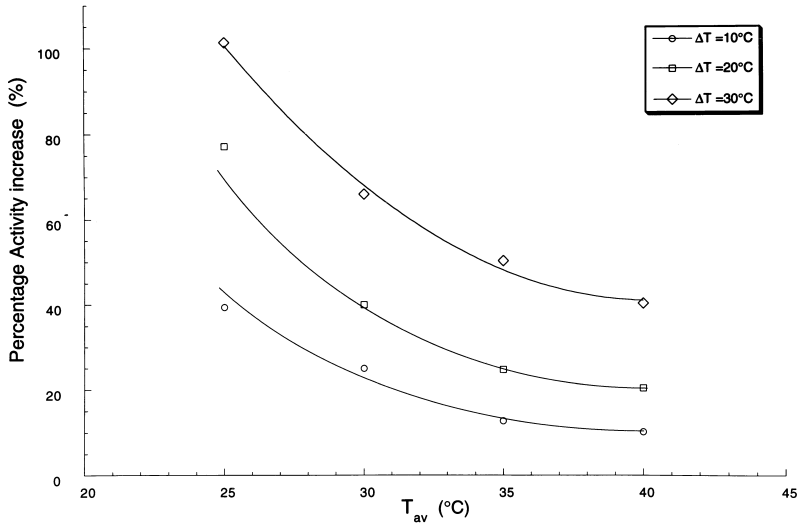

Fig. 6. Percentage activity increase as a function of T_{av}. Curve parameter is the temperature difference read by the thermocouples.

higher average temperature and temperature gradients, the latter conditions being less convenient.

All the results reported above clearly show the effect of the temperature gradient on the cephalexin hydrolysis by immobilized PGA in non-isothermal bioreactors. It is important now to measure the real magnitude of this effect. To do this, it is important to refer the catalytic membrane activities measured under non-isothermal conditions to the actual temperatures existing at the catalytic surfaces rather than to the ones measured by the thermocouples. As a consequence of the heat flow across the bioreactor, the actual temperature difference across the catalytic membrane ΔT^* will be smaller than the temperature difference read by the thermocouples, ΔT. This means that all the observed effects attributed to a macroscopic ΔT must be referred to the actual ΔT^*. Since it is impossible to measure the temperatures right at the faces of the catalytic membrane, they must be calculated from the ones measured at the thermocouples. It has been shown elsewhere [16] that the solution flow in each half-cell constituting the bioreactor is laminar. For this reason, the heat propagation in the two half-cell occurs by conduction between isothermal liquid planes perpendicular to the direction of heat flow. This allows the heat transport equation to be expressed by Fourier's law: $J_q = K_i(\Delta T/\Delta x)_i =$ const, where K_i is the thermal conductivity of the ith medium crossed by the heat flow and $(\Delta T/\Delta x)_i$ is the temperature gradient existing in the same medium, having a thicknesses Δx_i. In this way, knowing the thermal conductivities and thicknesses of the cephalexin solutions and

Table 1
Actual temperatures on membrane surfaces
Correspondence between the temperature values read by the thermocouples (indicated by the symbol T) and the ones calculated at the surfaces of the two catalytic membranes (indicated by T^*). The subscripts w and c refer to the warm solution and to the cold solution, respectively.

T_{av} (°C)	ΔT (°C)	T_c (°C)	T_w (°C)	T_c^* (°C)	T_w^* (°C)	T_{av}^* (°C)	ΔT^* (°C)
25	10	20	30	24.5	25.6	25	1.1
25	20	15	35	23.9	26.1	25	2.2
25	30	10	40	23.4	26.6	25	3.2
30	10	25	35	29.5	30.6	30	1.1
30	20	20	40	29.0	31.1	30	2.2
30	30	15	45	28.4	31.7	30	3.2
35	10	30	40	34.5	35.6	35	1.1
35	20	25	45	34.0	36.1	35	2.2
35	30	20	50	33.4	36.7	35	3.2
40	10	35	45	39.5	40.6	40	1.1
40	20	30	50	38.9	41.1	40	2.2
40	30	25	55	38.4	41.7	40	3.3

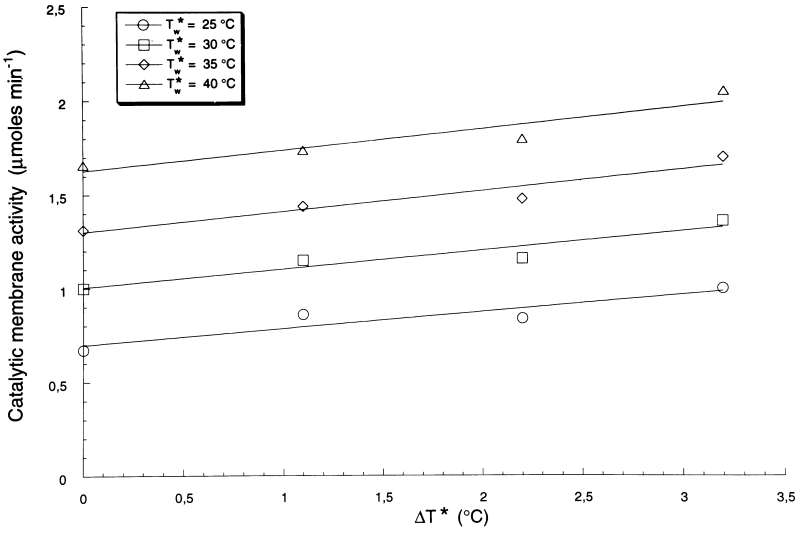

Fig. 7. Catalytic membrane activity as a function of ΔT^*, the actual temperature difference across the membrane. Curve parameter is the temperature on membrane surface facing the warm half-cell.

catalytic membrane, it is possible to calculate the temperature values at the membrane surfaces. In Table 1, the results of these calculations are listed, with reference to the experimental conditions employed in this study. From Table 1, it is possible to see that a ΔT of 10°C reduces to a ΔT^* of 1.1°C; similarly, a ΔT of 20°C to a ΔT^* of 2.2°C, and a ΔT of 30°C to a ΔT^* of 3.2°C. Inspection of the temperature values in the table enables us to write the following empirical equations:

$$\begin{cases} T_w^* = T_w - a\Delta T \\ T_c^* = T_c + b\Delta T \\ \Delta T^* = \Delta T\left[1 - (a+b)\right] \end{cases} \quad (2)$$

where a and b are constant. In this case, concerning a symmetric system, $a = b = 0.445$°C.

Accounting for the temperatures at the membrane faces listed in Table 1, one can re-plot the results of Fig. 5 to obtain the graph reported in Fig. 7. In Fig. 7, the catalytic membrane activity is reported as a function of the actual ΔT^* applied on the membrane. The curve parameter is T_w^* i.e., the actual temperature of the catalytic membrane surface facing the warm half-cell. The results clearly indicate that it is possible to increase the reaction rate of cephalexin hydrolysis keeping constant the temperature of the warm side of the catalytic membrane and lowering the temperature of its opposite face. The amount of the reaction increase depends on the ΔT^*. The linear behaviour evidenced by Fig. 7 allows us to write the following general equation:

$$\left[\text{Activity}\right]_{T_w^*}^{\Delta T \neq 0} = \left[\text{Activity}\right]_{T_w^*}^{\Delta T = 0}\left(1 + \alpha^* \Delta T^*\right) \quad (3)$$

for each value of T_w^*. The α^* coefficients represent the relative increase of catalytic membrane activity when a temperature difference of 1°C is applied across the membrane. α^* is the physical parameter indicating of the performance of the non-isothermal bioreactor. The α^* values calculated by means of Eq. (3) applied to the results in Fig. 7 are reported in Table 2.

Table 2
α^* coefficients at different T_w^*. α^* values calculated through Eq. (3) by the results reported in Fig. 7

T_w^* (°C)	$\alpha^* \times 100$ (°C^{-1})
25	13
30	10
35	9
40	7

These values clearly indicate the usefulness of executing cephalexin hydrolysis in non-isothermal bioreactors by means of immobilized PGA.

All the experimental results reported above can be explained considering the way by which the substrate reaches the catalytic site of the enzyme immobilized on and into the membrane.

Under isothermal conditions, only diffusion occurs. The diffusive substrate flux (mol cm^{-2} s^{-1}) is expressed by the equation:

$$J_S^D = D^* \frac{dc}{dx} \quad (4)$$

where D^* is the restricted diffusion coefficient (cm^2 s^{-1}) and dc/dx is the concentration gradient (mol cm^{-4}).

Under non-isothermal conditions, i.e., when a temperature gradient is applied across a hydrophobic membrane, catalytic or not, the membrane is crossed by differential matter fluxes, solvent and solutes, produced by the process of thermodialysis. Both matter fluxes are directly proportional to the magnitude of the temperature gradient applied across the membrane [18–22]. Under these conditions, two separate matter fluxes are observed: a volume flux (water) from warm to cold, measured in (cm^3 cm^{-2} s^{-1}) and expressed by the equation:

$$J_{H_2O} = L \frac{dT}{dx} \quad (5)$$

and a solute flux from cold to warm, measured in (mol cm^{-2} s^{-1}) and expressed by the equation:

$$J_S^{therm} = D'^* C_c \frac{dT}{dx}. \quad (6)$$

In these equations, dT/dx is the temperature gradient across the membrane (K cm^{-1}), C_c is the actual concentration of solute in the cold half-cell (mol cm^{-3}) and L is the thermoosmotic coupling coefficient (cm^2 s^{-1} K^{-1}).

In association with the volume flux, there is a solute flux, know as ‘‘solvent drag’’, given by:

$$J_S^{drag} = J_{H_2O} C_w = V_{H_2O} C_w \quad (7)$$

where C_w is the solute concentrations in cold half-cell, from which the water flux is coming and V_{H_2O} is the rate of water transport in cm s^{-1}.

Of course, also under non-isothermal conditions, the contribution of isothermal substrate diffusion still remains.

Summing up, under isothermal conditions, the only substrate traffic is given by that represented by Eq. (4); under non-isothermal condition, the substrate traffic across the membrane is due to three distinct contributions: from that expressed by Eq. (4) and from those indicated by Eqs. (6) and (7). A no-scale picture of substrate traffic across the catalytic membrane operating under isothermal and non-isothermal conditions is given in Fig. 8.

On the basis of all these considerations, it easy to conclude, therefore, that under non-isothermal conditions, the enzyme immobilized

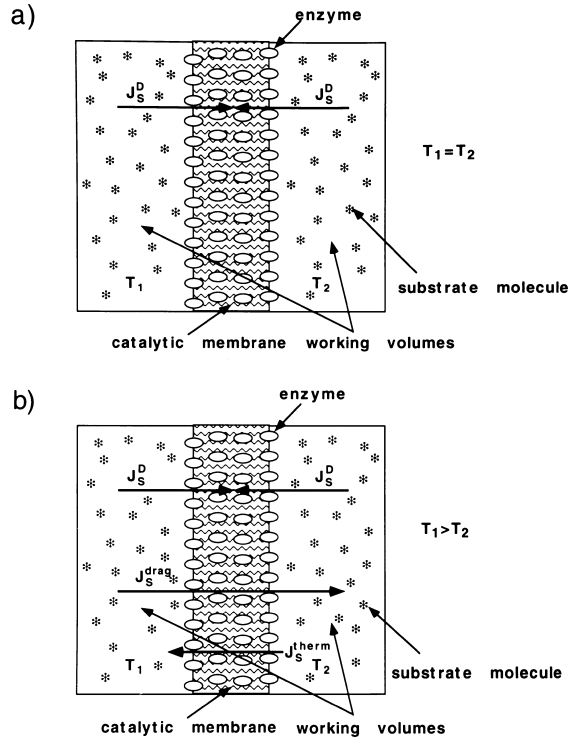

Fig. 8. A no-scale picture of substrate traffic across the catalytic membrane operating under isothermal (a) and non-isothermal (b) conditions.

onto the hydrophobic–catalytic membrane ''faces'' a higher substrate traffic and, hence, interacts with a substrate concentration higher than under isothermal conditions.

The same reasoning is applicable to the product removal from the active site, since the thermodialysis processes increase the removal speed from the catalytic site. As a consequence of the presence of a temperature gradient, the apparent turn-over number of the enzyme reaction appears to increase, thus increasing the rate of the enzyme reaction.

4. Conclusions

In the first place, it is possible to observe that the aim of this work has been reached since it has been demonstrated that the rate of cephalexin hydrolysis depends on whether the bioreactor is operating under isothermal or non-isothermal conditions.

The effect of temperature gradients on the catalytic membrane activity, and hence on the amount of hydrolyzed cephalexin, is evident from all the experimental data presented. The magnitude of this effect, measured through the α^* coefficient, appears attractive for practical applications. Incidentally, the α^* values found with the present membrane are smaller but of the same order of magnitude of the ones obtained with the two-membrane system used in the past. This means that the idea of obtaining a single membrane, catalytic and hydrophobic, able to work in non-isothermal bioreactors was right.

Experiments are in progress in our laboratory to construct other catalytic and hydrophobic membranes able to increase the yield of an enzymatic process executed in bioreactors operating under non-isothermal conditions.

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