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Urease immobilization on chemically grafted nylon membranes Part 2. Non-isothermal characterization

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Abstract

The behaviour of an urease loaded membrane, obtained by chemically grafting butyl methacrylate (BMA) on a nylon sheet, has been studied under non-isothermal conditions. Hexamethylenediamine (HMDA) and glutaraldehyde were also used as spacer and binding agent, respectively.

Results have shown that the catalytic activity of the membrane under non-isothermal conditions is increased when compared with the one found under comparable isothermal conditions. In addition, it has been found that the presence of temperature gradients decreases the apparent K_m and increases the apparent V_{max} with respect to the same values obtained under isothermal conditions.

The percentage activity increases induced by the presence of a temperature gradient have been found to decrease with increasing average temperature and urea concentration.

A parameter has also been identified correlating the percentage increase of enzyme activity under non-isothermal conditions with the hydrophobicity of the catalytic membrane, this parameter being the ratio between thermo-osmotic and hydraulic permeability.

Results have been discussed in terms of reduction of diffusion limitations for substrate and products movement towards or away from the catalytic site by the process of thermodialysis.

The usefulness of using non-isothermal bioreactors in industrial biotechnological processes and, in particular, in waste water treatment has been confirmed.

Keywords: Urease; Urea; Chemical grafting; Biocatalytic membranes; Non-isothermal bioreactors; Waste water treatment

1. Introduction

It is well known from the thermodynamics of irreversible processes [1,2] that temperature gradients generate matter fluxes in bulk solutions and across membranes separating liquid mixtures. In particular, when hydrophobic and unselective porous membranes are employed in a reactor to separate aqueous

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solutions kept at different temperatures, selective solvent and solute fluxes occur across it. This kind of mass transport has been called thermodialysis [3,4]. Water fluxes are directed towards the cold half-cell, while solute fluxes proceed towards the cold or the warm half-cell according to their nature and the dispersing phase. Both solvent and solute fluxes have been found to be proportional to the temperature gradient applied across the membrane.

When a hydrophilic catalytic membrane is coupled to a hydrophobic unselective membrane in a bioreactor operating under non-isothermal conditions, the process of thermodialysis affects the enzyme reaction rate [5–11]. The same occurs [12–19] when a single hydrophobic and catalytic membrane is used, since such a membrane gives simultaneously catalysis and thermodialysis. These membranes were obtained by chemical or by γ-radiation grafting of nylon or teflon membranes with suitable monomers, and by a subsequent enzyme immobilization. In both cases, namely by using a two-membrane system (one hydrophilic and the other hydrophobic) or a single catalytic and hydrophobic membrane, the efficiency increase of a non-isothermal bioreactor ranges from 10 to 30%, depending on the nature of the enzyme and on the immobilization method used.

For both the above mentioned catalytic systems, the presence of a hydrophobic membrane is of crucial importance to observe an increase in the yield of catalytic processes carried out under non-isothermal conditions. Under these conditions, the substrate traffic produced by the process of thermodialysis is added to the diffusive one. As a consequence, the catalyst immobilized on or into a membrane "encounters" a higher substrate concentration, thus, exhibiting a higher reaction rate with respect to that observed under comparable isothermal conditions. What is observed is an increase in the efficiency of a bioreactor [5–15] or a biosensor [16,17] proportional to the size of the temperature gradient applied across the catalytic membrane, as in the process of thermodialysis.

The aim of this work is to study under non-isothermal conditions the behaviour of a new membrane, catalytic and hydrophobic, obtained by chemically grafting butyl methacrylate (BMA) on nylon. As enzyme model we studied urease (E C 3.5.1.5) in view of the application of non-isothermal bioreactors in the treatment of urea polluted waste waters. The catalytic yield of these membranes under non-isothermal conditions was studied as a function of urea concentration, average temperature and magnitude of the applied temperature difference. The isothermal behaviour of the same catalytic membrane has been characterized in a separate paper [18].

2. Apparatus, materials and methods

2.1. The bioreactor

The apparatus (Fig. 1) was the same used for the experiments reported in [18], but with two differences: the possibility of working under non-isothermal conditions by thermostatting the two half-cells at different temperatures, and the possibility of working as permeability or thermodialysis cells. When the apparatus was working under isothermal conditions T_1 was equal to T_2, while under non-isothermal condition T_1 was different from T_2. Thermocouples, placed 1.5 mm from each of the membrane surfaces, measured the temperatures inside each half-cell and allowed to calculate the temperature profile across the catalytic membrane. The temperatures read by the thermocouples will be indicated by T, while the ones calculated at the membrane surfaces by T^*. Moreover, the temperatures of the warm or cold side are indicated by the subscript w or c, respectively. Under these assumptions, it follows that $\Delta T = T_w - T_c$ and $\Delta T^* = T_w^* - T_c^*$, as well as $T_{av} = (T_w + T_c)/2$, $T_{av}^* = (T_w^* + T_c^*)/2$, $T_w^* < T_w$, $T_c^* > T_c$ and $\Delta T^* < \Delta T$.

When the apparatus was working as a permeability cell or as a thermodialysis cell to measure hydraulic or thermo-osmotic water fluxes, alternative hydraulic circuits and devices were used. The functioning of the apparatus during these experiments will be described in the appropriate section.

2.2. Materials

As solid support to be grafted, nylon Hydrolon membranes by Pall (Pall Italia, Milan, Italy) were used. These hydrophobic membranes, 150 μm thick, have a nominal pore size of 0.2 μm.

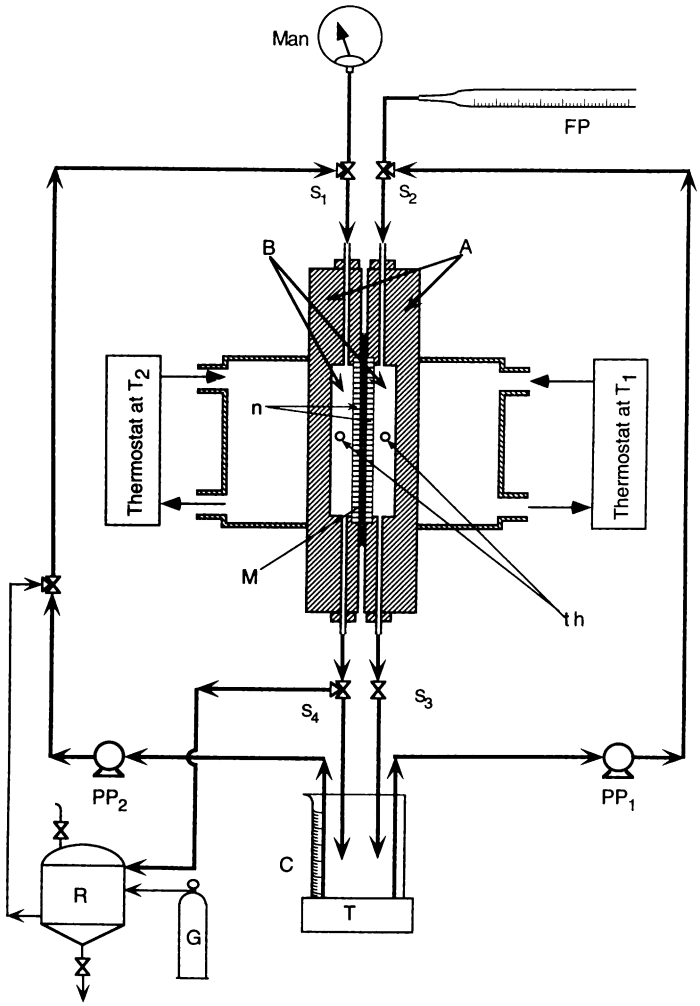

Fig. 1. Schematic (not to scale) representation of the bioreactor. (A) = half-cells; (B) = internal working volumes; (C) = external working volume; (M) = membrane; (n) = supporting nets; (th) = thermocouples; (S_i) = stopcocks; (T) = thermostatic magnetic stirrer; (PP_i) = peristaltic pumps; (Man) = manometer; FP = (flow-pipe); (R) = reservoir containing the working solution; (G) = pressurizing air tank.

BMA was used as the hydrophobic monomer to be grafted. Hexamethylenediamine (HMDA) and glutaraldehyde (Glu) were used as spacer and coupling agent, respectively.

Type III urease (E C 3.5.1.5) from jack beans was used as a catalyst. This enzyme was chosen in view of the employment of these catalytic membranes in the process of treatment of urea polluted waste waters.

All chemical products, including the enzyme, were purchased from Sigma (St. Louis, MO) and used without further purification.

2.3. Methods

2.3.1. Catalytic membrane preparation

The preparation of the catalytic membrane was done according to the procedure described in [18]. Grafting percentage ($X\%$) was determined by the difference between the membrane mass before (G_B) and after (G_A) the grafting process by the expression $X(\%) = (G_A - G_B)/G_B \times 100$. When not used, the membranes were stored at 4°C in a 0.1 M citrate buffer solution, pH 5.1.

2.3.2. Determination of the catalytic membrane activity

Membrane activity was assessed following the procedure described in [18].

2.3.3. Temperature profile across the catalytic membrane

To estimate the real effect of temperature gradients on the reaction rate of immobilized enzymes, the actual temperature at the membrane surfaces must be known. Since this measure is practically impossible, these temperatures have been calculated by applying the Fourier law for heat conduction trough the system composed by the catalytic membrane and the two non-isothermal solutions in the bioreactor. The calculation could be performed because in each half-cell the liquid motion, constrained at a rate of 2.5 $cm^3\ min^{-1}$ between two fins with rounded tips, was found to be not-turbulent [10,11]. In fact, the Reynolds number in our apparatus and under our experimental conditions resulted equal to 3.4. Thus, heat propagation in the bioreactor occurs by conduction between isothermal liquid planes (and the solid membrane) which are in motion perpendicularly to the direction of heat flow (Fig. 2a). In this way, starting from the consideration that

$$J_{q,i} = K_i \left(\frac{\Delta T}{\Delta x}\right)_i = J_q = \text{constant} \quad (1)$$

through all the media, liquids (solutions) and solids (membranes), confined between the two half-cells, the temperatures on each membrane surface constituting the membrane system are calculated. In Eq. (1) $J_{q,i}$ represents the heat flux across the ith medium, Δx_i in thickness, and K_i is the thermal conductivity of the same medium subjected to a temperature difference ΔT_i. Details on this methodology can be found in [10–15]. In Table 1 we report the temperatures read at the thermocouple positions and the corresponding ones on the surfaces of the catalytic nylon membrane interposed between the substrate solutions maintained at different temperatures. In Fig. 2b the real temperature profile for one of the cases reported in Table 1 is illustrated.

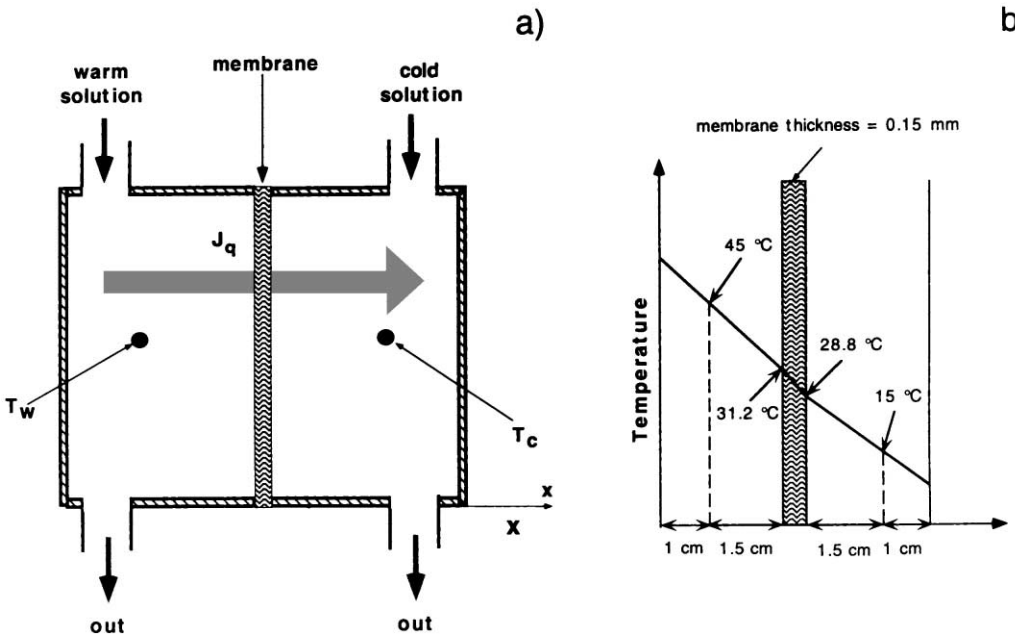

Fig. 2. (a) Side view of the cell showing position of thermocouples, membrane and heat flux (J_q). (b) Temperature profile in the bioreactor when the temperatures read by the thermocouples are $T_w = 45°C$ and $T_c = 15°C$. Magnification along the x-axis is 10.

Table 1
Correspondence between the temperatures T and T^*[a]

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	24.6	25.4	25	0.8
25	20	15	35	24.2	25.8	25	1.6
25	30	10	40	23.8	26.2	25	2.4
30	10	25	35	29.6	30.4	30	0.8
30	20	20	40	29.2	30.8	30	1.6
30	30	15	45	28.8	31.2	30	2.4
35	10	30	40	34.6	35.4	35	0.8
35	20	25	45	34.2	35.8	35	1.6
35	30	20	50	33.8	36.2	35	2.4

[a] Correspondence between the temperatures read by the thermocouples (T) and the ones calculated on the membrane surface T^*. Subscripts: w = warm side; c = cold side.

3. Results and discussion

Every experimental point in the Figures is the average value of five identical experiments performed under the same conditions. Each run lasted 18 min. The error did never exceed 5%.

Before illustrating the results relative to the enzyme reaction rate under non-isothermal conditions, the grafted membranes will be physically characterized by studying their hydraulic and thermo-osmotic permeability.

3.1. Physical characterization of the membranes

Grafted membranes are physically different from the untreated ones with reference to the mass transport occurring under pressure and temperature gradients [15]. Two physical parameters, controlling transmembrane mass transport, are the hydraulic permeability coefficient, A, and the thermo-osmotic coefficient, B. These coefficients are defined by the equations

$$J_{\text{volume}}^{\text{hydr.}} = A\frac{\Delta P}{\Delta x}, \tag{2}$$

$$J_{\text{volume}}^{\text{therm.}} = B\frac{\Delta T}{\Delta x} \tag{3}$$

describing the volume flow ($m^3\,m^{-2}\,s^{-1}$) across a membrane, Δx (m) in thickness, produced, under isothermal conditions, by a pressure gradient $\Delta P/\Delta x$ ($N\,m^{-3}$) or, under non-isothermal conditions, by a temperature gradient $\Delta T/\Delta x$ ($K\,m^{-1}$). A is expressed in $m^4\,s^{-1}\,N^{-1}$ and B in $m^2\,s^{-1}\,K^{-1}$.

With the aim of knowing the hydrophobicity of our pre-treated membranes, we have indirectly measured this parameter by studying, under pressure and temperature gradients, the hydraulic and thermo-osmotic volume fluxes. The membranes for these experiments were prepared in the same way of those for measuring the catalytic power of the immobilized urease.

Hydraulic fluxes have been calculated by pressurizing, as indicated in Fig. 1, one half-cell by a gas cylinder and measuring the rate of water transport to the other half-cell by means of a graduated pipe. The temperature of the apparatus was kept constant. In Fig. 3a, the hydraulic fluxes obtained across the membrane under a constant pressure difference of 30 mbar are reported as a function of temperature. The data in the Figure show that water fluxes increase with the increase of temperature. Since these fluxes are one order of magnitude larger [15] than those occurring under the same conditions across the untreated nylon membranes, one concludes that the grafting process increases the hydraulic membrane permeability by reducing its hydrophobicity. From Eq. (2), it is possible to calculate the coupling coefficient A.

Thermo-osmotic fluxes have been calculated by measuring, in the graduated pipe, the rate of water volume transport from the warm to the cold half-cell, in the absence of pressure gradients and in presence of temperature gradients. In Fig. 3b, the thermo-osmotic water fluxes are reported as a function of average temperature. The temperature difference measured by the thermocouple was $\Delta T = 30$°C. The Figure shows that thermo-osmotic fluxes increase with the increase of the average temperature. Since the thermo-osmotic

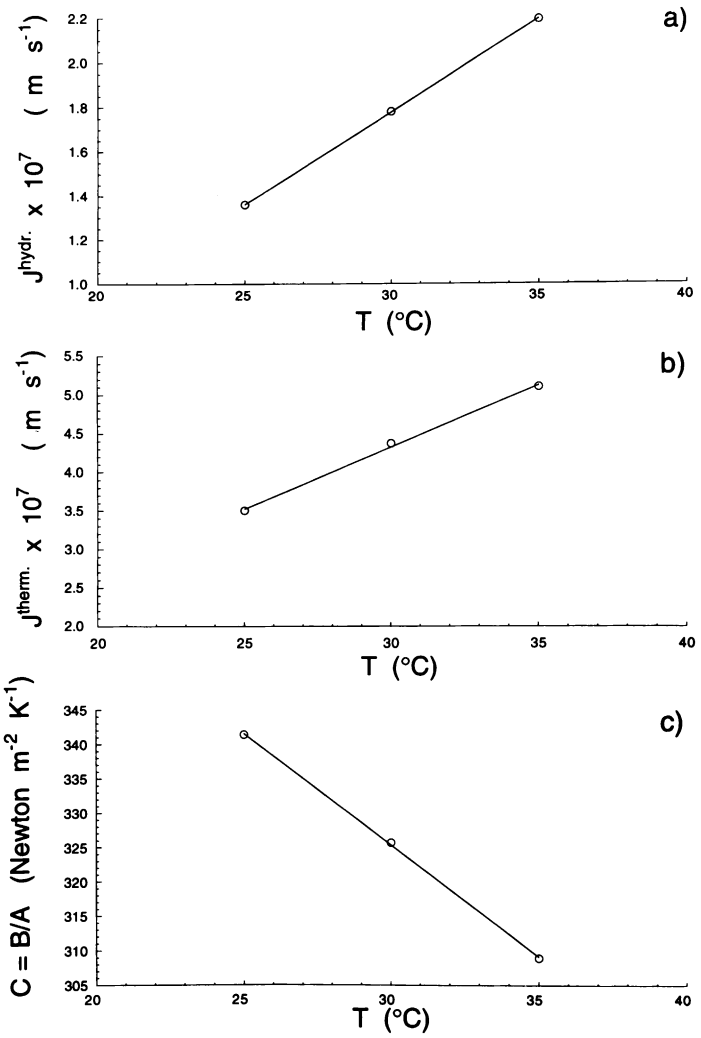

Fig. 3. Hydraulic (a) and thermo-osmotic (b) water fluxes as a function of temperature. Experimental conditions: $\Delta P = 30$ mbar and $T = 30^{\circ}$C for the data in (a); $\Delta T = 30^{\circ}$C and $T_{av} = 30^{\circ}$C for the data in (b). (c) Behaviour of the C parameter as a function of temperature.

fluxes are by one order of magnitude lower [15] than the ones occurring under the same conditions across the untreated nylon membranes, the results in Fig. 3b confirm that the grafting process reduces the membrane hydrophobicity. The latter determines the extent of the mass transport under non-isothermal conditions. From Eq. (3), it is possible to calculate the thermo-osmotic coupling coefficient B.

From the values found for the coefficients A and B, it is possible to calculate the values of the coefficient C (N m^{-2} K^{-1}) defined as

$$C = \frac{B}{A} = \frac{\Delta P}{\Delta T} \tag{4}$$

Coefficient C [15] can be viewed as a parameter able to foresee the behaviour of a catalytic membrane in non-isothermal bioreactors. For $C = 0$, i.e. in the absence of the process of thermodialysis, the yield of a catalytic membrane should be the same under isothermal or non-isothermal conditions. For $C > 0$,

i.e. with membranes giving transport by thermodialysis, the reaction rate of a catalytic membrane under non-isothermal conditions should increase with the increase of the C value. The C values calculated from the experiments in Fig. 3a and b are reported in Fig. 3c. Data from this Figure allow to test our working hypothesis. If the role of the C coefficient has been correctly identified, according to Fig. 3c we should observe under non-isothermal conditions percentage activity increases of the catalytic membrane decreasing with the increase of the average temperature.

3.2. Biochemical characterization of the catalytic membrane under non-isothermal conditions

In Fig. 4 the ammonia production under isothermal and non-isothermal conditions is reported as a function of time. The experiments were carried out with 15 mM urea concentration in 0.1 M citrate buffer at 25°C as average temperature. From the Fig. 4, it clearly emerges that ammonia production is linear during the experiment, and that the production measured under isothermal conditions is lower than that measured under comparable non-isothermal conditions. Moreover, ammonia production increases with the increase of the applied ΔT. The slopes of the straight lines in Fig. 4 give the activity of the catalytic membrane.

In Fig. 5 the activity of the catalytic membrane is reported as a function of substrate concentration. The curve parameter is the macroscopic temperature difference ΔT measured by the thermocouples. The average temperature of the experiments was 25°C, and 0.1 M citrate buffer solutions at pH 5.1 were used. The results in Fig. 5 show that at every urea concentration the activities of the catalytic membranes are higher than those found under comparable isothermal conditions. The comparison between the two different physical situations, isothermal and non-isothermal, is possible, since, even under a $\Delta T = 30$°C, the temperature at which the enzyme is actually operating can be considered equal to the average temperature, as one may deduce from the temperatures listed in Table 1. This means that the temperature at which the enzyme derivatives are exposed in the centre of the bioreactor is approximately equal to the average temperature. Two further assumptions have also been done: (i) the enzyme distribution on and into the membrane thickness is uniform; (ii) the actual temperature difference

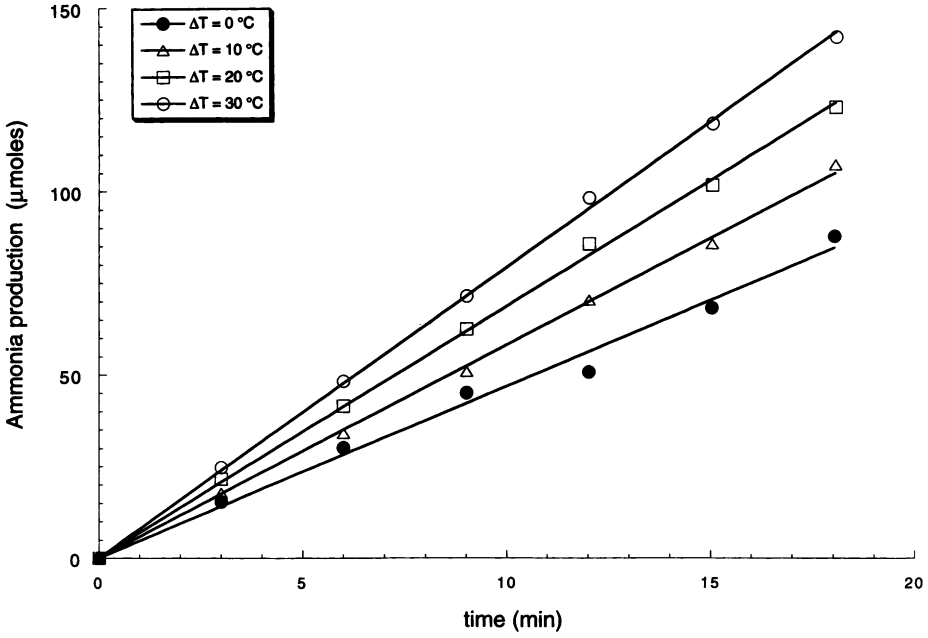

Fig. 4. Ammonia production as a function of time.

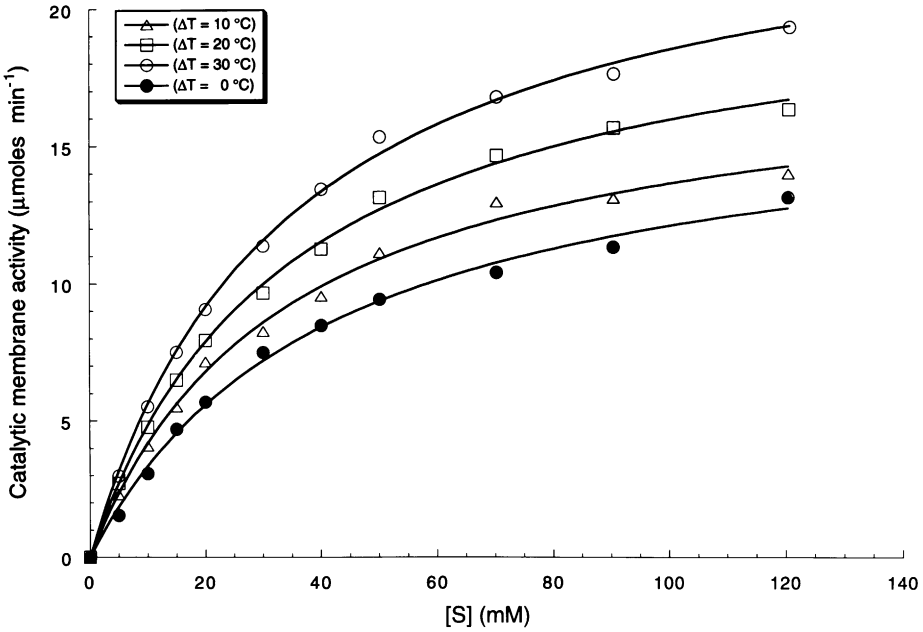

Fig. 5. Catalytic membrane activity as a function of urea concentration.

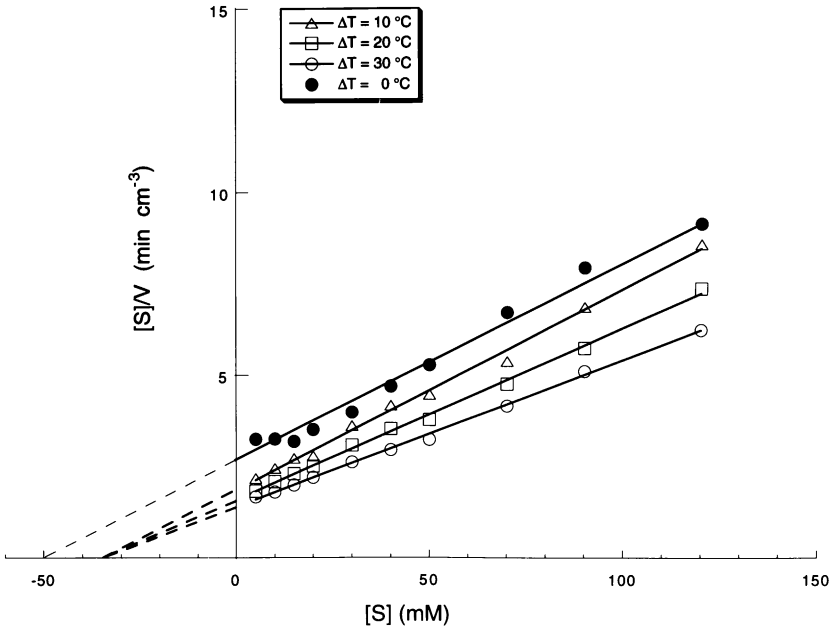

Fig. 6. Hanes plots of experimental points reported in Fig. 5.

Table 2
Kinetics parameters of immobilized urease[a]

ΔT (°C)	K_m (mM)	V_{max} (μmoles min^{-1})
0	50.0	18.7
10	34.4	20.3
20	34.4	22.0
30	34.4	25.4

[a] K_m and V_{max} values under isothermal and non-isothermal conditions for immobilized urease. Average temperature was 25°C.

across the membrane in the extreme case in which the measured ΔT is equal to 30°C is reduced to a $\Delta T^* = 2.4$°C and, accordingly, the enzyme activity onto the membrane is linear.

Since a Michaelis–Menten behaviour occurs either in the presence or in the absence of temperature gradients, it is possible to calculate the apparent values of the kinetic constants K_m and V_{max} for each physical situation. In Fig. 6 the experimental points of Fig. 5 are reported in forms of Hanes plot to calculate the values of the apparent K_m and V_{max}. These values, listed in Table 2, show that the apparent values of K_m under isothermal conditions are higher than the corresponding values under non-isothermal conditions and that the latter are independent on the macroscopic temperature difference applied across the membrane.

The first observation finds an explanation in the circumstance that the temperature gradient increases substrate and product fluxes across the catalytic membrane, reducing, in this way, the diffusion limitations [19] of these substances during their movement towards or away from the catalytic site. Moreover, the increase of the enzyme reaction rates with the applied ΔT finds an explanation with the analogous increases with the applied ΔT of mass fluxes induced by the process of thermodialysis across a membrane [3,4].

The second observation, concerning the independence of the K_m values on the macroscopic temperature difference across the catalytic membrane, can be justified with changes in the protein structure and dynamics, induced in the immobilized enzyme by the flux of thermal energy associated to the presence of the temperature gradient [6–8]. In our opinion the temperature gradient plays the same role than positive effectors on enzyme structure, each effector affecting in the same way the K_m value of the enzyme reaction, and consequently, the structure and dynamics of the protein, independently from its concentration.

In Fig. 7, the results relative to the isothermal and non-isothermal catalytic activity are reported as a

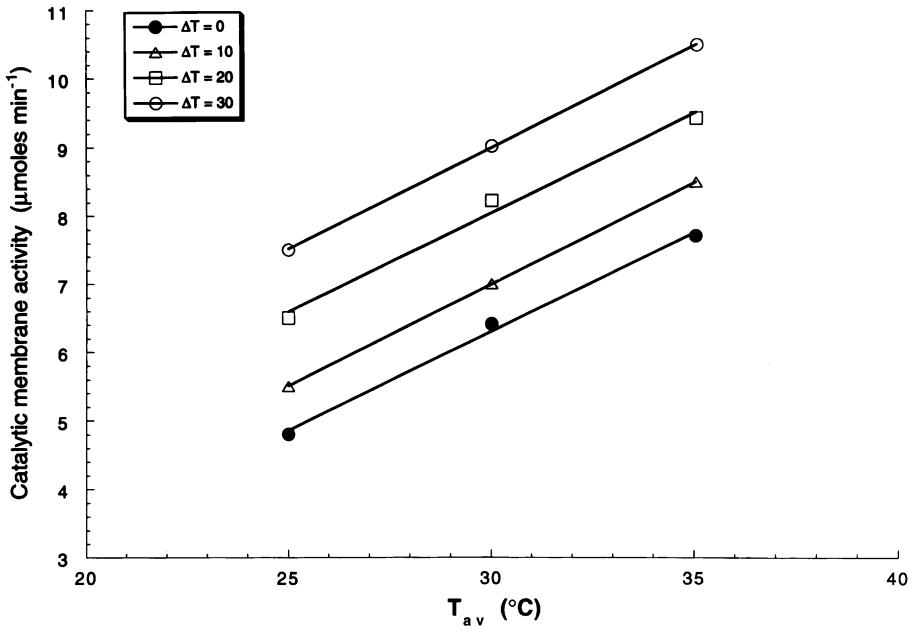

Fig. 7. Catalytic membrane activity as a function of average temperature.

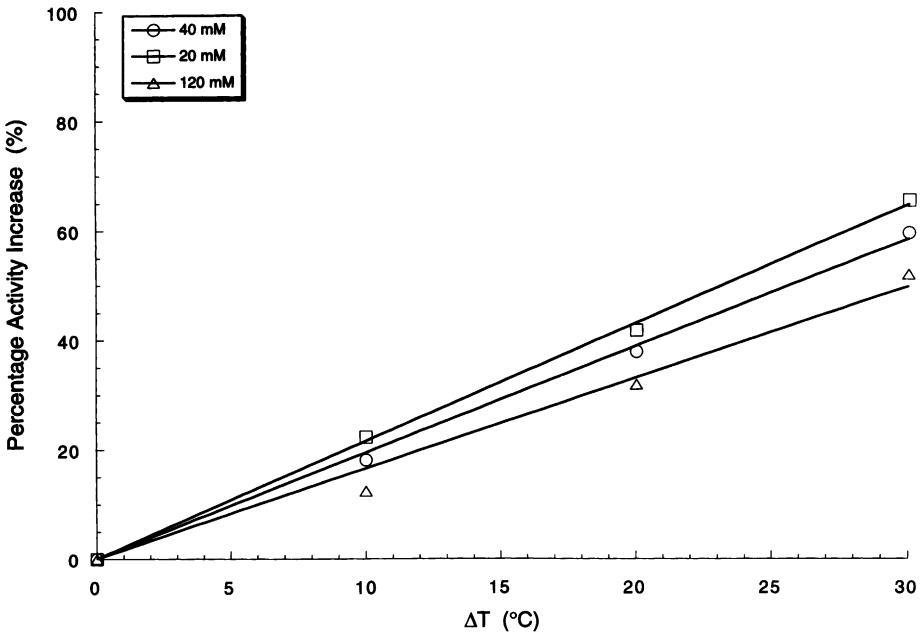

Fig. 8. PAI as a function of the macroscopic ΔT applied across the membrane. Curve parameter is the urea concentration.

function of the average temperature. The curve parameter is ΔT. Results in Fig. 7 show that at each average temperature the activity of the catalytic membrane increases with the applied temperature difference ΔT. All experiments were carried out with a 15 mM urea concentration in 0.1 M citrate buffer at pH 5.1.

From the results reported in Figs. 5 and 7, it is possible to obtain a parameter giving information on the usefulness of using non-isothermal bioreactors in industrial processes. This parameter is the percentage activity increase (PAI) defined as

$$\mathrm{PAI} = \frac{\mathrm{RR}|_{T_{\mathrm{av}}}^{\Delta T \neq 0} - \mathrm{RR}|_{T=T_{\mathrm{av}}}^{\Delta T=0}}{\mathrm{RR}|_{T=T_{\mathrm{av}}}^{\Delta T=0}} \quad (5)$$

where $\mathrm{RR}|_{T=T_{\mathrm{av}}}^{\Delta T=0}$ and $\mathrm{RR}|_{T_{\mathrm{av}}}^{\Delta T\neq 0}$ are the enzyme reaction rates at $T = T_{\mathrm{av}}$ under isothermal and non-isothermal conditions, respectively. The PAI values, calculated from the results of Fig. 5, are reported in Fig. 8 as a function of the applied temperature difference ΔT at three different substrate concentrations: small, average, high. The results in Fig. 8 show an increase of the PAI values with the increase of the applied ΔT.

In Fig. 9, the percentage activity increases as a function of the average temperature are reported. The results in Fig. 9 have been calculated by the experimental points of Fig. 7 in the case of $\Delta T = 30^{\circ}$. The similarity between the behaviour of the results shown in Figs. 9 and 3c confirms our working hypothesis about the role of the C parameter as a predictor of the efficiency increase of an enzymatic process performed in a non-isothermal bioreactor.

In Fig. 10 the PAI is reported as a function of substrate concentration for each of the temperature differences used. The decrease of the PAI values with the increase of the substrate concentration has already been found by us with β-galactosidase immobilized on differently grafted membranes. A simple phenomenological explanation for the decrease of percentage activity increase with increasing substrate concentration is that when the enzyme works appoximatively at its maximum rate, as at high substrate concentrations, the modulation of this rate by temperature gradients results less effective. A quantitative explanation of this behaviour has been recently published by us [20]. In this paper, starting from a substrate balance into the catalytic membrane under isothermal and

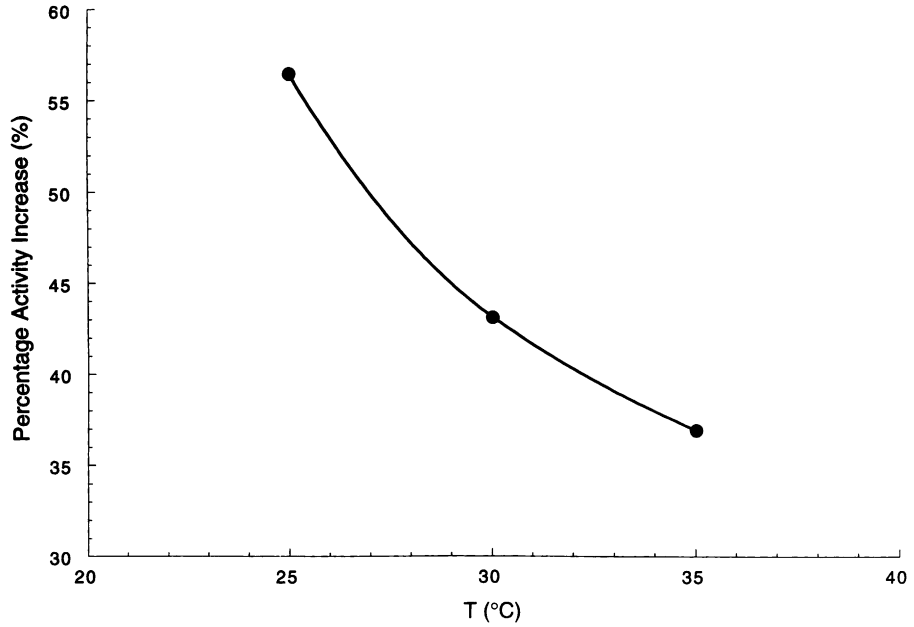

Fig. 9. PAI as a function of average temperature. Experimental conditions were: 15 mM in 0.1 M citrate buffer, pH 5.1, and $\Delta T = 30^{\circ}$C.

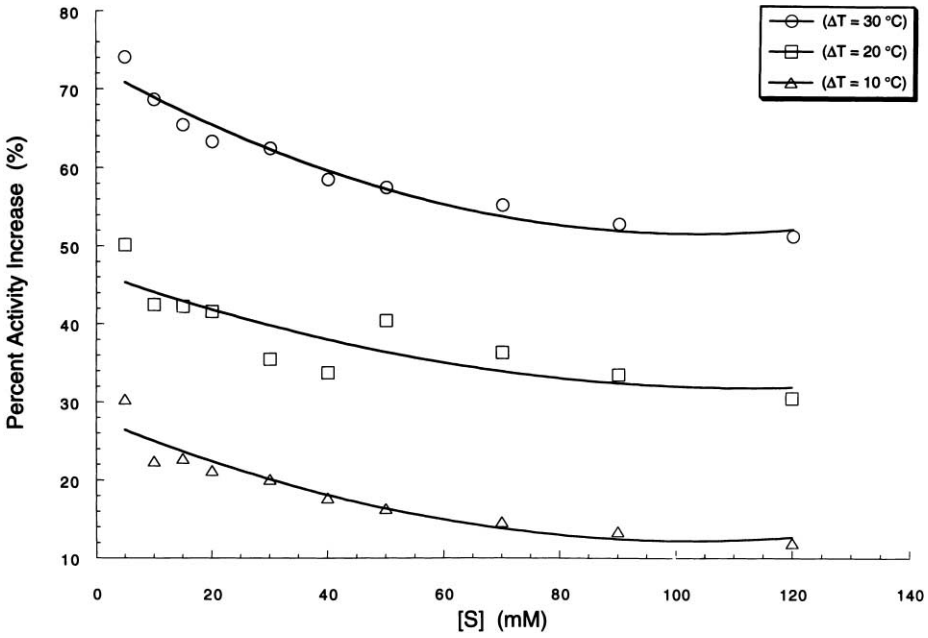

Fig. 10. PAI as a function of substrate concentration. Experimental conditions were: 0.1 M citrate buffer at pH 5.1, and $T_{av} = 25^{\circ}$C.

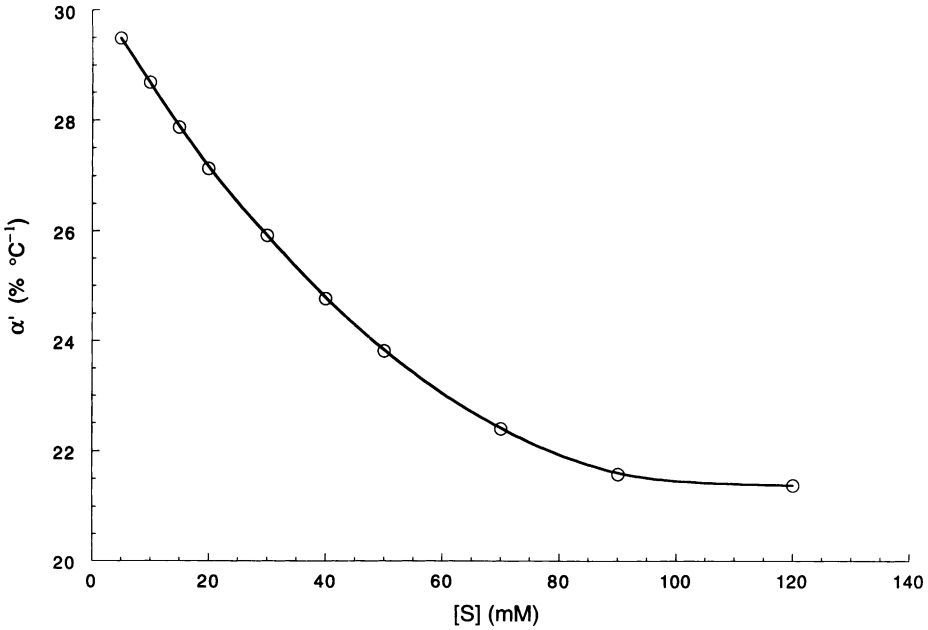

Fig. 11. The α' coefficient as a function of substrate concentration. Average temperature was 25°C.

non-isothermal conditions, the profiles of substrate concentration into the membrane have been derived and from these a parameter similar to PAI has been deduced. The dependence of this parameter on substrate concentration is similar to that of the PAI in Fig. 10.

The PAI values in Figs. 8–10, obtained with reference to the macroscopic temperature difference ΔT in the bioreactor, support the idea of employing non-isothermal bioreactors in biotechnological processes, such as the treatment of urea polluted waste waters. This observation is enhanced if attention is paid to the actual temperature difference across the membrane, which — as one can see in Table 1 — corresponds to an actual $\Delta T^* = 2.4$°C when a $\Delta T = 30$°C is measured by the thermocouples.

It is possible, now, to define a coefficient α', related to the PAI coefficient, by means of the equation

$$\alpha' = \frac{\text{PAI}}{\Delta T^*} \tag{6}$$

In this case α' represents the percentage increase of the enzyme reaction rate when an unit temperature difference is applied across the catalytic membrane. The α' values relative to the results in Figs. 5 and 8 are illustrated in Fig. 11.

The way by which temperature gradients affect the reaction rate of immobilized enzyme has been discussed elsewhere [14–17,20].

4. Conclusion

This work has demonstrated that under non-isothermal condition the hydrolysis of urea, by urease immobilized on grafted membrane, is increased when compared with the yield observed under comparable isothermal conditions.

A correlation between the thermodynamic coupling coefficients A and B, and the activity increase of a catalytic membrane in the presence of temperature gradients has been demonstrated, indicating that in order to obtain higher efficiency increases, membranes endowed with high values of the C coefficient have to be constructed.

Moreover, the advantage of using non-isothermal bioreactors has been confirmed, since also in the present study the bioreactors resulted more efficient

with respect to their functioning under comparable isothermal conditions.

Studies are in progress in our laboratory with the aim of constructing more efficient non-isothermal bioreactors, changing the planar geometry adopted so far.

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