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Advantages of Using Non-isothermal Bioreactors in Agricultural Waste Water Treatment by Means of Immobilized Urease. Study on the Influence of Spacer Length and Immobilization Method

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The behavior of three different catalytic membranes, obtained by immobilizing urease on nylon sheets chemically grafted with methyl methacrylate, was studied in a bioreactor operating under isothermal and non-isothermal conditions. Membrane activation was carried out by condensation or acyl azide reaction, and spacers of different lengths, such as hexamethylendiamine or hydrazine, were used. Under isothermal conditions, the activities of the catalytic membranes and soluble urease were characterized as a function of pH, temperature, and urea concentration. Both enzyme forms showed the same optimum pH, whereas the optimum temperature was lower for the immobilized enzymes. The spacer length appeared to determine broader pH− and temperature−activity profiles for the urease derivatives. The apparent K_m values of the insoluble urease were dependent on membrane type and were higher than those of the soluble counterpart, thus indicating an affinity loss for urea. Under non-isothermal conditions, all membranes exhibited an increase of percentage activity proportional to the applied temperature difference and decreasing with the increase of urea concentrations. A decrease of the apparent K_m was also observed. These results suggest that substrate diffusion limitations due to the immobilization process can be overcome in the presence of temperature gradients. In addition, the remarkable reduction of the production times supports the use of non-isothermal bioreactors for the treatment of urea-polluted waste waters.

KEYWORDS: Urea; urease; enzyme immobilization; nylon-grafted membrane; waste water treatment; non-isothermal bioreactors

INTRODUCTION

Urea has been recognized as a pollutant agent in agricultural waste waters (*1*). Its concentration can be reduced by means of an enzymatic reaction catalyzed by urease. This enzyme occupies a unique place in enzymology, in that it was the first enzyme to be crystallized (*2*). Besides its use in agricultural effluent treatment, immobilized urease has found applications in blood detoxification and urea removal from beverages and food (*3*). The broad range of applications of this enzyme promoted intensive work on the preparation and characterization of urease derivatives (*4*−*13*). The use of immobilized enzymes greatly reduces the expense of any biotechnological process requiring enzymes. For this reason, immobilized enzymes or whole cells are widely used in industrial processes (*14*−*16*), particularly in the food industry and ecology. As a result, enzyme immobilization has become one of the most important research area of biotechnology.

A grafting technique has been recently used by us to transform polymeric matrices into enzymatic carriers endowed with good mechanical strength and microbial resistance. β-Galactosidase has been immobilized on Teflon membranes, grafted with different monomers by γ-radiations (*17*−*21*). Nylon membranes, chemically grafted with different monomers, have also been used to immobilize β-galactosidase (*22*−*26*), urease (*9*, *10*), and penicillin G acylase (*27*, *28*).

One of the most serious problems for the industrial application of the technology of immobilized enzymes is the diffusional resistance to the transport of substrates. This limitation arises from steric hindrance of the solid matrix to the free diffusion of substrates and products toward or away from the catalytic site of immobilized enzymes. What is generally found is an

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increase of the value of the apparent kinetic parameter K_m, that is, an apparent decrease of the affinity of the enzyme for its substrate. In addition, the immobilization process induces twisting of the enzyme structure, with partial loss of activity. Despite these disadvantages, the use of bioreactors operating with immobilized enzymes has found several industrial applications (*14*−*16*), owing to the possibility of multiple reuse of the enzyme derivatives and easy separation of the reaction products from the catalyst. All of the bioreactors have so far been used under isothermal conditions.

Recently, a new technology has been proposed (*10*, *17*, *23*−*27*, *29*−*36*) to overcome the diffusional limitations and the apparent affinity loss of the enzyme for its substrate(s). This technology is based on the use of catalytic membranes in bioreactors operating under non-isothermal conditions. It has been found that, when a catalytic and hydrophobic membrane is interposed between two substrate solutions kept at different temperatures, an increase of the enzyme activity with respect to that measured under comparable isothermal conditions is observed. The activity increase was found to be proportional to the transmembrane temperature difference and to the hydrophobicity degree of the support. The activity increase was explained on the basis of the thermodynamics of irreversible processes (*37*, *38*), through the process of thermodialysis (*39*−*41*). In the presence of temperature gradients, a thermodiffusive transmembrane substrate flux is produced across a catalytic membrane, provided that the membrane is hydrophobic. This flux is added to the diffusive one, and the immobilized enzymes encounter a higher substrate concentration under non-isothermal conditions. The reaction rates are thus increased. A quantitative analysis, which accounts for substrate fluxes and substrate consumption by the enzyme reaction into the catalytic membrane, has been thoroughly described in some recent works (*23*−*26*).

To obtain catalytic and hydrophobic membranes, nylon or Teflon supports were grafted with suitable monomers. Factors affecting the hydrophobicity of membranes are the nature of the grafted monomers and the grafting degree when the same monomer is used. In our previous studies, the influence of the immobilization procedure on membrane activity was not assessed. In this paper, the biochemical and physical behavior of three catalytic membranes, obtained with different immobilization procedures and of different spacer lengths, is described under isothermal and non-isothermal conditions. Urease from Jack bean was used as a model enzyme, for its potential use in the treatment of urea-polluted agricultural waste waters.

MATERIALS AND METHODS

Materials. As solid support to be grafted, nylon Hydrolon membranes (a gift from Pall Italia, srl, Milano, Italy) were used. These hydrophobic membranes are 150 μm thick and have a nominal pore size of 0.2 μm. Pore size is related to the minimum diameter value of the smallest particles that the membrane retains; the membranes have no "classical" pores but irregular cavities across their thickness. All chemicals, including the enzyme, were purchased from Sigma (Sigma Aldrich, srl, Milano, Italy) and used without further purification. Methyl methacrylate (MMA) was used as a graft monomer. Hexamethylenediamine (HMDA) or hydrazine (HZ) was used as a spacer between the grafted membrane and the enzyme. Glutaraldehyde (GA) was used as a bifunctional coupling agent to covalently bind the enzyme to the preactivated membranes.

The enzyme was Sigma type III urease (EC 3.5.1.5) from Jack beans.

Bioreactor. The apparatus (**Figure 1**) consisted of two cylindrical half-cells, with a 35 mm diameter and a 2.5 mm depth, filled with substrate solution and separated by the catalytic membrane. Peristaltic pumps recirculated the working solutions in each half-cell, through

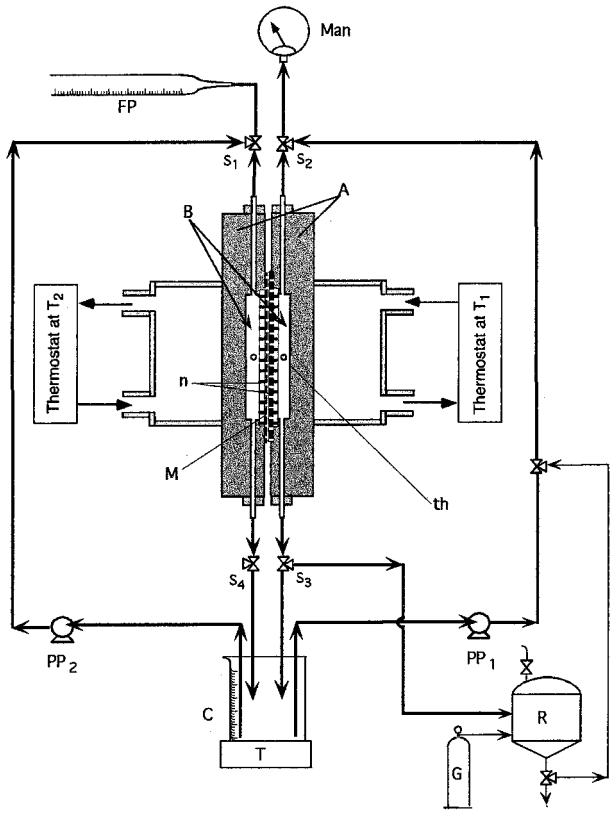

Figure 1. Schematic (not to scale) representation of the bioreactor: (A) half-cells; (B) internal working volumes; (C) external working volume; (M) membrane; (n) supporting nets; (th) thermocouples; (S_i) stopcocks; (T) thermostatic magnetic stirrer; (PP_i) peristaltic pumps; (Man) manometer; (FP) flow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

hydraulic circuits starting and ending in a common cylinder (C). Each half-cell was thermostated at a given temperature by thermostatic baths. Thermocouples were used to measure the temperatures T_i (i = 1, 2) inside each half-cell. When the apparatus worked under isothermal conditions, T_1 was equal to T_2. Under non-isothermal conditions T_1 was different from T_2. Thermocouples were placed 1.5 mm from each of the membrane surfaces. The temperature profile across the bioreactor and the catalytic membrane was calculated by starting from the T_1 and T_2 values, according to the procedure described under Temperature Profile across the Catalytic Membrane. The temperatures read by the thermocouples are indicated as T, whereas those calculated at the membrane surfaces are indicated as T^*. The temperatures of the warm or cold side are indicated by the subscript W or C, respectively. It follows that $\Delta T = T_W - T_C$, $\Delta T^* = T^*_W - T^*_C$, $T_{av} = (T_W + T_C)/2$, $T^*_{av} = (T^*_W + T^*_C)/2$, $T^*_W < T_W$, $T^*_C > T_C$, and $\Delta T^* < \Delta T$. Because the system is symmetric, $T_{av} = T^*_{av}$.

Methods. *Temperature Profile across the Catalytic Membrane.* To estimate the actual effects of temperature gradients on the activity of immobilized enzymes, the temperatures on the surfaces of the catalytic membrane must be known.

It has been demonstrated (*29*, *36*) that the fluid motion in each half-cell is laminar. Thus, heat flux J_q through the bioreactor occurs by conduction between isothermal liquid planes (**Figure 2a**), according to the equation

$$J_q = \lambda_i(\Delta T/\Delta x)_i = \text{constant} \qquad (1)$$

where λ_i is the thermal conductivity of the ith medium, $(\Delta T/\Delta x)_i$ the temperature gradient existing in the same medium, Δx_i thick.

By using computer simulation, it is possible to calculate the temperature values at each point of the apparatus and, hence, at the two surfaces of the catalytic membrane. In this calculation the thermal conductivity of our solutions has been taken as that of pure water,

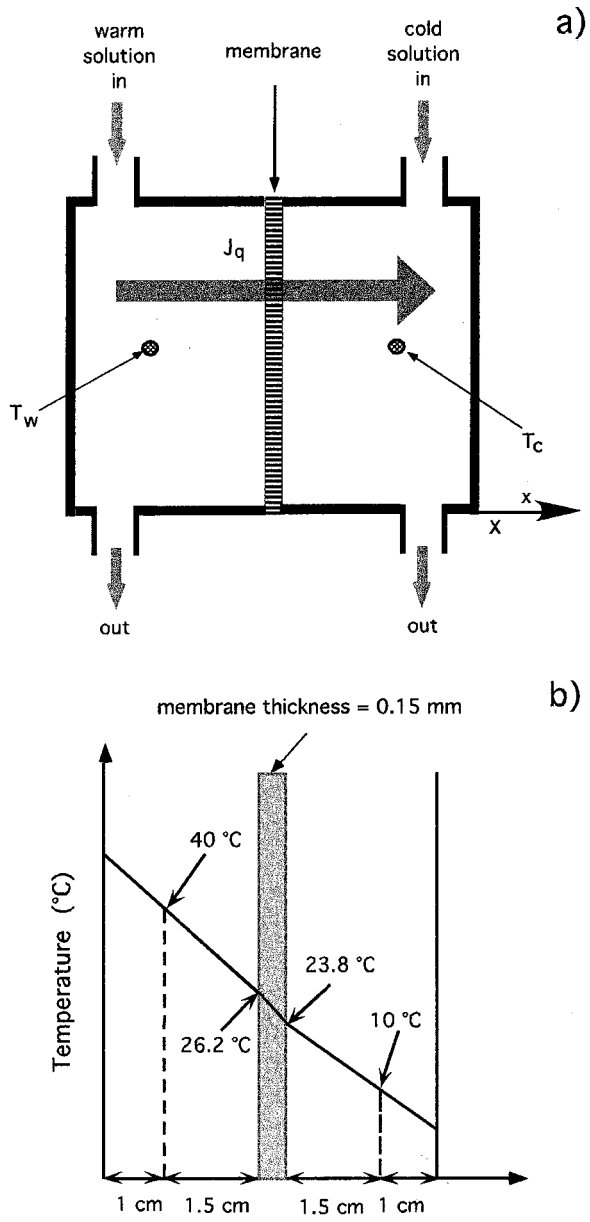

Figure 2. (a) Side view of the cell, showing the position of the thermocouples, the membrane, and the heat flux (J_q). (b) Temperature profile in the bioreactor, in the particular case when $T_W = 40$ °C and $T_C = 10$ °C. Magnification along the *x*-axis is 10.

Table 1. Correspondence between the Temperatures Read at the Thermocouple Positions (T) and Those Calculated on the Membrane Surface (T^*)[a]

T_{av}	ΔT	T_C	T_W	T^*_C	T^*_W	T^*_{av}	ΔT^*
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

[a] All temperatures are given in °C. Subscripts W and C refer to the warm and cold sides, respectively.

whereas the thermal conductivity of nylon membranes was taken from Touloukian (*42*). It was also assumed that the grafting process did not change the thermal conductivity of the untreated membrane. In **Table 1**, the temperatures measured at the thermocouple positions and those corresponding to the surfaces of the catalytic nylon membrane are reported. In **Figure 2b** the actual temperature profile for one of the cases reported in **Table 1** is shown. From the temperature values reported in **Table 1**, the following empirical equations can be derived:

$$T^*_W = T_W - a\Delta T$$

$$T^*_C = T_C + a\Delta T \quad (2)$$

$$\Delta T^* = \Delta T(1 - 2a)$$

In these equations a is a constant. In our case $a = 0.46$.

Preparation of the Catalytic Membranes. The preparation of the catalytic membranes was carried out in two steps: grafting copolymerization and enzyme immobilization.

Grafting copolymerization was carried out by using 0.093 M $K_2S_2O_8$ as initiating system. The membranes were immersed in a reaction vessel filled with a 1/1 water/ethanol solution containing 0.38 M MMA for 40 min at 60 °C. The membranes were then treated with acetone to remove the produced homopolymer and dried at 40 °C until a constant weight was obtained. At this point, nylon−poly(MMA) membranes were obtained. Grafting percentage (X, %) was calculated through the expression $X(\%) = [(G_A - G_B)/G_B] \times 100$, where G_B and G_A are the masses of the membrane before and after the grafting process, respectively.

Two different methods were used for urease immobilization onto the nylon−poly(MMA) membranes. Membranes were identified as M1, M2, and M3, according to the immobilization method and the type of spacer.

(a) Preparation of Membrane M1. Nylon−poly(MMA) membrane was activated by reaction with a 10% (v/v) HMDA aqueous solution for 1 h at room temperature. After washing with running water, the membrane was allowed to react with a 2.5% glutaraldehyde solution for 1 h at room temperature. After a further washing with running water, the membrane was immersed in a 0.1 M phosphate buffer solution, pH 6.5, containing urease at a concentration of 1 mg/mL, for 16 h at 4 °C. During this step, cross-linking between the aldheyde groups of the carrier and the amino groups of the enzyme occurred. The −SH groups of urease may cross-link with glutaraldehyde when all of the amine groups of the enzyme have been used. It is important to note that $-NH_2$ groups are more susceptible than −SH groups to reaction with glutaraldehyde (*8*). Washings with 0.1 M citrate buffer, pH 5.0, were carried out to remove the unbound enzyme.

(b) Preparation of Membrane M2. Nylon−poly(MMA) membrane was activated by reaction with a 1.0% (w/v) hydrazine aqueous solution for 20 min at room temperature. After this treatment, the procedure used for membrane M1 was followed to immobilize urease.

(c) Preparation of Membrane M3. The activation of nylon−poly(MMA) membrane was performed by hydrazinolysis (to form the hydrazide), using a 1% (w/v) hydrazine aqueous solution for 20 min at room temperature. The hydrazide was allowed to react with nitrous acid (2 N HCl + 4% $NaNO_2$ solution, volume ratio 2/1) for 1 h at 0 °C, to obtain acyl azide. After washing with ice-cold water, the membrane was allowed to react with a 0.1 M phosphate buffer solution, pH 8.5, containing urease at a concentration of 1 mg/mL, for 16 h and at a temperature of 4 °C. In this way, the acyl azide reacts with the nucleophilic groups of the enzyme, such as sulfhydryl, amino, or hydroxyl groups, to give thioester, amide, or ester linkage, respectively. The amount of immobilized protein was calculated by subtracting the amount of protein recovered in the solution at the end of the immobilization process and in the washing solutions from that initially present in the immobilization solution. Determination of urease concentration was performed according to the Lowry method (*43*).

Determination of Membrane Activity and Stability. Lactose and dithiothreitol (5 mM) were added to both the reaction mixture and the buffer solution to stabilize urease (*44*).

Membrane activity was determined by sampling, at regular time intervals, the urea solution interacting with the catalytic membrane and by measuring the ammonia concentration resulting from the reaction of urease with the urea. Ammonia concentration was measured by means of the phenol−hypochlorite method (*45*), through which a blue solution is developed. The ammonia concentration was spectrophotometrically determined at 625 nm by using a calibration curve. Membrane activity, indicated also as enzyme reaction rate, is expressed

Table 2. Comparison of the Physical–Chemical and Activity Parameters of Unsoluble Urease

enzyme form	spacer length	grafting (%)	urease (mg)	absolute activity (10^{-2}, μmol min^{-1} cm^{-2})	specific activity (μmol min^{-1} mg^{-1})
M1	long	14.1 ± 1.3	1.74	2.6	1.04
M2	short	13.9 ± 1.7	1.54	2.14	0.97
M3	none	14.4 ± 1.5	0.81	1.14	0.98

as micromoles per minute and is given by the slope of the linear plot of the ammonia production as a function of time.

The time stability of the three biocatalytic membranes was assessed by measuring their activity every day, under the same experimental conditions, that is, 15 mM urea in 0.1 M citrate buffer, at pH 5.0 and 25 °C. After a few days, during which the membrane lost part of its activity, a stable condition was reached for more than one month. Only these stabilized membranes were used for our study. When not used, the membranes were stored at 4 °C in 0.1 M citrate buffer, pH 5.0.

Treatment of Experimental Data. Every experimental point reported in the figures represents the average value of five experiments performed under the same conditions. The experimental errors did not exceed 10%. Each experiment lasted 24 min, but only the initial reaction rates were accounted for in the construction of the figures.

RESULTS AND DISCUSSION

Isothermal Characterization. Table 2 reports some of the physical and biochemical properties of the three membranes. The activity assays were carried out with 15 mM urea in 0.1 M buffer citrate, at pH 5.0 and 25 °C. Absolute membrane activity was calculated as the number of activity units (one unit is 1 μmol min^{-1}) per total membrane surface (two surfaces, each with a 35 cm^2 surface area), and the specific activity as activity units per milligram of immobilized urease. Results from this table show that the specific activities of the three membranes were the same, despite the differences in spacer length and amount of immobilized enzyme.

pH Dependence of Membrane Activity. The pH dependence of the activity of an immobilized enzyme is characteristic of the enzyme and carrier nature, as well as of the immobilization method. The support can change the pH value around the catalytic site, thus determining differences between the catalytic behavior of soluble and insoluble enzyme. This effect, known as the partitioning effect, is related to the chemical nature of the support (and of the grafted monomer) and arises from electrostatic or hydrophobic interactions between the matrix and the low molecular weight species present in solution. Partitioning effects cause differential concentrations of charged species (e.g., hydrogen and hydroxyl ions) in the microenvironment of immobilized enzymes. Thus, the pH−activity profile of immobilized enzymes may be displaced toward more alkaline or acidic pH values for negatively or positively charged matrices, respectively.

To know how the pH−activity profile was affected by the immobilization procedure and spacer length, we have studied the activity of free and immobilized urease in the pH range between 4.0 and 7.5. The results of this investigation are illustrated in **Figure 3**, in which the relative activity of each catalytic membrane is reported as a function of pH, together with that of the free enzyme as a comparison. Immobilization did not shift the position of the optimum pH of the enzyme, which occurred at pH 6.0 for both the soluble and insoluble forms of urease. Similar results were obtained by us (*9*) and other authors (*4*, *5*, *7*, *8*, *13*) with different types of immobilized urease. The "optimum pH range", however, was different not

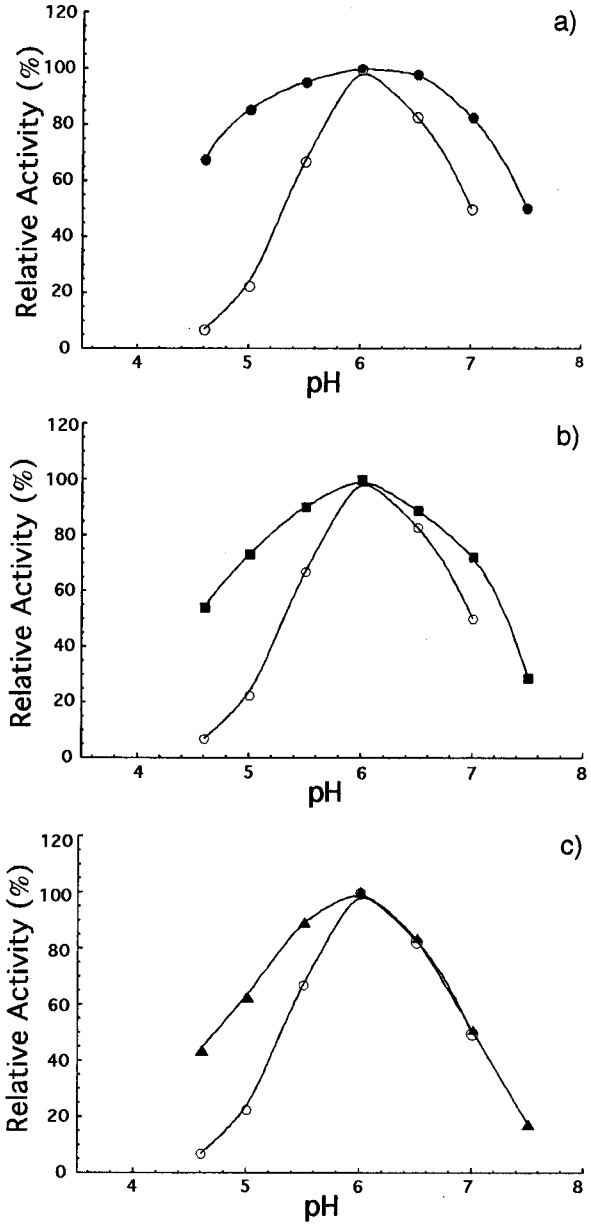

Figure 3. Relative enzyme activity as a function of pH for free (○) and immobilized urease: (a) membrane M1 (●); (b) membrane M2 (■); (c) membrane M3 (▲). Reactions were carried out in a 15 mM urea solution in 0.1 M citrate, at T = 25 °C.

only for free and immobilized urease but also for the three enzyme derivatives. The optimum pH range was defined as the range in which the relative activity is ≥95%. It occurred in the pH range 5.9−6.2 for the free enzyme, 5.5−6.6 for membrane M1, 5.7−6.2 for membrane M2, and 5.9−6.2 for membrane M3. Hence, the optimum pH range appeared to decrease with the decrease of spacer length. Moreover, at pH 5.0, the enzyme derivative retained ∼85% of its maximum activity in the case of membrane M1, ∼75% for membrane M2, and ∼60% in the case of membrane M3. Similarly, at pH 7.0 membrane M1 retained ∼80% of its maximum activity, membrane M2 ∼70%, and membrane M3 ∼50%. From **Figure 3** it also appears that all membranes were more resistant than free enzyme to acidic solutions and that membrane M1 retained most of its maximum activity under these extreme conditions.

Temperature Dependence of Membrane Activity. In **Figure 4**, the relative activity of each of the three catalytic membranes

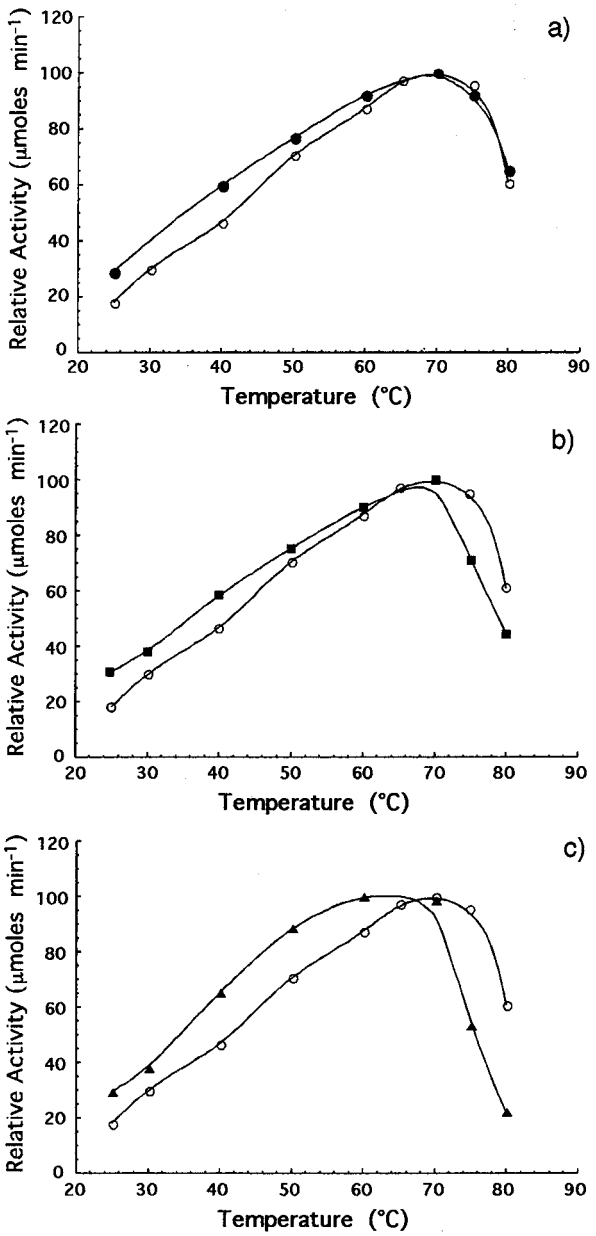

Figure 4. Relative enzyme activity as a function of temperature for free (○) and immobilized urease: (a) membrane M1 (●); (b) membrane M2 (■); (c) membrane M3 (▲). Reactions were performed in a 15 mM urea solution in 0.1 M citrate, at pH 5.

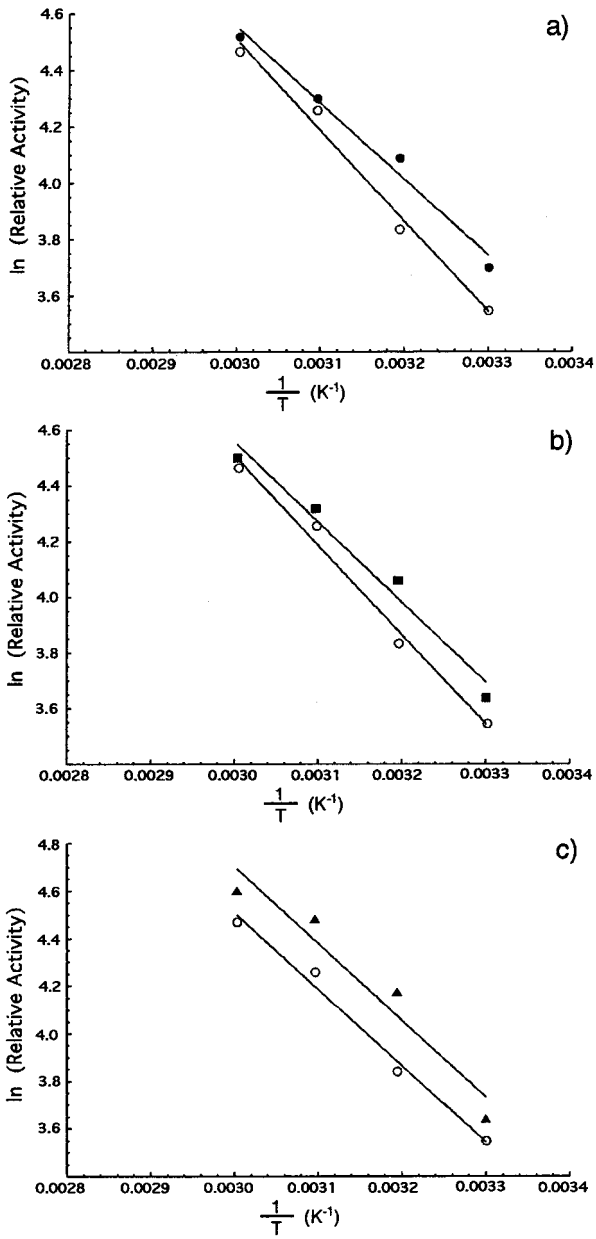

Figure 5. Arrhenius plots of the activity of the free (○) and immobilized urease: (a) membrane M1 (●); (b) membrane M2 (■); (c) membrane M3 (▲). Experimental conditions were the same as for the results reported in **Figure 4**.

as a function of temperature is reported. The relative activity of free urease is also reported as a comparison. Free urease showed an optimum temperature at ~70 °C, whereas the optimum temperature of immobilized urease appeared to be shifted toward lower temperatures, in a way inversely proportional to the spacer length. Indeed, the optimum temperature occurred at 68.5 °C for membrane M1, at 67.5 °C for membrane M2, and at 63.5 °C for membrane M3. The unusual shift toward lower temperatures was never observed by us with immobilized urease (*9*) or other enzyme derivatives (*18*, *19*, *22*, *24*, *25*). The decrease of the optimum temperature value with the decrease of the distance between the enzyme and membrane suggests that immobilization through the acyl azide method twists the enzyme structure more than the immobilization via a Schiff base. By defining the "optimum temperature range" as the range in which the relative enzyme activity is ≥95%, this range occurred between 63 and 75 °C for free urease, between 61.5 and 73.9 °C for membrane M1, between 63 and 71 °C for membrane M2, and between 55.1 and 69.2 °C for membrane M3.

In **Figure 5**, the results of **Figure 4** are reported as Arrhenius plots in the temperature range between 30 and 60 °C. The calculated values of the activation energies were 6.2 ± 0.1 kcal mol^{-1} for free urease, 5.3 ± 0.1 kcal mol^{-1} for membrane M1, 5.7 ± 0.2 kcal mol^{-1} for membrane M2, and 6.4 ± 0.8 kcal mol^{-1} for membrane M3. Except for membrane M3, the activation energy values of the catalytic membranes were lower than that of free urease. These results suggest that, only in the case of free urease and membrane M3, the rate of the enzyme reaction was kinetically rather than diffusion controlled (*46*).

Concentration Dependence of Membrane Activity. The catalytic activity of each of the three catalytic membranes was studied as a function of substrate concentration, in the range 0−120 mM, in 0.1 M citrate buffer solution, at pH 5.0 and 25 °C. A Michaelis−Menten behavior was found. The values of

Table 3. Kinetic Parameters of Urease Derivatives and Free Enzyme, Obtained under Isothermal ($T = 25$ °C) and Non-isothermal ($T_{av} = 25$ °C, $\Delta T = 30$ °C) Conditions

enzyme form	K_m (mM)		V_{max} (μmol min^{-1})	
	$\Delta T = 0$ °C	$\Delta T = 30$ °C	$\Delta T = 0$ °C	$\Delta T = 30$ °C
M1	54 ± 8	30 ± 2	8.8 ± 0.9	8.9 ± 0.6
M2	23 ± 5	16 ± 3	3.9 ± 0.6	4.7 ± 0.6
M3	31 ± 5	19 ± 3	2.8 ± 0.4	2.9 ± 0.5
free	15 ± 3		11.5 ± 0.5	

Table 4. Physical Parameters of the Catalytic Membranes

membrane	spacer length	A (10^{-15}, N^{-1} m^4 s^{-1})	B (10^{-12}, m^2 K^{-1} s^{-1})	C (10^2, Nm^{-2} K^{-1})
M1	long	6.0	2.4	4.0
M2	short	1.0	0.17	1.7
M3	none	8.5	2.1	2.5

the kinetic parameters K_m and V_m are reported in **Table 3**, together with that of the free urease. From the table it is possible to see that the apparent K_m values of the urease derivatives were higher than that of the free counterpart. The increase of the apparent K_m values upon immobilization clearly indicates an apparent lower affinity of the immobilized enzymes for the substrate compared to that of the free enzyme. This behavior may be attributed to changes in enzyme structure induced by the interaction of the macromolecule with the support (*47*). In addition, the increased diffusional resistance encountered by the substrate (products) toward (from) the catalytic site of immobilized enzymes could be responsible for the higher K_m values (*47*).

Non-isothermal Characterization. *Physical Characterization of the Membranes.* In **Table 4** are reported some physical parameters of the membranes M1, M2, and M3.

The hydraulic permeability coefficient A (N^{-1} m^4 s^{-1}) was calculated by the equation

$$J_{water}^{hydr} = A(\Delta P/\Delta x) \quad (3)$$

where J_{water}^{hydr} (ms^{-1}) is the isothermal hydraulic water flux produced by a pressure difference ΔP (N m^{-2}) across the membrane, Δx (m) thick.

The thermo-osmotic permeability coefficient B (m^2 K^{-1} s^{-1}) was calculated through the equation

$$J_{water}^{therm} = B(\Delta T/\Delta x) \quad (4)$$

where J_{water}^{therm} (ms^{-1}) is the non-isothermal water flux across a membrane Δx (m) thick, produced by a temperature difference ΔT (K), as measured at the position of the thermocouples.

Hydraulic fluxes were measured by pressurizing one half-cell by a gas cylinder and measuring the rate of water volume transport to the other half-cell by means of a graduate pipe (**Figure 1**). During these measurements, the temperature was kept constant. The experimental conditions were $\Delta P = 3 \times 10^3$ N m^{-2}, $T = 25$ °C, and $\Delta T = 0$. Under these conditions, the following hydraulic fluxes (J_{water}^{hydr}) were obtained: 1.2×10^{-7} ms^{-1} for membrane M1, 2.0×10^{-8} ms^{-1} for membrane M2, and 1.7×10^{-7}ms^{-1} for membrane M3.

Thermo-osmotic fluxes were determined by measuring, in a graduate pipe, the water volume transported from the warm to the cold half-cell, in the presence of temperature gradients and in the absence of pressure gradients. The experimental conditions for these experiments were $\Delta T = 30$ °C, $T_{av} = 25$ °C, and $\Delta P = 0$. The following thermo-osmotic fluxes (J_{water}^{therm}) were measured: 4.8×10^{-7} ms^{-1} for membrane M1, 3.3×10^{-8} ms^{-1} for membrane M2, and 1.4×10^{-7} ms^{-1} for membrane M3.

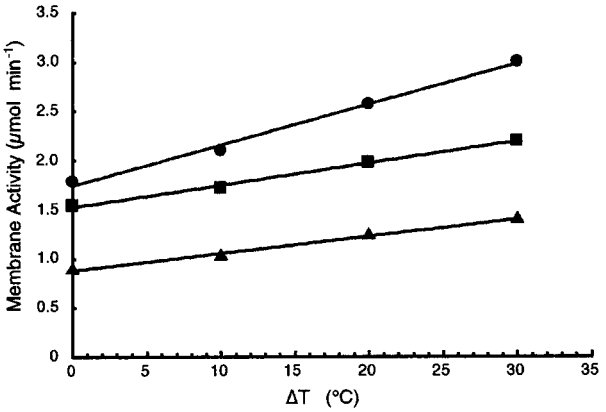

Figure 6. Activities of membrane M1 (●), M2 (■), and M3 (▲) as a function of the temperature difference ΔT measured at the thermocouple positions. Experimental conditions: 15 mM urea in 0.1 M citrate, at pH 5.0, and $T_{av} = 25$ °C.

The values of the coefficients A and B for M1, M2, and M3 membranes are listed in **Table 4**. In the same table, we have also reported the coefficient C, defined as

$$C = \frac{\Delta P_{S-S}}{\Delta T} = \frac{B}{A} \quad (5)$$

C represents the pressure difference ΔP_{S-S} to be applied to the cold half-cell to balance the thermo-osmotic water flux coming from the warm half-cell in the presence of a temperature difference ΔT measured at the thermocouple positions. C is indeed a measure of the effect produced by the process of thermodialysis across catalytic or untreated hydrophobic membranes. For $C \neq 0$, the process of thermodialysis takes place. $C = 0$ implies the absence of thermodialysis. Hence, higher C values correspond to larger matter fluxes produced by the process of thermodialysis. Because thermodialysis depends on membrane hydrophobicity, it follows that for $C = 0$, that is, in the absence of thermodialysis, the activity of a catalytic membrane should be the same under isothermal or non-isothermal conditions. By contrast, for $C > 0$, the reaction rates of a catalytic membrane working under non-isothermal conditions should be higher than those measured under comparable isothermal conditions.

Biochemical Characterization of the Catalytic Membrane under Non-isothermal Conditions. In **Figure 6** are reported the activities of the three catalytic membranes as a function of the temperature difference read at the thermocouple positions. As one can see, the catalytic activity increased in a linear fashion with the applied ΔT, for all membranes. These experiments were carried out with 15 mM urea in 0.1 M citrate, at pH 5.0 and $T_{av} = 25$ °C. Once the effect of the temperature gradients on the activity of the three membranes was assessed, we extended our investigation by testing under non-isothermal condition the membrane activities as a function of the urea concentration (**Figure 7**). Results in **Figure 7** show that, at every urea concentration, the non-isothermal activities of the three catalytic membranes were higher than those found under comparable isothermal conditions.

The apparent values of K_m and V_{max} for each of the three catalytic membranes under non-isothermal conditions are listed in **Table 3**. From the table it is possile to see that the apparent values of K_m under non-isothermal conditions were lower than

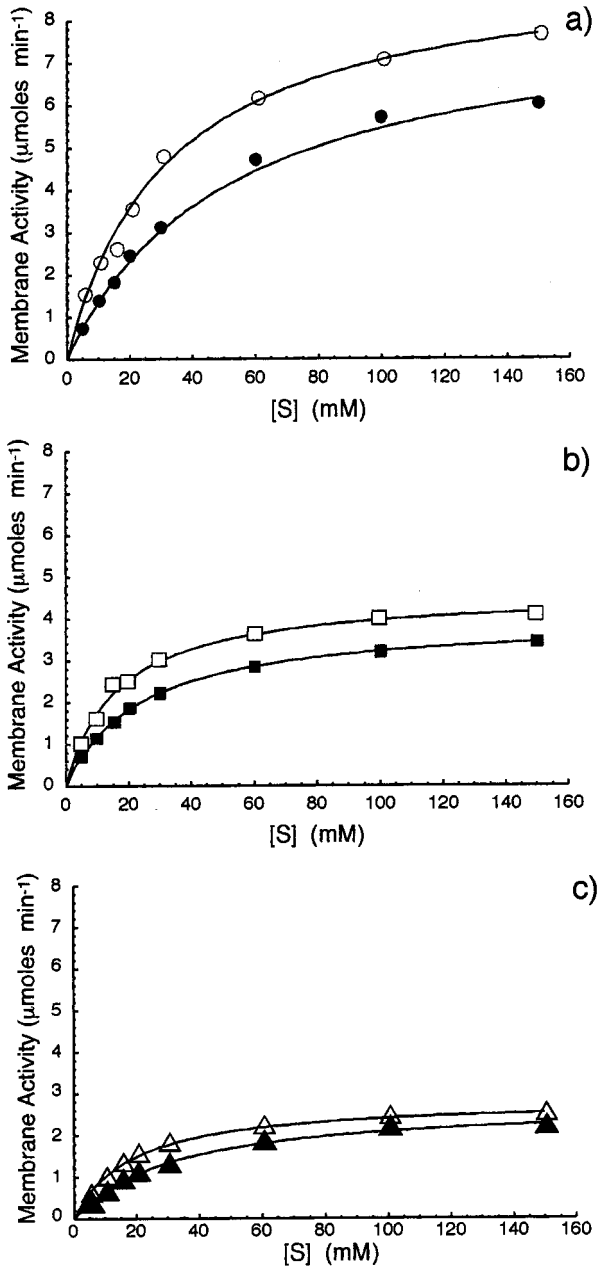

Figure 7. Michaelis–Menten plots relative to the activity of (a) membrane M1, (b) membrane M2, and (c) membrane M3, measured under isothermal (T = 25 °C, solid symbols) and non-isothermal (T_{av} = 25 °C, ΔT = 30 °C, open symbols) conditions.

the corresponding values under isothermal conditions, indicating a recovery of the affinity lost by the enzyme upon immobilization. This result suggests that the temperature gradient increases the substrate and product fluxes across the catalytic membrane, thus reducing the diffusion limitations toward or away from the catalytic site.

From the results reported in **Figure 7**, it is possible to calculate the percentage activity increase (PAI), a parameter giving information on the performance of non-isothermal bioreactors in industrial processes. It is defined as

$$\mathrm{PAI} = \frac{[\mathrm{RR}]_{T=T_{av}}^{\Delta T \neq 0} - [\mathrm{RR}]_{T=T_{av}}^{\Delta T=0}}{[\mathrm{RR}]_{T=T_{av}}^{\Delta T=0}} \qquad (6)$$

where $[\mathrm{RR}]_{T=T_{av}}^{\Delta T=0}$ and $[\mathrm{RR}]_{T=T_{av}}^{\Delta T\neq 0}$ are the reaction rates of the immobilized enzyme at $T = T_{av}$, under isothermal and non-isothermal conditions, respectively.

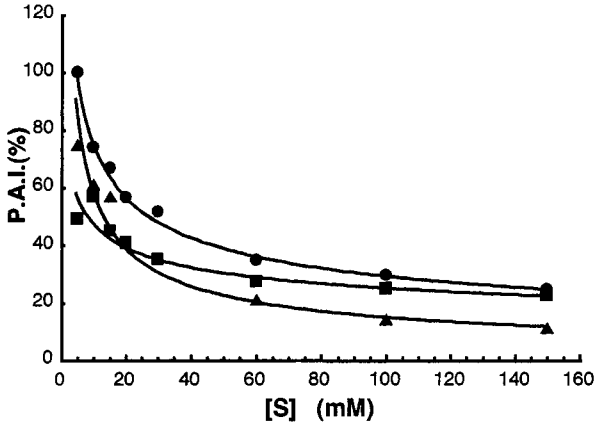

Figure 8. Percentage activity increase (PAI) of membrane M1 (●), membrane M2 (■), and membrane M3 (▲) as a function of the urea concentration. Experimental conditions: T_{av} = 25 °C and ΔT = 30 °C.

In **Figure 8** the PAI values for each of the three membranes are reported as a function of urea concentration. Decreases of the PAI values with the increase of substrate concentration have already been found by us with β-galactosidase immobilized on differently grafted membranes (*24*−*26*) and with urease immobilized on nylon membranes grafted with butyl methacrylate (*10*). An explanation for such a behavior is that when the immobilized enzyme works approximately at its maximum rate, such as at high substrate concentrations, the activity increases due to temperature gradients are less effective. A quantitative explanation of this behavior has been recently published by us (*26*). In this study, the substrate concentration profiles into a catalytic membrane have been derived under isothermal and non-isothermal conditions, and a parameter similar to the PAI has been calculated. The dependence of this parameter on substrate concentration was found to be very similar to that of the PAI shown in **Figure 8**. The PAI values from **Figure 8**, calculated with respect 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. The impact of our results is even more evident, if the actual temperature differences across the membrane are taken into consideration. As one can see from **Table 1**, a macroscopic ΔT = 30 °C corresponds to an actual ΔT^* = 2.4 °C. Hence, it is possible to define a new coefficient α', related to the PAI coefficient by the equation

$$\alpha' = \mathrm{PAI}/\Delta T^* \qquad (7)$$

α' represents the percentage increase of the enzyme reaction rate when a unit temperature difference is actually applied across the catalytic membrane. The α' values, relative to the results in **Figure 8**, are listed in **Table 5**. For each of the three membranes, the efficiency of the non-isothermal reaction decreased with the increase of the substrate concentrations. Membrane M1 was found to be the most efficient.

All of the results above-reported confirm the advantage of using non-isothermal bioreactors, because an increase of enzyme activity corresponds to a reduction of the production times. To demonstrate such a statement, we report in **Figure 9a**, as a function of the reaction time, the ammonia produc-

Table 5. α′ Coefficients of Urease Derivatives as a Function of Urea Concentration

	α′ (% °C^{-1})		
[urea] (mM)	M1	M2	M3
5	41.7	24.5	37.7
10	31.2	20.2	25.4
15	27.1	17.7	19.6
20	23.5	16.4	16.4
30	20.0	14.6	12.8
60	15.2	12.1	8.4
100	12.2	10.5	6.4
150	10.4	9.2	5.1

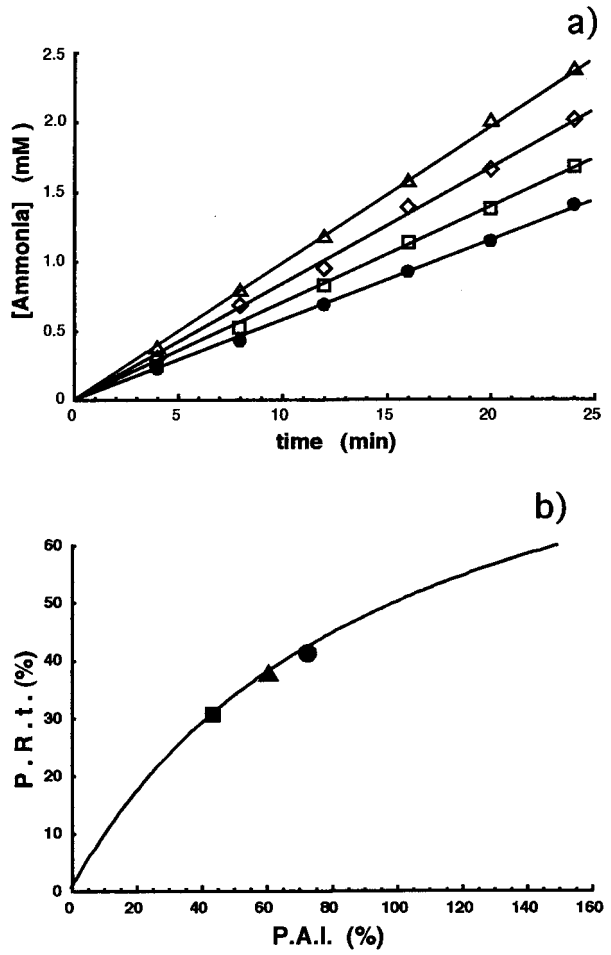

Figure 9. (a) Ammonia production as a function of time for membrane M1. The reactions were carried out with 15 mM urea in 0.1 M citrate, at pH 5.0 and $T = 25$ °C under isothermal (●) and non-isothermal conditions with $\Delta T = 10$ °C (□), $\Delta T = 20$ °C (◇), and $\Delta T = 30$ °C (△). (b) Percentage reduction of the production times of membrane M1 (●), membrane M2 (■), and membrane M3 (▲) as a function of the PAI. Curve is derived from eq 8.

tion under isothermal and non-isothermal conditions when a 15 mM urea solution is treated with membrane M1. From this figure, it is possible to see that, to obtain a urea conversion of 9.3% (corresponding to an ammonia concentration of 1.4 mM), 24 min was required under isothermal conditions, 20 min with a $\Delta T = 10$ °C, 16.5 min with a $\Delta T = 20$ °C, and 14 min with a $\Delta T = 30$ °C. This clearly indicates a decrease of the urea conversion time with increasing ΔT values. We can, therefore, define the percentage reduction of production time (PRt) related to the PAI by the equation

$$\mathrm{PRt} = \frac{t_{\mathrm{iso}} - t_{\mathrm{non\text{-}iso}}}{t_{\mathrm{iso}}} = \frac{\mathrm{PAI}}{\mathrm{PAI} + 100} \qquad (8)$$

where t_{iso} and $t_{\mathrm{non\text{-}iso}}$ are the times required to obtain a given yield of an enzymatic process under isothermal and non-isothermal conditions, respectively. A plot of eq 8 is reported in **Figure 9b**, where it is possible to appreciate how the percentage reductions of the production times of an enzyme process performed under non-isothermal conditions are related to the PAI and, consequently, to the applied ΔT. **Figure 9b** reports the percentage reduction of the production times relative to the PAI values obtained with each of the three membranes at a 15 mM urea concentration and with a temperature difference $\Delta T = 30$ °C. From **Figure 9b**, it is possible to observe how the reduction times for the membranes M1, M2, and M3 increase with the increase of the value of the *C* coefficient. These results emphasize the role of membrane hydrophobicity in the process of thermodialysis and thus for catalytic processes carried out in non-isothermal bioreactors. The dependence of the PAI on enzyme immobilization procedure and spacer length is also evident. Similar considerations can be done from the average PAI values calculated from **Figure 8**. These values are ∼60% for membrane M1, 40% for membrane M2, and 45% for membrane M3; the average PAI values of the three membranes are somehow related to the corresponding *C* coefficients and, hence, to their performance in the process of thermodialysis.

Conclusions. This work has shown that the hydrolysis of urea by urease immobilized on nylon grafted membranes is increased under non-isothermal conditions, thus confirming the advantage in using non-isothermal bioreactors in industrial processes employing immobilized enzymes. The α′ values reported in **Table 5** also indicate that the increase of urea hydrolysis in the presence of a fixed transmembrane temperature difference depends on membrane type, that is, in the present case on the immobilization procedure and spacer length. Also the pH, temperature, and concentration dependence of the membrane activities under isothermal conditions showed a clear dependence on these two parameters.

In view of further industrial applications of our technology we are currently trying to construct more efficient non-isothermal bioreactors by changing the planar geometry of our supports and using capillary hollow fiber systems.

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