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Influence of the spacer length on the activity of enzymes immobilised on nylon/polyGMA membranes Part 2: Non-isothermal conditions

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Abstract

β-Galactosidase has been immobilised through spacers of different length on nylon membranes grafted with glycidyl methacrylate. Hexamethylendiamine, ethylendiamine or hydrazine have been separately used as spacers.

The behaviour of the catalytic membranes has been studied in a bioreactor operating under non-isothermal conditions as a function of the applied temperature difference ΔT.

Comparison of the enzyme reaction rates under isothermal and non-isothermal conditions resulted in percentage activity increases (PAI) and reduction of the production time (τ_r) proportional to the size of the applied ΔT. Both these parameters increased with the increase of the spacer length.

Results have been discussed in the frame of reference of the process of thermodialysis which reduces the limitations to the diffusion of substrate and reaction products across the catalytic membrane, limitations introduced by the grafting and immobilisation process.

The advantages of employing non-isothermal bioreactors in biotechnological productive processes have been outlined.

Keywords: β-Galactosidase; Chemical grafting; Biocatalytic membranes; Non-isothermal bioreactors; Nylon membranes; Thermodialysis

1. Introduction

It is well-known that the catalytic activity of enzymes immobilised on a solid carrier is lower than that of an equal amount of soluble enzyme [1–3]. The activity loss is attributed to several factors, among which the changes in the structure of the macromolecule and/or the limitations to the free diffusion of substrate and products towards or away from the catalytic site. Both causes are related to the interactions between the carrier and the enzyme. To minimise the effects of these interactions, a spacer can be introduced between the support and the enzyme, provided that the attachment to the spacer does not interfere with the aminoacidic residues of the catalytic site [4].

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In a separate paper [5], it has been reported the influence of the spacer length on the isothermal activity of β-galactosidase immobilised on nylon membranes grafted with glycidyl methacrylate (GMA). The different spacers had the structure NH_2-$(CH_2)_i$-NH_2, with $i = 0$, 4 or 6. Under isothermal conditions, the behaviour of the immobilised enzyme was found to be dependent on the spacer length when compared with that of the soluble counterpart. In particular, the size of the shifts of the optimum pH and optimum temperature values were found to be correlate to the spacer length. It was concluded that a soluble enzyme can be considered as an enzyme immobilised on a solid support through a spacer of infinite length. Similar conclusions were derived when the values of the apparent K_m of the immobilised enzyme were compared with that of the free enzyme. Moreover, the apparent K_m of the immobilised enzymes resulted higher than that of the soluble β-galactosidase. This result confirmed that the loss of catalytic activity of the enzyme derivatives was mainly due to diffusional limitations.

To overcome this drawback, some tricks have been attempted, such as enzyme immobilisation onto soluble–insoluble matrices [6–11] or in hydrogels thermally reversible [12,13] or pressure sensitive [14]. Recently, a new strategy to improve the yield of a catalytic process has been proposed [15–24] and to reduce the differences between the K_m values of the soluble and insoluble enzyme. This new technology is based on the use of catalytic membranes, porous and hydrophobic, in non-isothermal bioreactors. Under these conditions a termodiffisive substrate flux adds to the diffusive one. As a consequence, the immobilised enzyme "encounters" a substrate concentration higher than that occurring under isothermal conditions, and the apparent value of the K_m decreases. It follows that it is possible to measure increases of the activity of a catalytic membrane, proportional to the applied temperature differences. A quantitative explanation of this effect has been done [19,21] on the basis of the matter transport induced by temperature gradients across hydrophobic and porous membrane, i.e. on the basis of the process of thermodialysis [25,26]. Coupling between matter and heat fluxes is one of the main topics of the thermodynamics of irreversible processes [27,28].

In this paper, we will report the influence of the spacer length on the non-isothermal activity of β-galactosidase immobilised on nylon membranes grafted with glycidyl methacrylate. It will be demonstrated that under non-isothermal conditions the percentage activity increases of the membranes are dependent on the spacer length, the applied temperature difference and membrane hydrophobicity.

2. Apparatus, materials and methods

2.1. The bioreactor

The apparatus (Fig. 1) consisted of two cylindrical half-cells, 2.5 mm in depth and 35 mm in diameter, filled with the working solution containing the substrate and separated by the catalytic membrane. Substrate solutions were recirculated in each half-cell at a rate of 3.5 mL min^{-1} by means of peristaltic pumps through hydraulic circuits starting and ending in a common cylinder C. Each half-cell was separately thermostatted at the required temperature by circulation in external jackets of water coming from a thermostatic bath. Thermocouples placed 1.5 mm from each of the membrane surfaces were used to measure the temperatures T_i ($i = 1, 2$) inside each half-cell. Under isothermal conditions T_1 was equal to T_2, while under non-isothermal condition T_1 was different from T_2.

When the apparatus was used as permeability cell or thermodialysis cell to measure the hydraulic or thermoosmotic water fluxes, alternative hydraulic circuits and devices were used. The functioning of the apparatus during these measurements will be described in the following.

2.2. Materials

As solid support to be grafted, we used nylon Hydrolon membranes, a gift of Pall Italia (Pall Italia srl-Milano-Italy). These membranes, 150 μm in thickness, are hydrophobic and have a nominal pore size of 0.2 μm.

All chemicals, including the enzyme, were purchased from Sigma (Sigma–Aldrich srl-Milano-Italy) and used without further purification. As the monomer to be grafted, we used glycidyl methacrylate (GMA). Hexamethylendiamine (HMDA) or hethylendiamine (HTDA) or hydrazine (HZ) were separately used

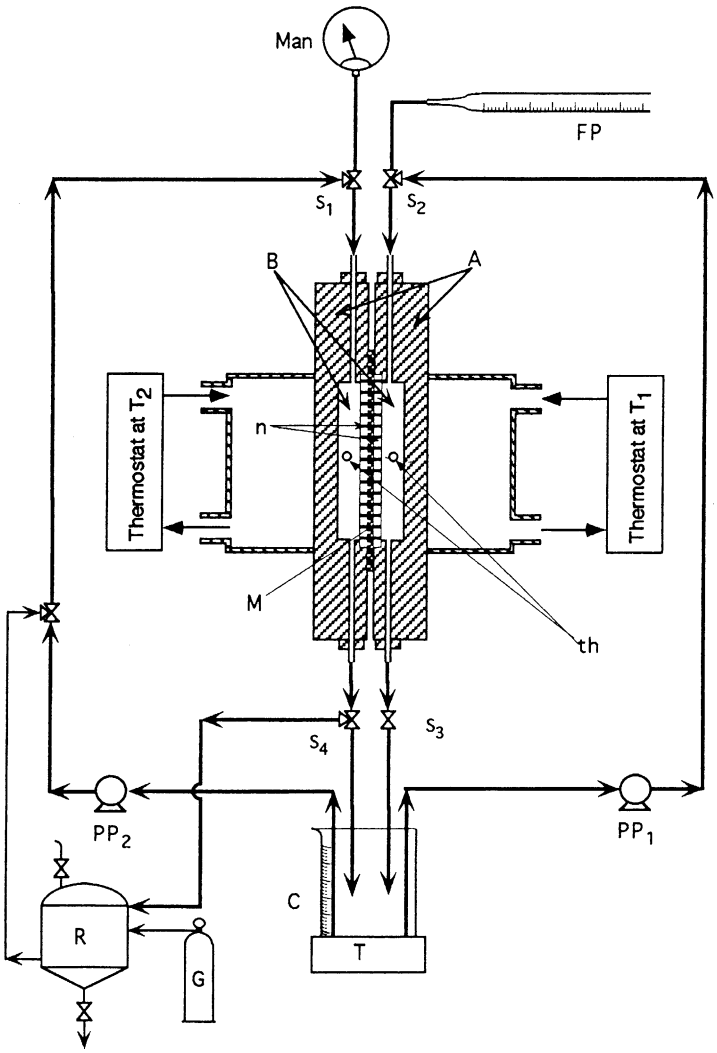

Fig. 1. Schematic (not to scale) representation of the bioreactor. A, half-cells; B, internal working volumes; C, external working volume; M, membrane; n, supporting nets; th, thermocouples; S_i stopcocks; T, thermostatic magnetic stirrer; PP_i, peristaltic pumps; Man, manometer; FP, flow-pipe; R, reservoir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

as spacers between the grafted membrane and the enzyme. A 2% glutaraldehyde (GA) aqueous solution was also employed as bifunctional coupling agent for covalently binding the enzyme to the pre-activated membrane.

The enzyme employed was β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*. β-Galactosidase hydrolyzes glucose to lactose and galactose. This enzyme has been chosen in view of the employment of these catalytic membranes in the process of lactose hydrolysis in milk [29] or in the treatment of the waste waters coming from the dairy industry.

2.3. Methods

2.3.1. Catalytic membrane preparation

The preparation of the three different catalytic membranes employed in this research, named hence-

forward M_0, M_4 and M_6, was done according to the procedures described in reference [5]. All membranes had a common backbone structure constituted by a nylon sheet grafted with GMA, but spacers of different length through which the enzymes were attached to the solid support via glutaraldehyde. On membrane M_0, the spacer was hydrazine, on membrane M_4 the spacer was hethylenediamine, and on membrane M_6 the spacer was hexamethylenediamine. Enzyme was attached to the pre-treated support by condensation, i.e. through a reaction between the amino groups of the arginine residues and the carboxylic groups of glutaraldehyde. During the reaction, water molecules are released. The various steps for the construction of the membranes, together with the methodology for their preparation, were described in detail in [5].

2.3.2. Determination of the activity and stability of the catalytic membranes

Activity and stability of each of the three membrane types were assessed following the procedures described in reference [5]. When not in use, the membranes were stored at 4 °C in 0.1 M phosphate buffer, pH 6.5.

2.3.3. Temperature profile in the bioreactor

When the experiments are carried out under non-isothermal conditions, a non-constant temperature profile is present in the bioreactor and across the membrane. The profile depends on both the thickness' and thermal conductivities of the membrane and of the solutions filling the two half-cells. Since under our experimental conditions the solution motion in each half-cell is laminar [30,31], it is possible to calculate the temperatures on the surfaces of the catalytic membrane by the knowledge of the temperatures at the position of the thermocouples and by applying the Fourier's law. Calling T_w and T_c the temperatures read by the thermocouples in the warm and cold half-cell, respectively, and T_w^* and T_c^* the temperatures on the membrane surfaces facing the warm and the cold substrate solutions, it follows that: $\Delta T = T_w - T_c$; $T_{av} = (T_w + T_c)/2$; $\Delta T^* = T_w^* - T_c^*$; $T_{av}^* = (T_w^* + T_c^*)/2$; $T_w^* < T_w$; $T_c^* > T_c$. Being our system symmetric it follows that. $T_{av} = T_{av}^*$.

By applying the Fourier's law it is possible to derive that:

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

where a is a constant depending on the thickness' and thermal conductivities of the membrane and the solutions filling the two half-cells. In previous works [20–24], we have demonstrated that, with the same substrate solutions and with nylon membranes, a is equal to 0.445. This means that a transmembrane $\Delta T^* = 1.1\,^\circ$C corresponds to a macroscopic $\Delta T = 10\,^\circ$C; $\Delta T^* = 2.2\,^\circ$C corresponds to a macroscopic $\Delta T = 20\,^\circ$C; and $\Delta T^* = 3.3\,^\circ$C corresponds to a macroscopic $\Delta T = 30\,^\circ$C. In the calculation, we assumed as value of thermal conductivity of our solution that of pure water [32], whereas the value of thermal conductivity of the nylon membrane was taken out by Touloukian [33].

2.3.4. Determination of the hydraulic and thermoosmotic permeability of the membranes

The hydraulic permeability of the catalytic membranes has been determined by over-pressurising one half-cell and by measuring in a graduated pipe, the solution volume transported under a pressure gradient and in the absence of temperature gradients.

The thermoosmotic permeability of the catalytic membranes has been determined by measuring in a graduated pipe attached to the cold half-cell, the solution volume transported under a temperature gradient and in the absence of pressure gradients. It must be noticed that when a hydrophobic membrane separates two aqueous solutions or two pure water volumes kept at different temperatures, water is always transported into the cold half-cell and the volume transported is proportional to the size of the transmembrane temperature gradient [25,26]. Solute fluxes generally take place in opposite direction, i.e. from cold to warm. Transport of solute and solvent mediated by temperature gradients is known as thermodialysis.

2.3.5. Experimental data treatment

Every experimental point reported in the figures represents the average value of five experiments

performed under the same conditions. Each experiment lasted 30 min. Only the initial reaction rates were accounted for in the construction of the figures. The experiment duration, the composition of our solutions, and the hydrophobic nature of the membrane, excluded the occurrence of membrane fouling. In any case, to avoid membrane fouling due to reuse, a cleaning 0.1 M phosphate buffer solution was recirculated for 60 min through the bioreactor and the membrane between two subsequent experiments. Effects due to concentration polarisation, even is present, have not been taken in account for considering the hydrophobic nature of the membrane.

3. Results

In Table 1, the values of some physical and biochemical parameters characterising each of the three membranes are reported. Grafting degree and amount of immobilised enzyme were calculated according to the methodology described in reference [5]. The absolute activity of the membrane was calculated in the presence of a 200 mM lactose solution, at pH 6.5 and $T = 25\,^{\circ}C$. Absolute activity is referred to as the total surface of the catalytic membrane, i.e. two surfaces of 35 cm^2. The specific activity was obtained upon division of the absolute activity by the amount of immobilised enzyme, calculated according to the procedure described in reference [5].

By considering that the grafting degree and the amount of the immobilised enzymes are practically the same for the three membrane types, it is evident that the absolute and specific activities of the catalytic membranes are strongly dependent on the spacer length, being increasing with its length. Since the attachment of the enzyme to the membrane occurs through the same aminoacidic residues, the differences in the catalytic activity must be attributed exclusively to the different diffusion limitations introduced by the length of the different spacers and to the consequent different electrostatic interactions between the density of negative charges on the nylon support and the enzyme.

3.1. Physical characterisation of the membranes

The knowledge of the transmembrane hydraulic and thermoosmotic fluxes is very important to forecast the behaviour of a catalytic membrane in a bioreactor operating under non-isothermal conditions [18–23]. In these papers it was identified a coefficient "*C*", function of the hydraulic and termoosmotic fluxes, whose value is related to the activity increase of a catalytic membrane interposed between two substrate solutions kept at different temperatures. For $C = 0$, the catalytic activity under non-isothermal conditions is equal to that measured under comparable isothermal conditions. For $C > 0$, the higher is its value, the greater is the increase of the activity of the catalytic membranes under non-isothermal conditions. C is given by the ratio B/A, where B and A are the coefficients of thermoosmotic and hydraulic permeability of the membrane, respectively. From the values of the fluxes reported in Table 2, it is possible to calculate the A and B values for each of the three membranes employed in this experimentation.

Isothermal hydraulic fluxes have been measured in the pipe connected to one half-cell when the other is overpressured by means of a gas tank, as in Fig. 1, under the conditions $\Delta P = 3 \times 10^3\,N\,m^{-2}$, $T = 25\,^{\circ}C$, and $\Delta T = 0$. The hydraulic permeability coefficient A (measured in $N\,m^4\,s^{-1}$), is calculated through the equation [26,27]:

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

Table 1
Constitutive properties of the three catalytic membranes

Membrane type	Grafting degree (%)	Immobilized enzyme (mg)	Absolute activity ($\mu mol\,min^{-1}$)	Specific activity ($\mu mol\,min^{-1}\,mg^{-1}$)
M_0	13.96	2.8	0.12	0.043
M_4	14.03	3.1	0.41	0.132
M_6	13.86	2.9	1.99	0.686

Table 2
Physical characterisation of the catalytic membranes

Membrane type	$J^{\text{hydr.}}_{\text{water}} \times 10^5$ (m s^{-1})	$J^{\text{therm.}}_{\text{water}} \times 10^5$ (m s^{-1})	$A \times 10^{12}$ ($\text{m}^4\,\text{N}^{-1}\,\text{s}^{-1}$)	$B \times 10^9$ ($\text{m}^2\,\text{K}^{-1}\,\text{s}^{-1}$)	$C \times 10^{-3} = B/A$ ($\text{N m}^{-2}\,\text{K}^{-1}$)
M_0	2.91	9.06	1.45	4.11	2.83
M_4	2.04	9.39	1.20	4.27	4.18
M_6	1.62	9.83	0.81	4.46	5.51

where $J^{\text{hydr.}}_{\text{water}}$ (m s^{-1}), is the isothermal hydraulic water flux produced by a pressure difference ΔP (N m^{-2}) across the membrane, Δx (m) thick.

Non-isothermal thermoosmotic fluxes have been measured in the pipe connected to the cold half-cell under the conditions $\Delta T = 30\,^{\circ}\text{C}$, $T_{\text{av}} = 25\,^{\circ}\text{C}$ and $\Delta P = 0$. The thermoosmotic permeability coefficient B, measured in $\text{m}^2\,\text{K}^{-1}\,\text{s}^{-1}$, is calculated through the equation [26,27]:

$$J^{\text{therm.}}_{\text{water}} = B\frac{\Delta T^*}{\Delta x} \tag{3}$$

where $J^{\text{therm.}}_{\text{water}}$, expressed in m s^{-1}, is the non-isothermal water flux produced by the temperature difference ΔT^* (K) across the membrane thickness.

The values of the coefficient A, B, and C are reported in Fig. 2a as a function of the number of CH_2 groups into the spacer chain. More interesting is the linear dependence observed for all the three parameters. The equations of the straight lines best fitting the experimental points of Fig. 2a are of the types $y = ax + b$, where x is the number of the CH_2 groups in the spacer molecule. In the case of the permeability coefficient A, the equation is: $y = -0.108x + 1.454$ (with $R = 0.99$). In the case of the thermoosmotic permeability coefficient B the equation is: $y = 0.055x + 4.101$ (with $R = 0.97$). In the case of the parameter C the equation is: $y = 0.432x + 2.738$.(with $R = 0.98$). It is easy to see that "a" assumes positive values for the straight lines relative to the coefficients B and C, and negative value for the straight line relative to the coefficient A. The positive value of "a" in the equation of the parameter "B", and the negative value in the equation of the parameter "A" indicate an increase of the membrane hydrophobicity with the spacer length. Indeed while the hydraulic membrane permeability decreases, the corresponding thermoosmotic permeability increases.

In Fig. 2b the percentage decreases of each of the three parameters A, B, and C are separately reported as a function of the number of CH_2 groups in the spacer. As 100% the highest value of each series of measurements has been taken. These results indicate that the spacer length affect in different way the hydraulic and thermoosmotic permeabilities of the membrane. The influence on the hydraulic permeability is greater than that on the thermoosmotic permeability. The values of the C parameter for the three membranes indicate that membrane M_6 would exhibit the highest activity increase under non-isothermal conditions, followed by membrane M_4, which in turn would have a greater activity increase in comparison with membrane M_0.

3.2. Biochemical characterisation of the membranes

To verify the previsions coming out from the data in Table 2, the experiments reported in Fig. 3 have been carried out under isothermal and non-isothermal conditions. The substrate concentration was 200 mM, a value more than double of the highest value of the apparent K_m found in a separate paper [5] for the three membranes. In Fig. 3, the values of the glucose production by each of the three catalytic membranes is reported as a function of time. Fig. 3a refers to membrane M_0, Fig. 3b to membrane M_4, Fig. 3c to membrane M_6. The angular coefficient of each straight line, multiplied by the solution volume (30 mL), gives directly the value of the activity of each membrane, expressed as $\mu\text{mol min}^{-1}$. Results in Fig. 3 show that: (i) the activity of the three membranes, under comparable isothermal and non-isothermal conditions, increases with the increase of the spacer length, as activity of membrane M_0 is lower than that of membrane M_4, in turn lower than that of membrane M_6; (ii) for each of the three membranes the catalytic activity under non-isothermal conditions is higher than that measured

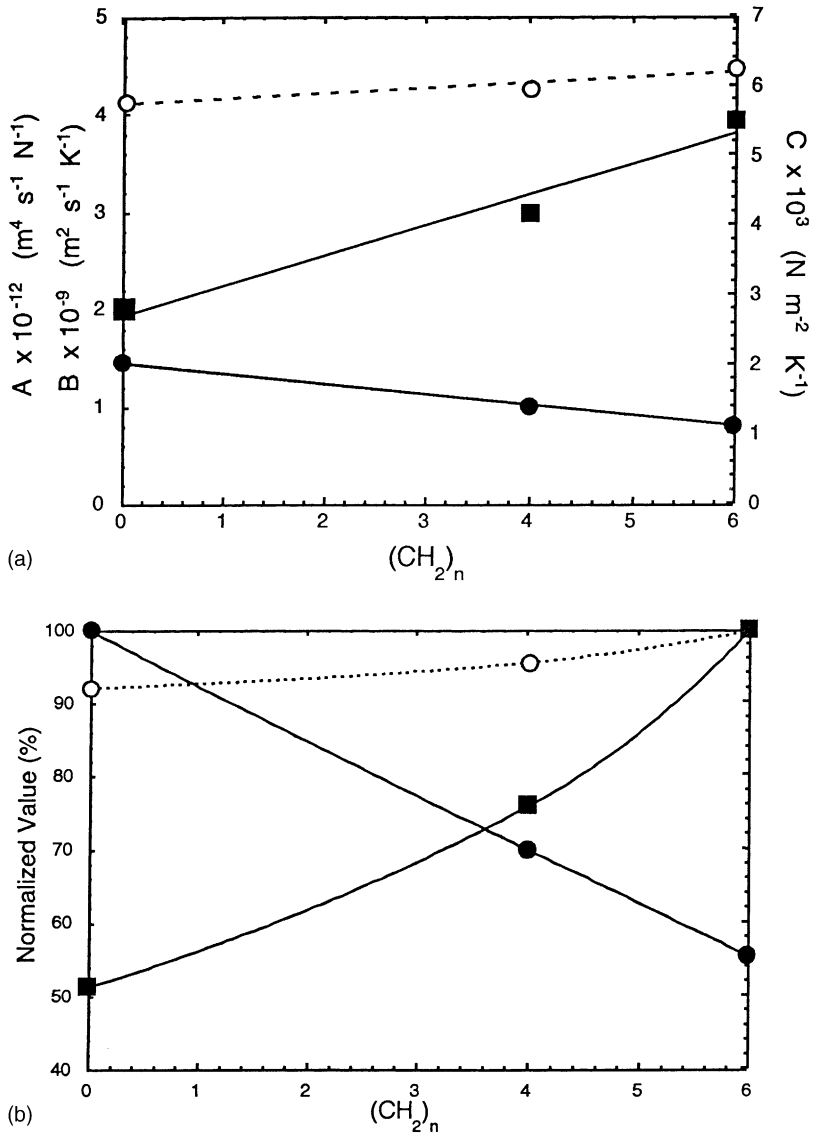

Fig. 2. (a) Hydraulic permeability coefficient A (●, external left scale), thermoosmotic permeability coefficient B (○, internal left scale), and C coefficient (■, right scale) as a function of the number of CH_2 groups in the spacer chain. (b) Normalised percentage values for the hydraulic A (●) and thermoosmotic (○) permeability coefficients, and for the C coefficient (■) as a function of the number of CH_2 groups in the spacer chain.

under isothermal conditions and increases with the increase of the temperature difference ΔT measured at the thermocouple positions. In Fig. 4, the catalytic activity of each membrane is reported as a function of the applied ΔT. Data in the Fig. 4 show that the activity increases are directly proportional to the size of the applied ΔT. Each straight line fitting the experimental points is expressed by an equation of the type $y = y_0 + a\Delta T$, where, at least in the range of the temperature differences employed in this investigation, y represents the value of the non-isothermal activity of the membrane under a determined value of ΔT, y_0 is

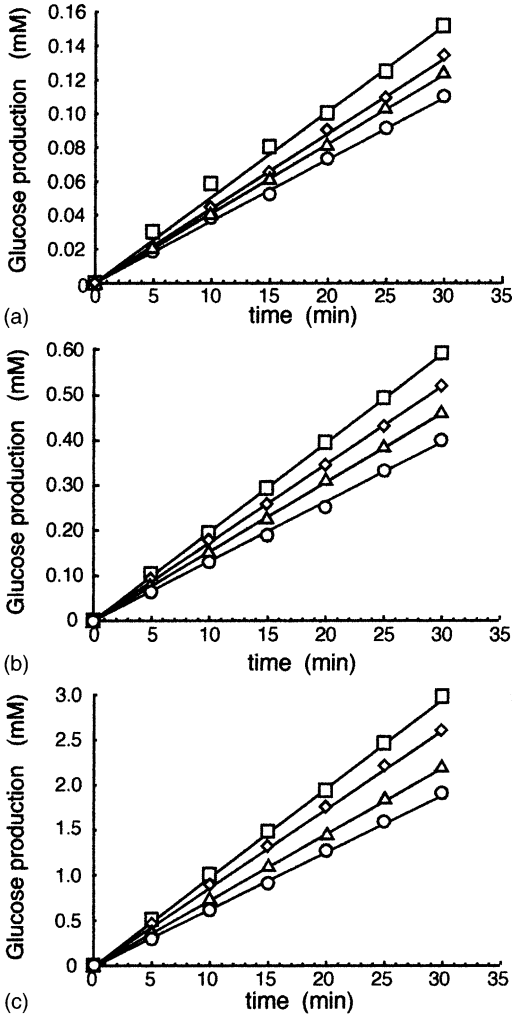

Fig. 3. Isothermal and non-isothermal glucose production as a function of time for membrane M_0 (a), membrane M_4 (b) and membrane M_6 (c). Symbols: $\Delta T = 0\,^{\circ}C$ (○); $\Delta T = 10\,^{\circ}C$ (△); $\Delta T = 20\,^{\circ}C$ (◇); $\Delta T = 30\,^{\circ}C$ (□).

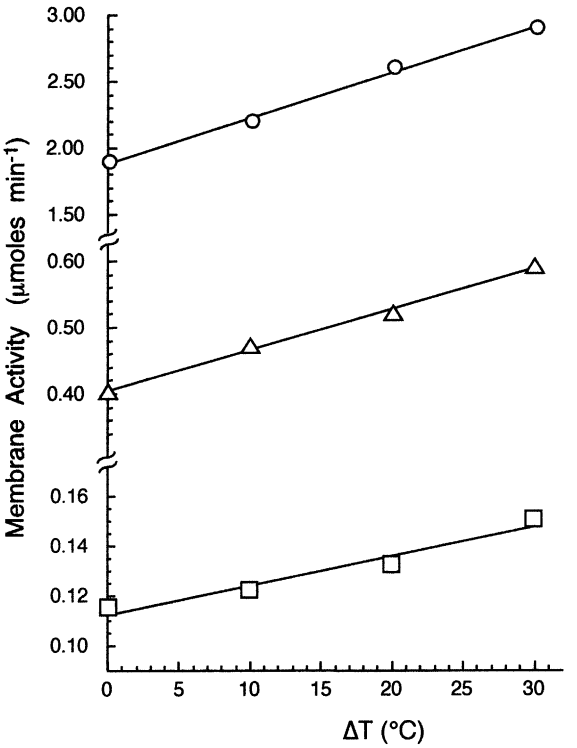

Fig. 4. Membrane activity as a function of the applied macroscopic ΔT. Symbols: membrane M_6 (○); membrane M_4 (△); membrane M_0 (□).

the value of the isothermal activity, and $(y - y_0)$ represents the activity difference when 1 °C of temperature difference is measured at the thermocouple positions.

More interesting is the value of the percentage activity increase (PAI), defined as:

$$\mathrm{PAI} = \frac{y - y_0}{y_0} = \frac{a_0}{y_0}\Delta T = \alpha \Delta T \tag{4}$$

where α represents the percentage activity increase when a temperature difference of 1 °C is measured at the thermocouple positions. In Table 3, the values of

Table 3
Efficiency parameter of a non-isothermal catalytic process

Membrane type	a_0 (μmol min^{-1} °C^{-1})	y_0 (μmol min^{-1})	α (% °C^{-1})	PAI (%)
M_0	0.00115	0.1125	1.0	30
M_4	0.0062	0.402	1.5	45
M_6	0.0340	1.890	1.8	54

a_0, y_0 and α are listed for each of the three membranes. From Table 3 it clearly appears that membrane M_6 gives the highest percentage activity increases among the three membranes, and that the α values, as well as those of the PAI, agree with the C values characterising each membrane.

When the advantages of using non-isothermal bioreactors are considered, a new parameter τ_r can be taken in consideration. This parameter is the reduction of the production time and is defined as:

$$\tau_r = \frac{\tau_{\text{non-iso}} - \tau_{\text{iso}}}{\tau_{\text{iso}}} \quad (5)$$

where $\tau_{\text{non-iso}}$ and τ_{iso} are the time required to obtain equal amounts of reaction products under non-isothermal and isothermal conditions, respectively. On the basis of this definition and considering the glucose production obtained under isothermal conditions at the end of each run of Fig. 3, it is possible to obtain Fig. 5. In this figure, for membrane M_0 the times requested to obtain a 0.11 mM glucose production, under isothermal and non-isothermal conditions, have been taken into account; for membrane M_4 the times for a 0.40 mM glucose production; for membrane M_6 the times for a 1.80 mM glucose production. Once again, membrane M_6 gives a reduction of the production time greater than membranes M_4 and M_0, in agreement with the C values in Table 2.

Having ascertained that under non-isothermal conditions at 0.2 M lactose concentration the catalytic activity of the enzyme derivatives is affected by the length of the spacer, we have extended these studies to the whole concentration range from zero to 300 mM to know how this parameter modifies the kinetics constants. The results of this investigation are reported in Fig. 6, where the catalytic activities of the

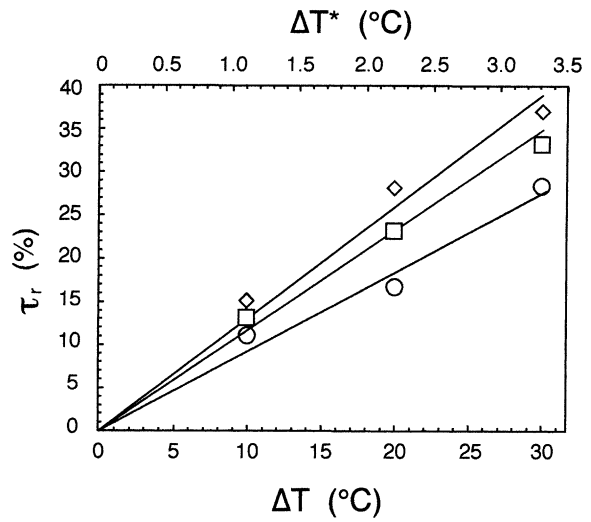

Fig. 5. Reduction of the production time (τ_r) as a function of ΔT (lower scale) or ΔT^* (upper scale). Symbols: membrane M_6 (◇); membrane M_4 (□); membrane M_0 (○).

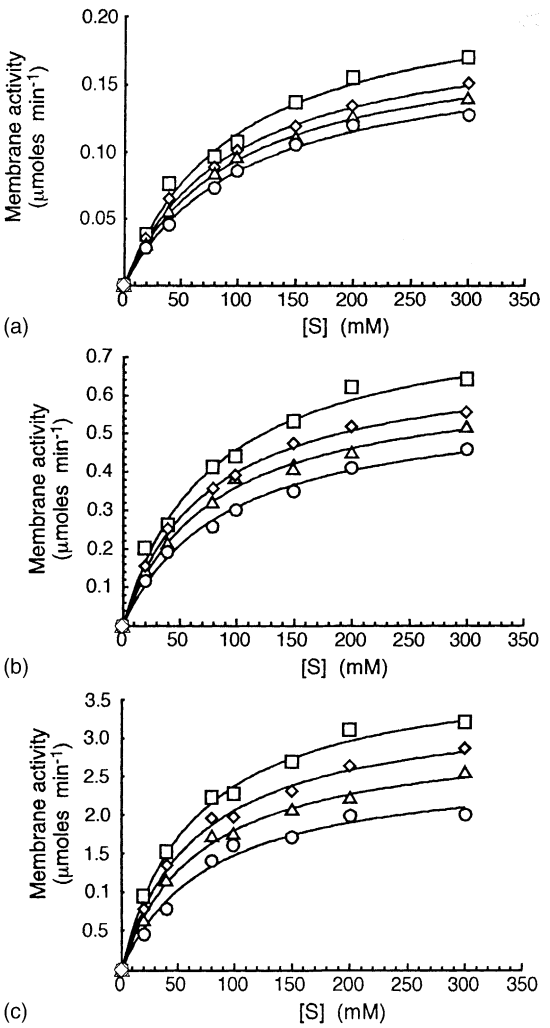

Fig. 6. Membrane activity as a function of substrate concentration for membrane M_0 (a), membrane M_4 (b) and membrane M_6 (c). Symbols: $\Delta T = 0\,^{\circ}C$ (○); $\Delta T = 10\,^{\circ}C$ (△); $\Delta T = 20\,^{\circ}C$ (◇); $\Delta T = 30\,^{\circ}C$ (□).

three membranes are reported as a function of lactose concentration. The curve parameter in each Figure is the temperature difference ΔT measured by the thermocouples, whereas the average temperature is 25 °C. Fig. 6a refers to membrane M_0, Fig. 6b to membrane M_4, and Fig. 6c to membrane M_6. Results in Fig. 6 show that: (i) the activity of the three membranes, under comparable isothermal and non-isothermal conditions, increases with the increase of the spacer length, as activity of membrane M_0 is lower than that of membrane M_4, in turn lower than that of membrane M_6; (ii) for each of the three membranes the catalytic activity under non-isothermal conditions is higher than that measured under comparable isothermal conditions and increases with the increase of the temperature difference ΔT measured at the thermocouple positions; (iii) at each glucose concentration the activity increases are proportional to the size of the applied ΔT how it is possible to see when plots similar to that in Fig. 4 are considered; (iv) all the curves interpolating the experimental points exhibit a Michaelis–Menten behaviour either in the presence or in the absence of temperature differences. In Fig. 7 the experimental points of Fig. 6 are reported in forms of Hanes plot to calculate the values of the apparent K_m and V_{max}. These values are listed in Table 4 together with the ones relative to the soluble β-galactosidase. Fig. 8a refers to membrane M_0, Fig. 8b to membrane M_4, and Fig. 8c to membrane M_6. The data in Table 4

Table 4
Kinetic parameters

Enzyme status	Experimental conditions		K_m (mM)	V_{max} (μmol min^{-1})
	T_{av} (°C)	ΔT (°C)		
Free	25	0	21.40	3.20
Membrane M_0	25	0	112.00	0.170
	25	10	97.61	0.193
	25	20	97.61	0.206
	25	30	97.61	0.220
Membrane M_4	25	0	92.00	0.60
	25	10	78.74	0.67
	25	20	78.74	0.75
	25	30	78.74	0.82
Membrane M_6	25	0	81.00	2.67
	25	10	72.70	3.13
	25	20	72.70	3.62
	25	30	72.70	4.00

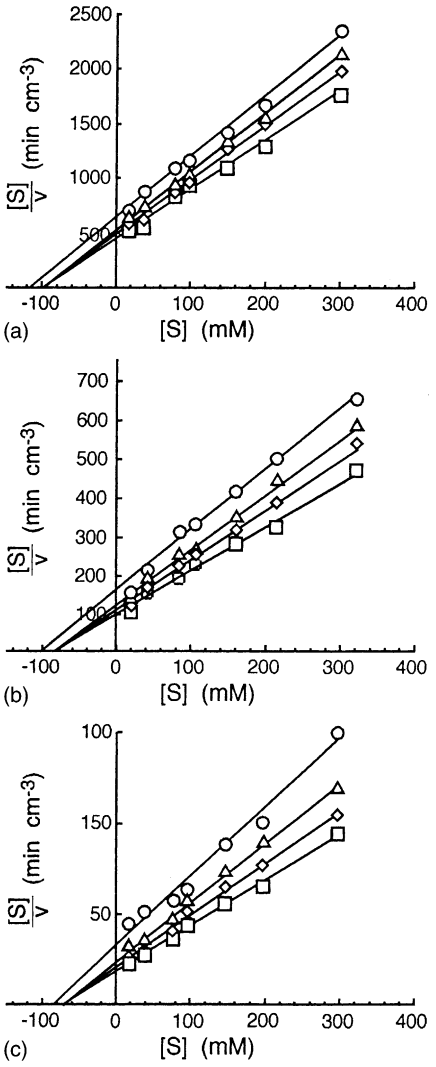

Fig. 7. Hanes plots for membrane M_0 (a), membrane M_4 (b) and membrane M_6 (c). Symbols: $\Delta T = 0$ °C (○); $\Delta T = 10$ °C (△); $\Delta T = 20$ °C (◇); $\Delta T = 30$ °C (□). In all experiments $T_{av} = 25$ °C.

show that: (i) the immobilised enzymes have apparent K_m values higher than that of the free counterpart; (ii) the apparent values of K_m under non-isothermal conditions are lower than the corresponding values under isothermal conditions but higher than that of the soluble enzyme; (iii) for each membrane type, the K_m values under non-isothermal conditions appear independent on the macroscopic temperature difference measured by the thermocouples.

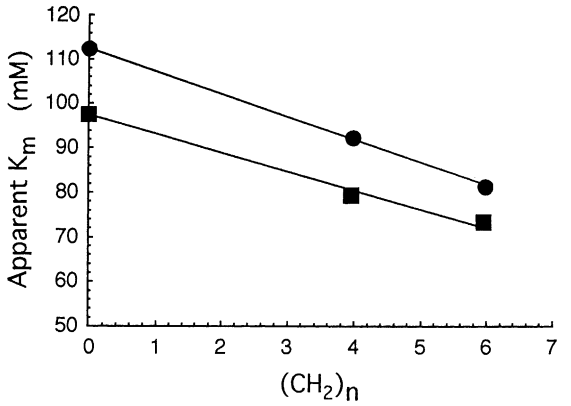

Fig. 8. Apparent value of K_m as a function of the number of CH_2 groups in the spacer chain. Symbols: (●) isothermal conditions; (■) non-isothermal conditions.

The first observation finds explanation in the diffusive limitations introduced by the immobilisation process to substrate and products traffic towards or away from the catalytic site.

The second observation finds explanation in the circumstance that the temperature gradient increases substrate and product fluxes across the catalytic membrane reducing, in this way, the diffusion limitations for these substances during their movement towards or away from the catalytic site. Moreover, the increase of the enzyme reaction rates with the applied ΔT are analogous to the increase of non-isothermal transmembrane mass fluxes with the applied ΔT induced by the process of thermodialysis [25,26]. A detailed analysis of these fluxes and of the substrate concentrations profiles into a catalytic membrane under isothermal (diffusion) and non-isothermal (thermal diffusion/thermodialysis) conditions, in the presence or in the absence of catalysis, has been recently published [19,21].

The third observation, concerning the independence of the K_m values on the macroscopic temperature difference across the catalytic membrane, is explained on the basis of the changes in the protein structure and dynamics induced on the immobilised enzyme by the flux of thermal energy associated to the presence of the temperature gradient [23]. The temperature gradients should play on the enzyme structure the same role than positive effectors, which affect the K_m value of an enzyme reaction independently on their concentration. In this analogy, the size of the temperature gradient results equivalent to the size of the effector concentration.

In Fig. 8, the apparent K_m under isothermal and non-isothermal conditions are reported as a function of the number of CH_2 groups in the spacer chain. Once again, these values decrease with the spacer length and approach to the value of the soluble enzyme, which, consequently, can be considered as immobilised on a solid support through a spacer of infinite length.

Coming back to the results of Fig. 7, it is evident that at all the lactose concentrations studied it is possible to apply the approach used for the experiments reported in Figs. 3 and 4. In this way it is possible to calculate, at all the concentrations, the PAI values, and consequently the α values, which correspond to the PAI values with $\Delta T = 1\,^{\circ}\mathrm{C}$. In Fig. 9 the α values relative to the results of Fig. 7 are reported as a function of lactose concentration. From the figure it clearly emerges that the α values decrease with the increase of the substrate concentration. From Eq. (4), it follows that the PAI values also decrease with the increase of the lactose concentration. Also, this behaviour has been analytically explained [19,21]. Similar dependence of the α values on the substrate concentration has been found with different enzymatic derivatives [21–24]. Also the α values, at each substrate concentration, show a dependence on the spacer length. The values relative to membrane M_0 are always lower that those relative to membrane M_4 and the latter in

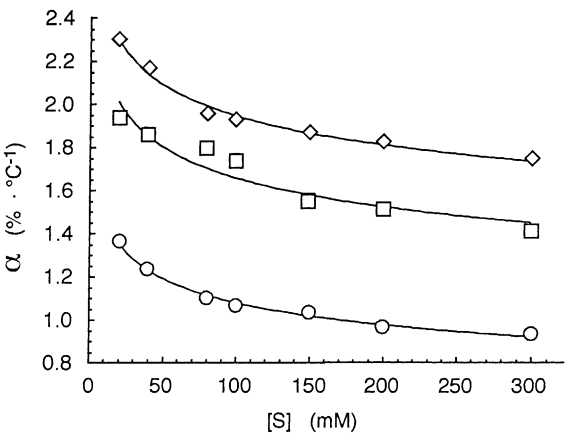

Fig. 9. α-values as a function of substrate concentration. Symbols: membrane M_0 (○); membrane M_4 (□); membrane M_6 (◇).

Table 5
α' values of the catalytic membranes as a function of substrate concentration

Lactose concentration (mM)	$(\alpha')_{M_0}$ (% °C^{-1})	$(\alpha')_{M_4}$ (% °C^{-1})	$(\alpha')_{M_6}$ (% °C^{-1})
20	12.7	18.2	20.9
40	10.9	16.4	19.5
80	10.0	15.5	18.2
100	9.7	15.1	17.6
150	9.1	14.5	17.3
200	8.7	13.6	16.4
300	8.5	13.2	15.7

turn are lower than the ones relative to membrane M_6.

The α values of Fig. 9 confirm the usefulness of employing the three membranes in non-isothermal bioreactors, since it is evident that a macroscopic temperature difference of 30 °C produces average PAI values equal to 35% for membrane M_0, to 51% for membrane M_4, and to 61% for membrane M_6.

A more interesting parameter for the industrial application of the technology of the non-isothermal bioreactors is the α' coefficient which is the percentage activity increase when an actual temperature difference $\Delta T^* = 1\,^\circ\mathrm{C}$ is applied across the catalytic membrane. This new coefficient is defined as:

$$\alpha' = \alpha\left(\frac{\Delta T}{\Delta T^*}\right) = \frac{\mathrm{PAI}}{\Delta T^*} \tag{6}$$

In Table 5, the α' values for the three membranes are listed.

4. Conclusions

All the results reported above have shown that under non-isothermal conditions, the percentage activity increases of the three catalytic membranes were strongly dependent on the spacer length. At the same time, the results strengthened also the interest in the employment of the technology of the non-isothermal bioreactors in productive biotechnological processes.

To this aim, one must be sure that it is possible to compare, as we have done, the results obtained under two different physical situations, i.e. in the presence or in the absence of temperature gradients, particularly when large temperature gradients are applied in the bioreactor. Such a comparison is right since the actual temperature differences across the catalytic membranes are reduced to small values, also in the presence of the highest temperature differences read by the thermocouples. The macroscopic temperature difference $\Delta T = 30\,^\circ\mathrm{C}$, indeed, reduces to 3.3 °C. The temperatures between the two membrane surfaces vary linearly within the membrane thickness and are slightly different from the temperature at the centre of the membrane, which is equal to the macroscopic average temperature. This implies that by considering only the dependence of the enzyme activity on the temperature, the total activity of the catalytic membrane under non-isothermal conditions can be considered equal to that obtained at the corresponding T_{av}. In fact, by considering the enzyme uniformly distributed through the membrane thickness, the activity loss at temperatures lower than the average one is balanced by the activity increase at temperatures higher than the average. Accordingly, in the absence of specific effects due to the temperature gradients, the activity of the membrane under non-isothermal condition should be considered equal to that of the corresponding average temperature. These considerations allow the comparison between the results obtained under non-isothermal conditions with those obtained under comparable isothermal conditions and confirm the positive role of the temperature gradients on the enzyme activity of a catalytic and hydrophobic membrane.

In the light of the last consideration and considering the α' values listed in Table 5, one can conclude that it is advantageous to use hydrophobic catalytic membranes in non-isothermal bioreactors.

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