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Modulation of immobilized enzyme activity by altering the hydrophobicity of nylon-grafted membranes Part 1. Isothermal conditions

M.M. El-Masry [a,1] A. De Maio [a], S. Di Martino [a,b], N. Diano [a,b], U. Bencivenga [a], S. Rossi [a], V. Grano [a], P. Canciglia [a,2] M. Portaccio [b], F.S. Gaeta [a], D.G. Mita [a,b,*]

[a] *International Institute of Genetics and Biophysics of CNR, via Guglielmo Marconi, 12, 80125 Naples, Italy*

[b] *Department of Human Physiology and Integrated Biological Functions of the Second University of Naples, via S.M. di Costantinopoli, 16, 80138 Naples, Italy*

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Abstract

The catalytic behaviour under isothermal conditions of two different membranes loaded with β-galactosidase was investigated. One membrane (M_1) was constituted by a nylon sheet grafted with methylmethacrylate by means of chemical grafting. The other, (M_2), was prepared by a double chemical grafting: the first one with styrene (Sty) and the second one with methylmethacrylate. Membrane activity was characterized as a function of temperature, pH and substrate concentration. The role of Sty in increasing membrane hydrophobicity has been discussed. Membrane M_2 was found to be better suited for employment in non-isothermal bioreactors.

Keywords: β-Galactosidase; Grafted membranes; Immobilized enzymes; Lactose hydrolysis; Bioreactors

1. Introduction

Recently, it has been shown that catalytic membranes in a non-isothermal bioreactor increase their activity with respect to that exhibited under comparable isothermal conditions [1–6]. The activity increase is proportional to the temperature difference applied across the catalytic membrane. These results were obtained with a composite membrane system: a catalytic hydrophilic membrane coupled to a hydrophobic unselective porous membrane. The presence of the hydrophobic membrane is required since it induces transmembrane substrate and products transport by the process of thermodialysis [7–12]. This is considered to be one of the causes of the increased enzyme activity under non-isothermal conditions. Thermodialysis is the process by which selective transport of solvent and solutes occurs across a hydropho-

* Corresponding author. International Institute of Genetics and Biophysics of CNR, via Guglielmo Marconi, 12, 80125, Naples, Italy. Tel.: +39-81-2395887; fax: +39-81-2395887.

E-mail address: mita@iigbna.iigb.na.cnr.it (D.G. Mita).

[1] Permanent address: Department of Polymers and Pigments, National Research Center, Dokki, Cairo, Egypt.

[2] Institute of General Physiology, University of Messina, Salita Sperone, 31, 98166, Messina, Italy.

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bic, unselective, porous membrane separating two aqueous solutions kept at different temperatures. The transmembrane temperature gradient drives the mass fluxes.

With the aim of simplifying the composite membrane system employed so far, we investigated the possibility of obtaining a single membrane, catalytic and hydrophobic, utilizing the technique of grafting by γ-radiation [13–24]. With this purpose we immobilized enzymes on Teflon or nylon membranes grafted with suitable monomers by means of γ-radiations [25–31]. Two of these membranes have given good results when employed in non-isothermal bioreactors [27,31].

Grafting by γ-radiation is expensive, takes a lot of time and allows treatment of small samples, but not of whole reactors. For this reason we changed strategy employing chemical grafting, which is also used [32–34] in activating polymer structures for enzyme immobilization.

The aim of this work is to obtain, by chemical grafting, a single membrane, catalytic and hydrophobic, which could be usefully employed in non-isothermal reactors. Since hydrophobicity is important for the process of thermodialysis we will discuss, in this paper, the isothermal behaviour of two different catalytic nylon membranes. These membranes were both copolymerized with methylmethacrylate (MAA), one of them being different due to a previous grafting of a hydrophobic monomer, such as styrene (Sty). These membranes will be indicated in the following by M_1 and M_2, respectively, M_2 being M_1 previously copolymerized with Sty. As enzyme model a β-galactosidase has been used. The non-isothermal behaviour of these membranes is illustrated in a separate paper [35].

2. Materials and methods

2.1. Materials

As solid support to be grafted we used nylon Hydrolon membranes (Pall Italia, Milano, Italy). These membranes, 150 μm in thickness, are hydrophobic and have a nominal pore size of 0.2 μm. The pore size is related to the size of the minimum value of the diameter of the smallest particles that the membrane retains, since the membrane has no ''classical'' pores but irregular cavities crossing the membrane thickness.

All chemicals, including the enzyme, were purchased from SIGMA (Sigma Aldrich, Milano, Italy) and used without further purification. As monomer to be grafted we used Sty and/or methylmethacrylate (MMA). Hexamethylenediamine (HMDA) (70% aqueous solution) was used as spacer between the grafted membrane and the enzyme. A 2.5% glutaraldehyde (Glu) aqueous solution was employed as a bifunctional coupling agent for covalently binding the enzyme to the activated membrane.

The enzyme employed was a β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*. The enzyme hydrolyzes lactose in glucose and galactose. The β-galactosidase activity was colorimetrically assayed by the GOD-Perid method for glucose determination (Boehringer, D-68298 Mannheim, Germany) and expressed as μmol min^{-1}.

2.2. Methods

2.2.1. Grafting copolymerization

Grafting polymerization was carried out by using $K_2S_2O_8/Na_2S_2O_3$ in the ratio 1/5 as initiating system. Since the initiation is enhanced by copper ion [36], the nylon membranes were pre-treated for 3 h in a 0.5 (w/v) $(CH_3COO)_2Cu$ aqueous solution at room temperature. After this treatment the membranes were placed for 30 min in a reaction vessel containing an aqueous solution 0.93 M in MMA, 3.66 mM in $K_2S_2O_8$ and 18 mM in $Na_2S_2O_3$. The reaction temperature was 55°C. Later on, the membranes were treated with chloroform to remove the homopolymers produced. At this point a Nylon/Poly-MMA membrane was obtained, ready to be directly loaded with the enzyme to obtain membrane M_1.

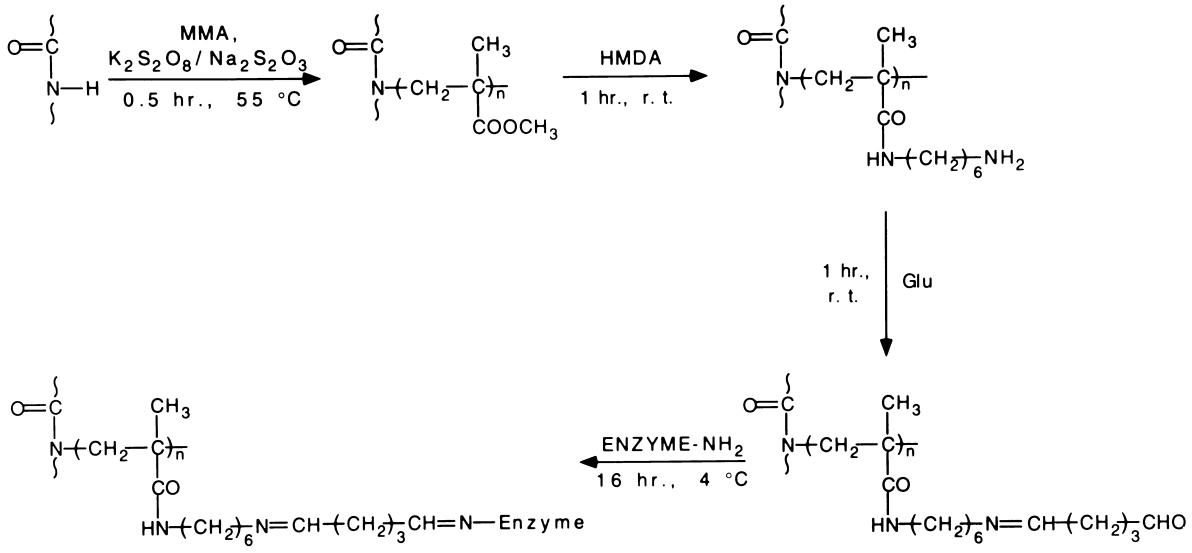

Fig. 1. Schematic sequence of the processes of grafting and enzyme immobilization to obtain membrane M_1.

Nylon/Poly-Sty/Poly-MMA membranes, i.e., M_2 membranes, were obtained with an experimental procedure similar to that described above. After a pre-treatment of the nylon membrane with the same $(CH_3COO)_2Cu$ aqueous solution, a subsequent treatment for 1 h at 55°C with a 1:1 water/ethanol solution 500 mM in Sty, 3.66 mM in $K_2S_2O_8$ and 18 mM in $Na_2S_2O_3$, was performed. Later on the membranes were treated with tetrahydrofuran to remove the produced homopolymers. Once obtained, the Nylon/Poly-Sty membranes were copolymerized with methylmethacrylate following the procedure described above for M_1.

2.2.2. Determination of grafting degree

As for the determination of percentage of grafting degree we adopted the classical definition for this parameter. The degree of grafting (X, %) was determined by the difference between membrane masses before, G_B, and after, G_A, the grafting by the formula:

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

2.2.3. Activation of the grafted membranes

The activation of grafted membranes was done by two successive treatments each one for 1 h at room temperature. The first one was carried out by immersing the membrane in a 10% (w/v) hexamethylenediamine aqueous solution. After washing with running water to remove unreacted amines, the membranes were treated in a 2.5% (v/v) glutaraldehyde aqueous solution.

2.2.4. Enzyme immobilization

Both Nylon/Poly-MMA or Nylon/Poly-Sty/Poly-MMA membranes were loaded with β-galactosidase by immersing for 16 h at 4°C the pre-activated membrane in a 0.1 M buffer phosphate solution containing the enzyme at a concentration of 3 mg/ml, at pH 6.5. At the end of the immobilization period the membranes were washed several times with the same buffer solution. The overall processes of membrane grafting and enzyme immobilization are represented in a schematic form in Figs. 1 and 2. Fig. 1 refers to the preparation of membrane M_1; Fig. 2 to membrane M_2.

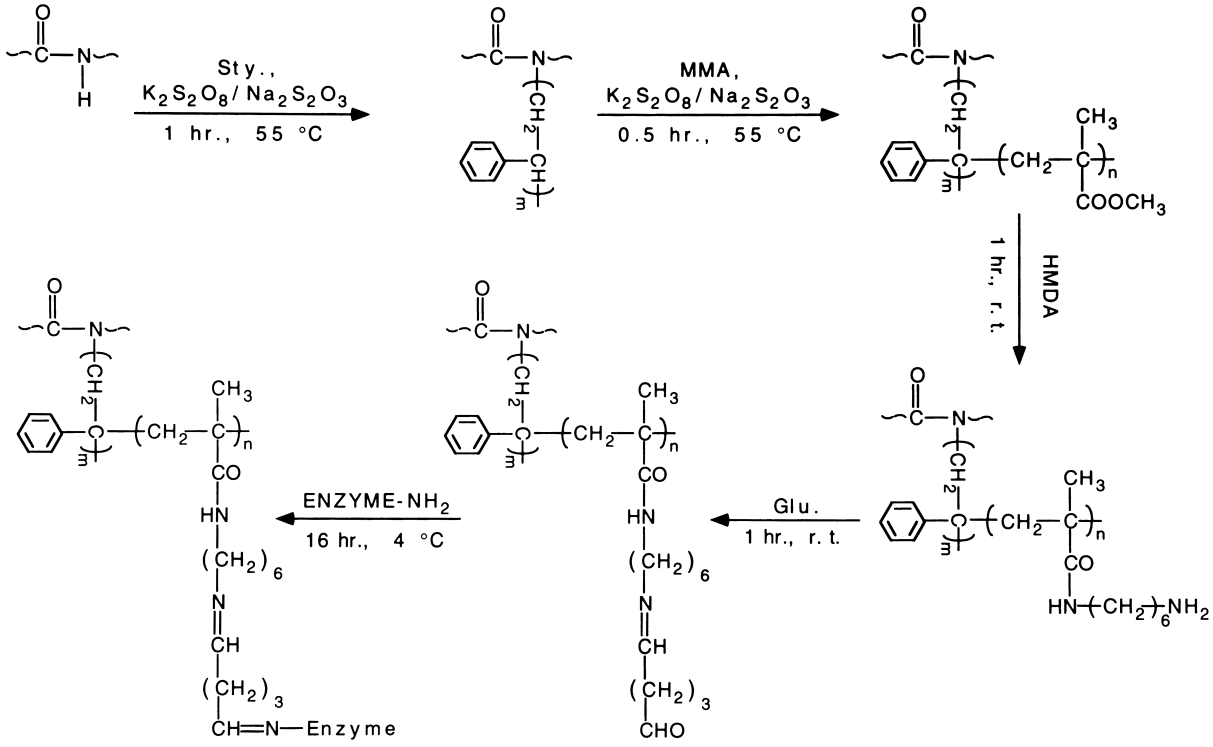

Fig. 2. Schematic sequence of the processes of grafting and enzyme immobilization to obtain membrane M_2.

2.2.5. Determination of the catalytic membrane activity

Enzyme activity was determined by sampling, at regular time intervals, the solution in contact with the membrane and measuring the glucose concentration by the GOD-Perid test. According to the scheme

$$\text{glucose} + O_2 + H_2O \overset{\text{GOD}}{\rightarrow} \text{gluconate} + H_2O_2,$$

$$H_2O_2 + \text{ABTS} \overset{\text{POD}}{\rightarrow} \text{coloured complex} + H_2O,$$

the test uses a coupled enzyme reaction by which a coloured solution is obtained. Glucose concentration, proportional to the intensity of solution colour, is spectrophotometrically determined. Membrane activity, expressed as μmol min^{-1}, is given by the slope of the linear plot of the glucose production as a function of time.

2.2.6. Membrane stability

The time stability of the biocatalytic membranes was assessed by analyzing every day their activity under the same experimental conditions, i.e., 0.2 M lactose in 0.1 buffer phosphate solution, pH 6.5 and $T = 30$°C. After 3–4 days, during which the membranes lost some activity, a stable condition was reached, remaining unchanged for over 2 months. No significant differences were found in the rate by which both membranes approach and maintain the time stability. Only these stabilized membranes were used in the comparative experiments reported in the following. Under the standard conditions defined above, the absolute membrane activity was 814 and 209 μmol min^{-1} m^{-2} of membrane surface area, for M_1 and M_2, respectively. When not in use the membranes were stored at 4°C in 0.1 M buffer phosphate solution, pH 6.5.

2.2.7. Treatment of experimental data

Every experimental point reported in the figures represents the average value of four experi-

ments performed under the same conditions. The experimental errors did not exceed 6%. Each experiment lasted 30 min, but only the initial reaction rates were accounted for in the construction of the figures.

3. Results and discussion

To compare the behaviour of membranes M_1 and M_2 it is important to know the amount of enzyme immobilized on each membrane. This can be done by measuring the enzyme activity in the initial solution used for the immobilization, the residual activity of this solution after the immobilization process and the activity of all washing solutions. Since at constant substrate concentration the catalytic activity is proportional to enzyme concentration, it is easy to evaluate the amount of immobilized enzyme through a calibration curve of the catalytic activity of the free enzyme as a function of its concentration. This is done by means of the equation: $a = b - c - \sum_{i=1}^{n} d_i$, where a is the amount of immobilized enzyme, b and c the amount of enzyme in the initial and final solution used for immobilization, respectively, and d_i the amount of enzyme found in the i-th washing, n being the number of washing processes. The washing ends when d_n becomes zero. The result of this procedure provides the amount of enzyme on each membrane. It was found that the amount of enzyme immobilized on the unit surface of M_1 was 0.545 ± 0.006 mg cm^{-2}, while on M_2 was 0.509 ± 0.005 mg cm^{-2}. Grafting percent values were (15 ± 2)% for the MMA, and (12 ± 3)% for Sty.

3.1. Temperature dependence

The isothermal characterization of membrane activity as a function of the temperature is one of the principal parameters required to know how the immobilization procedures affect the enzyme activity. In Fig. 3 the glucose production as a function of time is reported. The curve parameter is the temperature of the experiments. All experiments were performed with 0.2 M lactose concentration in a 0.1 M buffer phosphate solution, pH 6.5. Fig. 3a gives the behaviour of membrane M_1, while Fig. 3b refers to membrane M_2. Two general considerations can be done observing the results in Fig. 3: the linearity of glucose production during the experiments and the higher activity of membrane M_1 in respect to membrane M_2.

Calling $[M_1]/[M_2]$ the ratio of the amount of enzyme immobilized on M_1 and M_2, respectively, and $[A_1]/[A_2]$ the ratio between the activity of the membranes M_1 and M_2 at a given temperature, it is possible to observe that $[M_1]/[M_2] = 1.07$ and $[A_1]/[A_2] \approx 3.9$. This means that the presence of Sty on membrane M_2 reduces the absolute activity of the catalytic membrane. This effect can be attributed to the circumstance that Sty somewhat induces a hydrophobic environment around the enzyme, thus reducing the rate of substrate diffusion towards the catalytic site. This observation is confirmed by the calculation of the activation energies for the enzyme reaction when the catalyst is immobilized on M_1 or M_2. An Arrhenius plot of the enzymes reaction rates, deduced from the data of Fig. 3a and b allows this calculation. We have found for membrane M_1 an E_a value of 6.1 kcal/mol and for membrane M_2 a value of 8.0 kcal/mol. These small values of the activation energies suggest that in our case the apparent enzyme reaction rate may be controlled by a diffusive process. The higher E_a value found in the case of membrane M_2 confirms the circumstance that the presence of Sty reduces the diffusion rate of substrate towards the catalytic site.

In Fig. 4 the relative activities of the membranes as a function of temperature are reported, together with that of the free enzyme, for comparison. Fig. 4a refers to membrane M_1 and Fig. 4b to membrane M_2. Both membranes exhibit an equal shift of the optimum activity towards higher temperatures, even if the curves activity/temperature for the immobilized catalyst

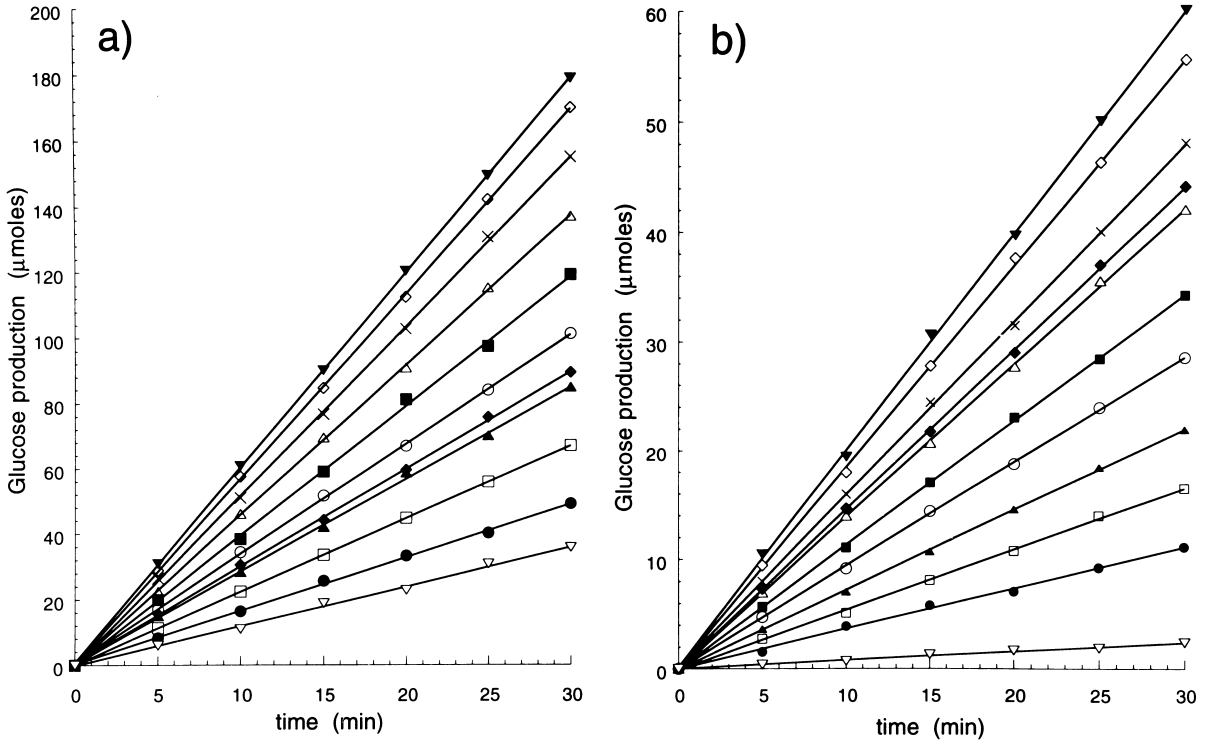

Fig. 3. Isothermal glucose production as a function of time. Symbols: (●) $T = 20°C$; (□) $T = 25°C$; (▲) $T = 30°C$; (○) $T = 35°C$; (■) $T = 40°C$; (△) $T = 45°C$; (X) $T = 50°C$; (◇) $T = 55°C$; (▼) $T = 60°C$; (◆) $T = 65°C$; (▽) $T = 70°C$. (a) membrane M_1; (b) membrane M_2.

show a different shape. The optimum temperature for the free form of the enzyme occurs at 48°C, while for the immobilized ones at about 60°C. This means that the immobilization procedure strengthens the enzyme structure.

Calling ''optimum temperature range'' the range in which the relative enzyme activity is higher than 95%, it is possible to see that this range is between 43.4°C and 53.5°C, for the free enzyme; between 55.0°C and 61.2°C, for the enzyme on M_1; between 54.8°C and 62.1°C, for the enzyme on M_2. From these data it is possible to deduce that the immobilization procedure and enzyme structure are somewhat affected by the presence of Sty which enlarges the optimum temperature range beside to induce limitations to the diffusion of the substrate towards the catalytic site. The simultaneous existence of a large range of thermal stability and of the shift of the optimum temperature position suggest the use of either M_1 and M_2 membranes in processes requiring high working temperatures, M_2 appearing more resistant than M_1 This conclusion is confirmed also by the values of the activation energies for enzyme disactivation, which resulted equal to 36.6 kcal/mol for M_1 and 72.0 kcal/mol for M_2.

3.2. Effect of pH

It is well known that the pH plays a relevant role on enzyme activity. This role is more clearly evidenced when the activities of soluble and immobilized enzymes are compared. In the latter case the support itself can change the pH value around the catalytic site thus determining different catalytic performances between the free and bound state of the catalyst. This effect is known as partitioning effect.

We have investigated the activity of the β-galactosidase in the free and immobilized states as a function of pH in the range between 2 and

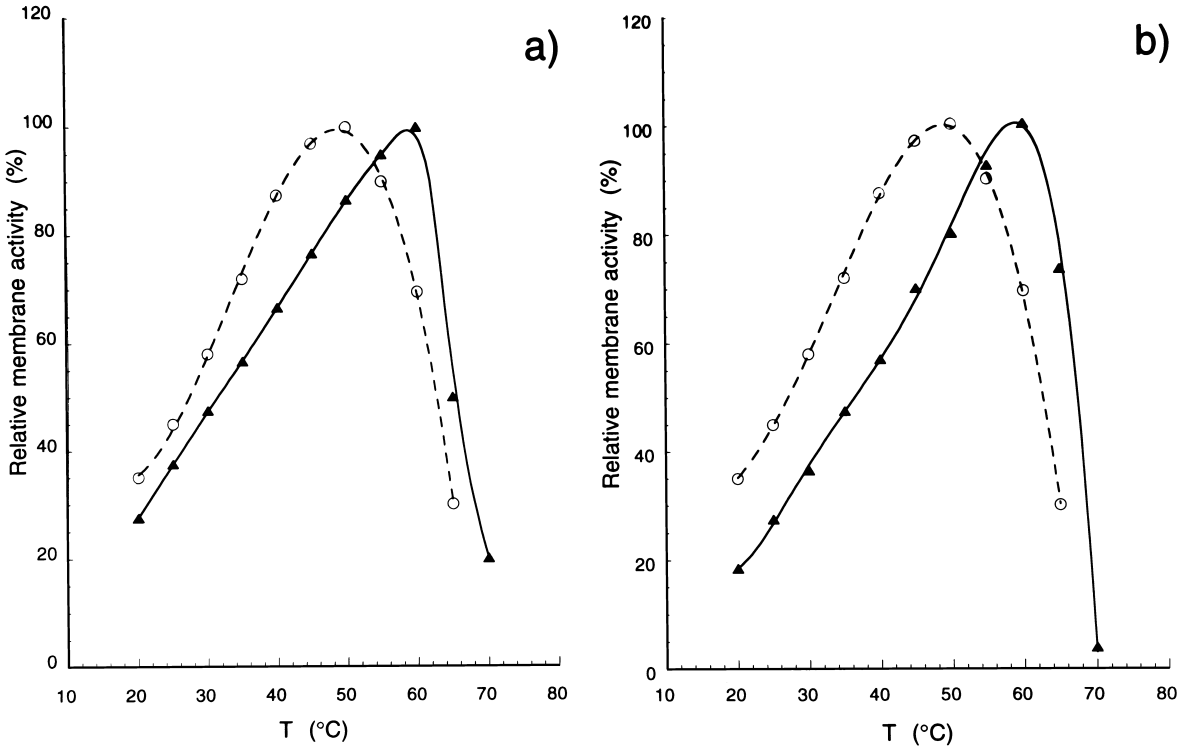

Fig. 4. Relative activity for the free (○) and immobilized form (▲) of β-galactosidase as a function of temperature. (a) membrane M_1; (b) membrane M_2.

7. We used 0.1 M NaCl–HCl buffer solution for pH 2, 0.1 M citrate buffer solution for the 3–5 pH range, and 0.1 M phosphate buffer solution for the 6–7 pH range. The results of this investigation are reported in Fig. 5a and b, where the relative activities of M_1 and M_2 membranes are respectively reported as a function of pH. In the figures the relative activity of the free enzyme is also reported as reference curve. All the experiments were performed with a 0.2 M lactose concentration in the appropriate buffer solutions, the temperature being constant at 30°C. No great differences between the free and immobilized form of the enzyme are observed. The same bell-shaped behaviour is obtained, only a shift of about 0.5 pH units towards more acid pH values is found with both membranes. This means that the spacer (HMDA), keeping the enzyme distant from the electric field of the nylon membranes, creates the conditions by which the microenvironment around the immobilized enzyme is quite similar to that around the free form. In a similar way, Sty, being neutral, does not affect the microenvironment.

As for the temperature, it is possible to define ''optimum pH range'' the range in which the relative enzyme activity is higher than 95%. From Fig. 5a and b it is possible to see that this range occurs between 4.25 and 5.00 pH for the free enzyme, and between 3.70 and 4.60 for both M_1 and M_2. This means that enzymes immobilized on M_1 and M_2 show more resistance with respect to the free form when employed in more acid solutions.

3.3. Kinetic parameters

When a biocatalyst is immobilized, the kinetic parameters K_m and V_{max} undergo variations with respect to the corresponding parame-

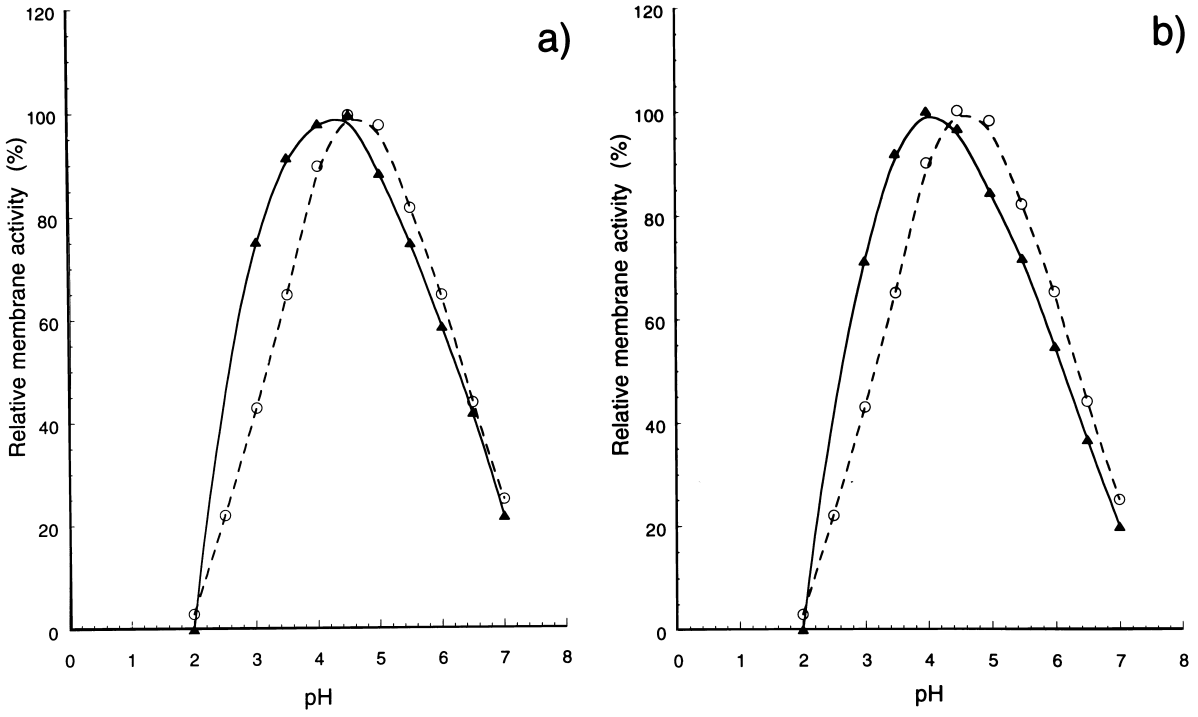

Fig. 5. Relative activity for the free (○) and immobilized form (▲) of β-galactosidase as a function of pH. (a) membrane M_1; (b) membrane M_2.

ters of the free form, revealing an affinity change for the substrate. These variations are attributed to several factors such as protein conformational changes induced by the support, steric hindrances and diffusional effects. These factors may operate simultaneously or separately, altering the microenvironment around the immobilized enzyme. Consequently the apparent K_m value of the immobilized form may increase [37,38] or decrease [39,40]. A decrease in the K_m value leads to a faster reaction rate, whereas an increase of the K_m implies the use of a higher substrate concentration in order to get the same reaction rate observed for the free enzyme. The apparent K_m decreases if, for example, the electric charges on support and substrate are opposite. The contrary occurs if the support and the substrate have electric charges of the same sign.

Also the V_{max} values are affected by the immobilization process. In general similar values of V_{max} have been found for the free and the immobilized form of the enzyme, even if increases [41] or decreases [42] have also been reported.

To determine the kinetic parameters for β-galactosidase immobilized on M_1 and M_2 the activity of the catalytic membranes was studied as a function of substrate concentration. The pH and temperature of the solutions were 6.5 and 30°C, respectively. The results are reported in Fig. 6a. Again the activity of M_1 results higher than the activity of M_2 in all the concentration range explored.

A Hanes plot of the results of Fig. 6a allows the calculation of the apparent kinetic parameters for β-galactosidase immobilized on M_1 and M_2. This was done in Fig. 6b. The apparent values of K_m and V_{max} were reported in Table 1, together with the ones relative to the free enzyme. Surprisingly, very different values of K_m and V_{max} have been found. The enzyme immobilized on M_2 has a higher affinity for the substrate than when immobilized on M_1. This

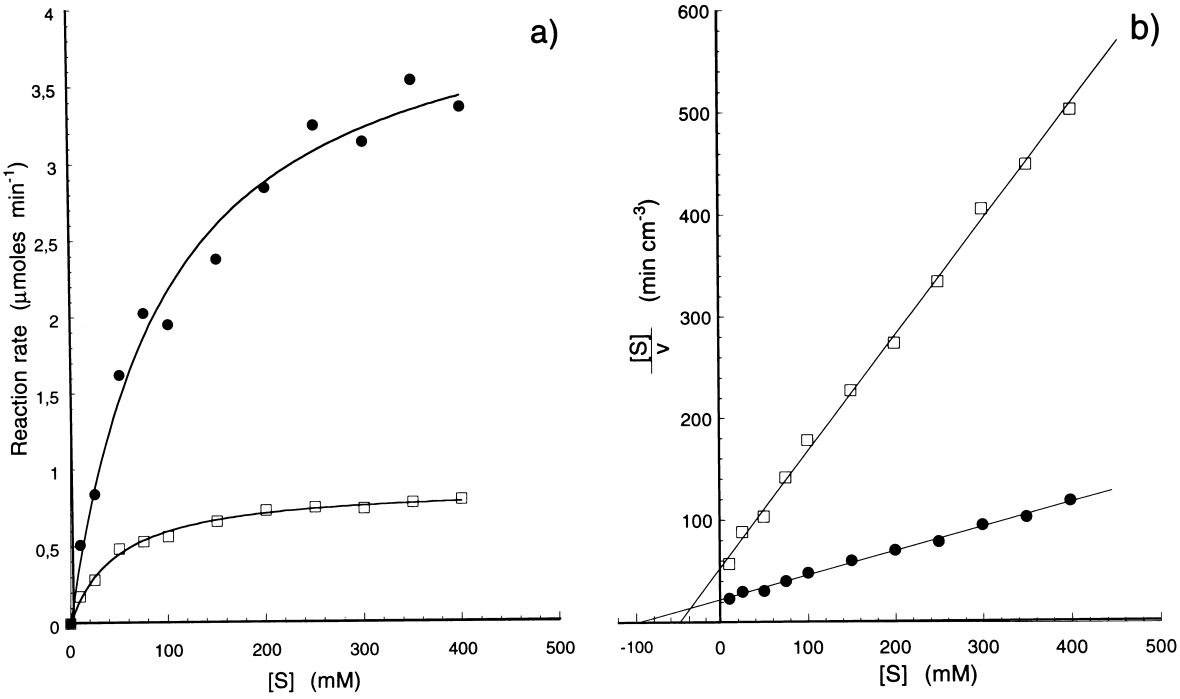

Fig. 6. (a) Relative activity of membranes M_1 and M_2 as a function of substrate concentration. (b) Hanes plot of the experimental points reported in (a). Symbols: (●) = membrane M_1; (□) = membrane M_2.

means that the presence of the Sty layers grafted on the membrane in somewhat protects the enzyme structure from the modification induced by the electric charges present on the nylon membrane. This protective effect is reduced when the enzyme is directly bound to the membrane via MMA.

3.4. Membrane hydrophobicity

In view of the employment of M_1 and M_2 membranes in non-isothermal bioreactors we have measured their hydrophobicity, since membrane hydrophobicity is required to have thermodialysis. Membrane hydrophobicity has been measured operatively, i.e., by measuring water transport induced by the process of thermodialysis, i.e., in presence of temperature gradients across the membranes.

A schematic representation of the apparatus employed for this measurements is reported in Fig. 7.

It is well known from the thermodynamics of irreversible processes [43,44] that non-isothermal water fluxes ($m^3\ m^{-2}\ s^{-1}$) across membranes are expressed by the equation:

$$J_{\text{water}} = B\frac{\Delta T}{\Delta x}; \qquad (1)$$

where $(\Delta T)/(\Delta x)$ is the temperature gradient ($K\ m^{-1}$) applied across a membrane having thickness Δx (m). B is a coupling phenomenological coefficient ($m^2\ s^{-1}\ K^{-1}$), representing

Table 1
Apparent K_m and V_{max} values for the free and immobilized form of β-galactosidase in membranes M_1 and M_2. The value for the free enzyme were calculated by us in a previous work

System	Kinetic parameters	
	$K_m^{(app)}$ (mM)	$V_m^{(app)}$ (μmol min^{-1})
Free enzyme	21.4	3.2
Membrane M_1	94.2	4.24
Membrane M_2	46.1	0.87

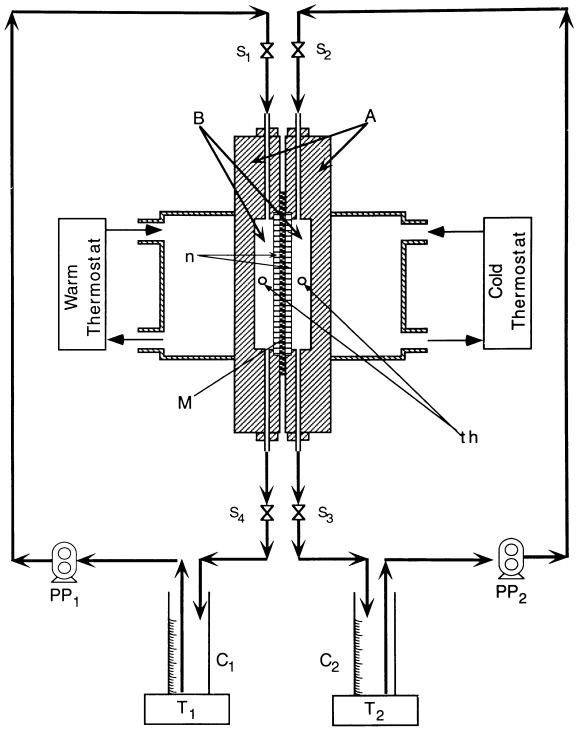

Fig. 7. Schematic representation of the bioreactor. Symbols: A: half-cells; B: internal working volumes; C_i: external working volumes; M: membrane; n: supporting nets; th: thermocouples; S_i: stopcocks; T_i: thermostatic magnetic stirrers; PP_i: peristaltic pumps.

the rate of water transport for unit temperature gradient. Water transport by thermodialysis occurs from warm to cold.

Since thermodialysis occurs only when hydrophobic membranes are used, we can introduce into Eq. (1) an adimensional coefficient σ^* given by:

$$B = \sigma^* B^*, \tag{2}$$

with $0 \leq \sigma^* \leq 1$. When σ^* is 0, thermodialysis does not occur and J_{water} is zero. σ^* is related to the hydrophobicity degree of the membrane in a similar way in which the Staverman coefficient σ is related to the permeability of a membrane for a determined solute. When $\sigma = 0$ the membrane is completely permeable, while when $\sigma = 1$ the membrane is completely impermeable.

We are unable to measure σ^*, but we can measure B related to σ^* by Eq. (2). The apparatus employed is schematically represented in Fig. 7.

J_{water} is determined by measuring the increase (or decrease) of liquid volume ΔV in C_2 (or C_1) during the time interval Δt through the equation

$$J_{water} = \frac{\Delta V}{S \Delta t}, \tag{3}$$

where S is the membrane surface area. Once known J_{water} and $(\Delta T)/(\Delta x)$, B is calculated through Eq. (1).

Under the experimental conditions $T_w = 45°C$, $T_c = 15°C$, i.e., $\Delta T = 30°C$ and $T_{av} = 30°C$, we have found a B value of 1.89×10^{-12} and 5.74×10^{-12} for M_1 and M_2 membranes, respectively, and of 8.20×10^{-12} for the untreated nylon membrane. This means that the membranes after the grafting treatment still re-

main hydrophobic. As foreseen, M_2 membrane has a higher value of B than M_1, owing to the increased hydrophobicity by Sty. These results encourage the employment of membrane M_2 in non-isothermal bioreactors.

4. Conclusions

The aim of this work has been reached as demonstrated by the results. Catalytic and hydrophobic membranes to be employed in non-isothermal bioreactors have been constructed.

Under isothermal conditions the copolymerization of Sty does not affect the general behaviour of the enzyme immobilized on M_1 or M_2. The presence of Sty only reduces the absolute activity of the M_2 membrane with respect to that of the membrane M_1, since a restricted substrate diffusion occurs in presence of a hydrophobic medium. This circumstance is a disadvantage in the use of membrane M_2 in respect to membrane M_1 under isothermal conditions, even if the apparent enzyme affinity for the substrate results higher for the β-galactosidase immobilized on M_2. The increase of the hydrophobicity in the Sty-grafted membranes, instead, represents an advantage in the use of M_2 rather than M_1 in presence of temperature gradient. This indication is confirmed by the results obtained employing M_1 and M_2 membranes in non-isothermal bioreactors, reported in a separate paper [35].

Acknowledgements

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