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Influence of the immobilization process on the activity of β-galactosidase bound to Nylon membranes grafted with glycidyl methacrylate Part 1. Isothermal behavior

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Abstract

β-Galactosidase from *Aspergillus oryzae* has been immobilized via diazotization or condensation on Nylon membranes grafted with glycidyl methacrylate. Immobilization via diazotization occurs through tyrosine residues, while immobilization via condensation involves multipoint attachment of the enzyme to the membrane through arginine residues. It was found that the immobilization via condensation strengthens the enzyme structure in contrast to the immobilization via diazotization, giving to the membranes prepared according to the first method higher resistance to temperature and acidic solutions in comparison to those prepared with the second method.

The solvent accessibility to the residues of the amino acid constituting the enzyme was studied and the 3D structure of the catalytic site was obtained by computer simulation using β-galactosidase from *Escherichia coli* as template. The interpretation of the results was based on the 3D structure of the catalytic site and the arginine and tyrosine density around it.

The apparent K_m values of β-galactosidase immobilized on both membrane types are higher than those of the free enzyme, thus reducing the advantages of employing these new catalytic membranes in industrial processes. A way to overcome this drawback is indicated.

Keywords: β-Galactosidase; Graft membranes; Enzyme immobilization; Diazotization; Condensation; Bioreactors

1. Introduction

In the last years, we focused our research on enzyme immobilization on polymeric membranes activated by a grafting technique [1–5]. By this technique, we immobilized β-galactosidase by entrapment

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or covalent binding on Teflon membranes grafted with different monomers by means of γ-radiations [6–10]. Nylon membranes chemically grafted with similar or different monomers have also been used to immobilize β-galactosidase [11–16], urease [17,18], penicillin G acylase [19,20] and glucose oxidase [21,22]. The behavior of all these membranes was studied under isothermal conditions, while for those exhibiting hydrophobic properties, we studied the enzyme reaction rates also under non-isothermal conditions. Under these conditions it was found that the rate of the catalytic process is increased [8,10,13–16,18,19,21,22] proportionally to the applied temperature gradients. Provided that the catalytic membranes were hydrophobic, average percentage increases of the activity ranging from 10 to 30% were observed with 1 °C of transmembrane temperature difference. The values of the percentage activity increases were dependent on the degree of hydrophobicity of the membrane, operatively measured by the ratio of the thermo-osmotic permeability coefficient to the hydraulic permeability coefficient. Factors affecting the hydrophobicity of catalytic membranes derived from Teflon or Nylon were the nature of the graft monomers and the grafting degree, when the same monomer was used.

In our previous studies, no attention was paid to the immobilization method. In this paper, we report the isothermal activity of catalytic Nylon membranes, having the same hydrophobicity and loaded with the same enzyme amount, but obtained with different immobilization methods: diazotization or condensation. Immobilization by diazotization involves the binding of the enzyme to the activated membranes through the tyrosine residues of the macromolecule, whereas the immobilization by condensation occurs through arginine residues. The dependence of the catalytic performance of both membrane types on pH, temperature, and substrate concentration has been investigated. When possible, comparison between the behavior of the immobilized and free enzyme has been done. To better understand the differences between the catalytic behavior of the free and immobilized form of the β-galactosidase from *Aspergillus oryzae*, a 3D model of the catalytic site has been built up starting from its amino acid sequence and the homology of this sequence with that of the same enzyme from *Escherichia coli*.

In a separate paper [23], the performance of both membranes in a bioreactor operating under non-isothermal conditions is discussed.

2. Apparatus, material 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 and separated by the catalytic membrane. Substrate solutions were recirculated in each half-cell by means of a peristaltic pump through hydraulic circuits starting and ending in a common cylinder C. Each half-cell was termostatted at a temperature T_i ($i = 1, 2$). Under isothermal conditions T_1 was equal to T_2. Thermocouples, placed at 1.5 mm from each surface of the membrane, measured the temperatures inside each half-cell and allowed to calculate the temperature profile across the catalytic membrane when the apparatus is kept under non isothermal conditions.

When the apparatus is employed to physically characterize the membranes, i.e. in the study of their hydrophobicity with measurements of transmembrane water transport under pressure or temperature gradients, the flow pipe and the pressure air tank are added to the bioreactor and different hydraulic circuits are consequently used.

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. The pore size is related to 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, srl-Milano, Italy) and used without further purification. As monomer to be grafted we used glycidyl methacrylate (GMA). Phenylenediamine (PDA) was used as spacer between the grafted membrane and the enzyme. For

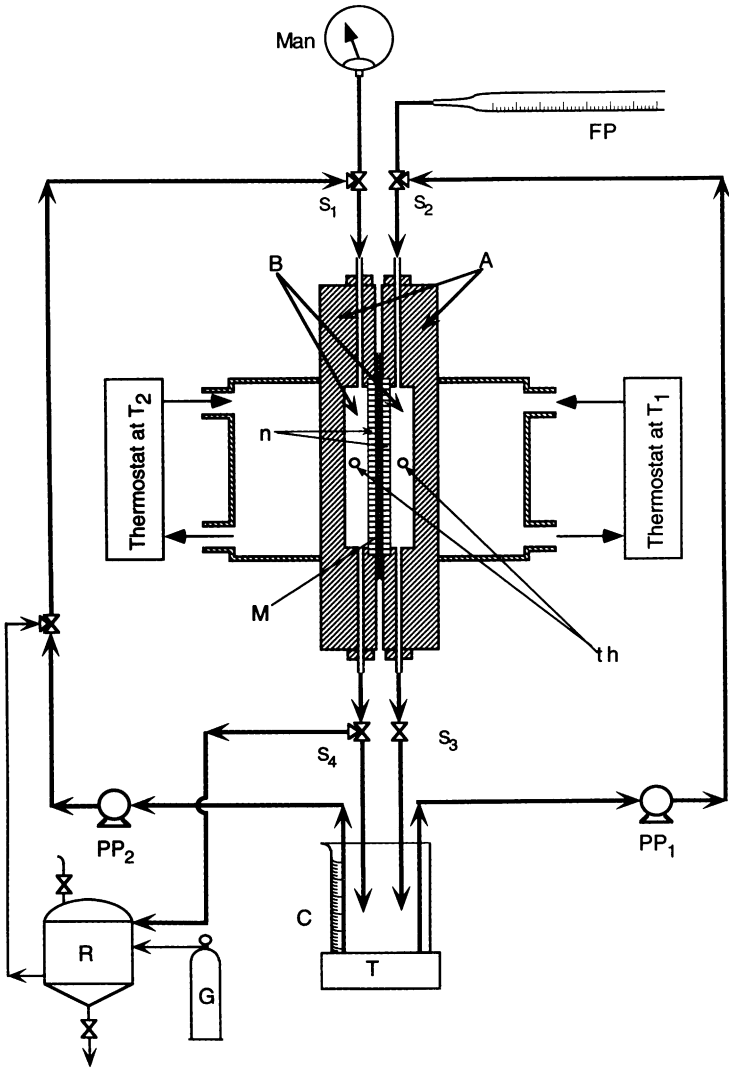

Fig. 1. Schematic (not to scale) representation of the bioreactor. (A): half-cells; (B): internal working volumes; (C): external working volume; (M): membrane; (n): supporting nets; (th): thermocouples; (S_i): stopcocks; (T): thermostatic magnetic stirrer; (PP_i): peristaltic pumps; (Man): manometer;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

the preparation of the catalytic membrane via condensation a 2.5% glutaraldehyde aqueous solution was also employed as 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 *A. oryzae*. β-Galactosidase has been used in view of the employment of these catalytic membranes in the lactose hydrolysis in milk as well as in the treatment of the waste waters coming from dairy industry.

2.3. Methods

2.3.1. Preparation of the catalytic membranes

The preparation of the catalytic membranes was carried out by means of two steps: (a) grafting copolymerization; and (b) enzyme immobilization.

2.3.1.1. Grafting copolymerization. Grafting copolymerization was carried out by using as initiating system $K_2S_2O_8/Na_2S_2O_3$ in the ratio 1/1. The membranes were immersed, for 1 h at 40 °C, in a reaction vessel filled with a 1/1 water/ethanol solution containing 0.3 M GMA, 0.008 M $K_2S_2O_8$ and $Na_2S_2O_3$ and in the presence of 0.004% (W/V) copper acetate. Later on the membranes were treated with methylethyl ketone to remove the produced homopolymer, then dried at 40 °C until a constant weight was measured. At this point, a Nylon/polyGMA membrane was obtained.

2.3.1.2. Enzyme immobilization. Two different methods have been used for immobilization of β-galactosidase onto Nylon/polyGMA membranes: diazotization and condensation. For both methods *p*-phenylenediamine was used to obtain aminoaryl derivatives on Nylon/polyGMA membrane. To this aim, the membranes were treated, for 45 min at room temperature, with a 2% *p*-phenylenediamine solution in 0.1 M sodium carbonate buffer, pH 9. After washing with water, a different procedure was used in dependence on the method used for the enzyme immobilization. In the case of enzyme immobilization by diazotization, the aminoaryl derivatives were treated for 30 min at 0 °C with an aqueous solution containing 2 M HCl and 4% $NaNO_2$. In the case of enzyme immobilization by condensation, the aminoacryl derivatives were treated for 90 min at room temperature in 2.5% glutaraldehyde aqueous solution. At the end of each of the two treatments described above, each membrane type was washed at room temperature with double distilled water and 0.1 M phosphate buffer solution, pH 6.5, then treated for 16 h at 4 °C with the same buffer solution containing β-galactosidase at a concentration of 3 mg ml^{-1}. At the end of this step, the membranes were washed with the buffer solution to remove the unbound enzymes. These conditions were found to be optimal in preliminary experiments aimed to obtain the two membrane types with comparable hydrophobicity and amount of immobilized enzyme.

Catalytic membranes obtained by diazotization were named M_1; the other ones, obtained by conden-

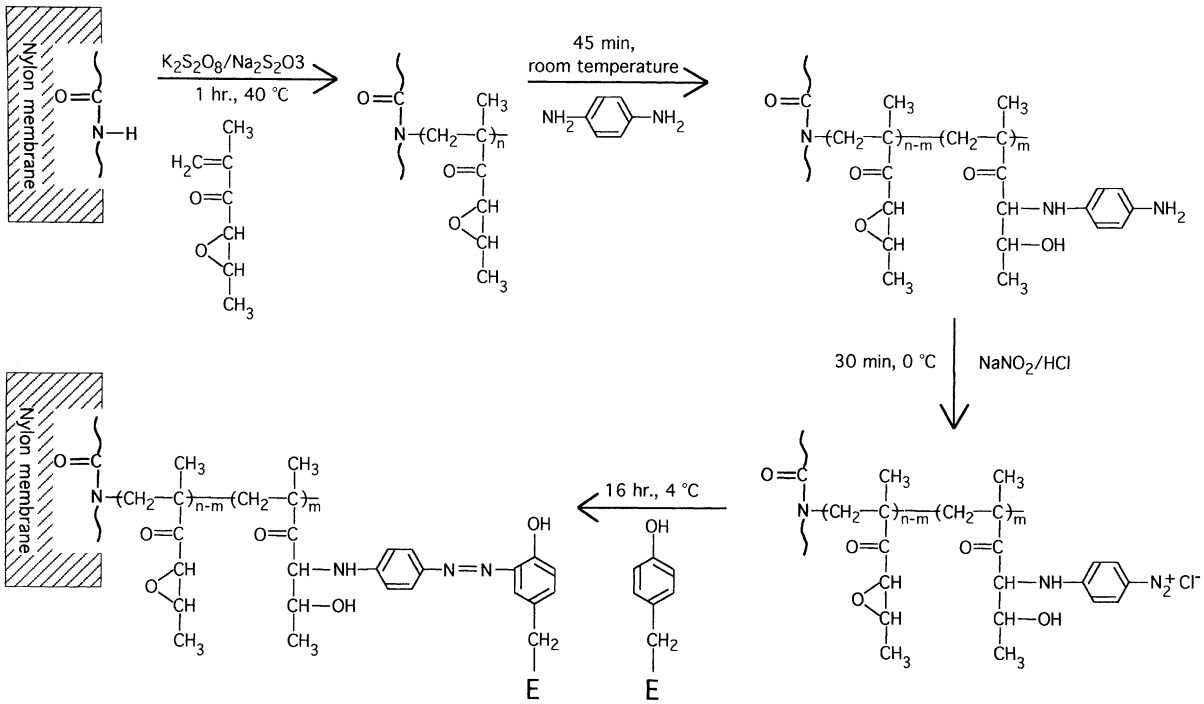

Fig. 2. Schematic sequence of grafting and enzyme immobilization processes to obtain membrane M_1.

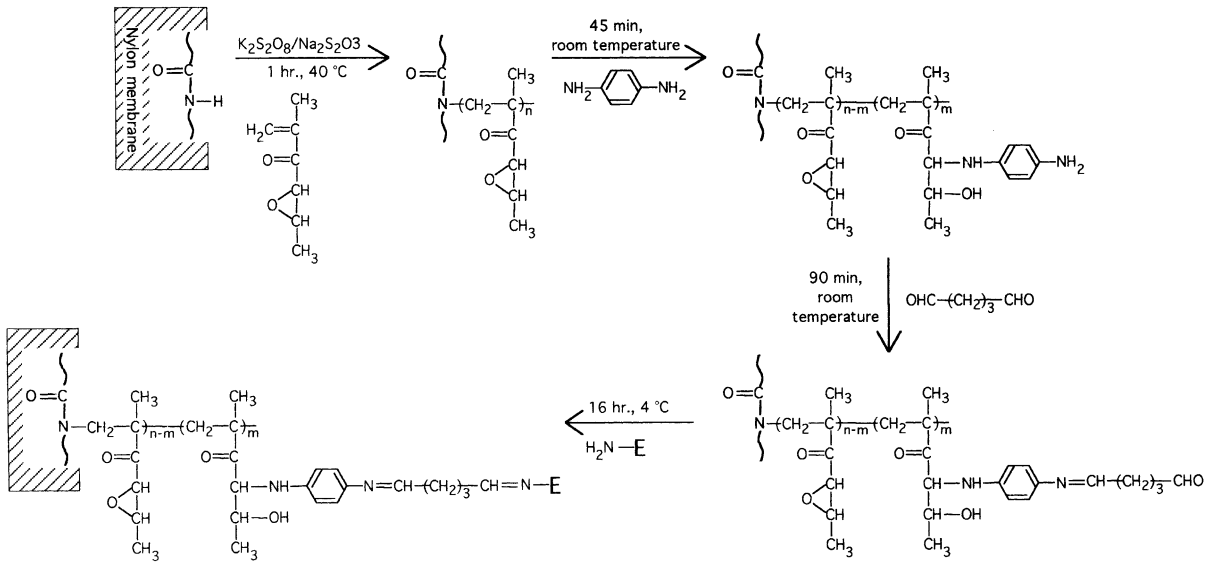

Fig. 3. Schematic sequence of grafting and enzyme immobilization processes to obtain membrane M_2.

sation, M_2. The steps for the preparation of the two types of catalytic membranes are reported in Figs. 2 and 3.

2.3.2. Determination of membrane activity and stability

β-Galactosidase hydrolyzes lactose to glucose and galactose. Enzyme activity was determined by sampling every three minutes in the cylinder C the solution in contact with the membrane and by 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. The glucose concentration, which is proportional to the intensity of the colour solution, is spectrophotometrically determined. Membrane activity, expressed as $\mu\text{mol min}^{-1}$, is given by the slope of the linear plot of the glucose production as a function of time. The working volume of the lactose solution was 30 ml and was circulated through the two half-cell of the bioreactor and the cylinder C at a rate of $3\,\text{ml min}^{-1}$ by means of two peristaltic pumps.

Time stability of both biocatalytic membranes was assessed by analyzing every day their activity under the same experimental conditions, i.e. 0.2 M lactose in 0.1 phosphate buffer solution, pH 6.5 and $T = 30\,^{\circ}\text{C}$. After 3 or 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 types approached and maintained their long term stability. Only these stabilized membranes were used in the comparative experiments reported in the following. When not in use, the membranes were stored at $4\,^{\circ}\text{C}$ in 0.1 M phosphate buffer solution, pH 6.5.

2.3.3. Determination of hydraulic and thermo-osmotic permeability of the membranes

The hydraulic permeability of the catalytic membranes has been determined by overpressurizing 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 thermo-osmotic 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.

2.3.4. Treatment of experimental data

Every experimental point reported in the figures represents the average value of five experiments performed under the same conditions. Each experiment lasted 30 min, but only the initial reaction rates were accounted for in the construction of the figures. The duration of each experiment and the hydrophobic nature of the membrane excluded the occurrence of membrane fouling. In any case, to avoid membrane fouling owing to membrane reuse a cleaning 0.1 M phosphate buffer solution was circulated for 20 min through the bioreactor and the membrane between two subsequent experiments. Effects due to concentration polarization, even is present, have not been taken in account for considering the hydrophobic nature of the membrane. The experimental errors did not exceed 4%.

2.3.5. Modelling procedure

In order to detect, the residues bound to the membrane, the relative solvent accessibility to the amino acids sequence was predicted with AccPRO (http://promoter.ics.uci.edu/BRNN-PRED/), as described by Pollastri et al. [24].

Secondary structure of β-galactosidase from *A. oryzae* has been predicted with SecPred (http:/prion.biocomp.unibo.it/secpred.html) and according to Jacoboni et al. [25], while the secondary structure of β-galactosidase from *E. coli* has been computed with the DSSP program [26].

The active site of β-galactosidase of *A. oryzae* (BGAL_ASPOR; sequence available at www.ncbi.nlm.nih.gov, Patent no. US5736374) was modelled using the structure of β-galactosidase from *E. coli* (BGAL_ECOLI; PDB code: 1BGL) as template. Residues involved in the catalytic activity of BGAL_ASPOR were identified by sequence comparison with the program CLUSTALW available at www.ebi.ac.uk[27]. 3D modeling was performed using MODELLER [28]. The quality of the 3D structure of the active site of the enzyme was checked with PROCHECK [29] (biotech.embl-ebi.ac.uk:8400). Model structures were visualized using RASMOL [30].

3. Results and discussion

3.1. 3D model of β-galactosidase from A. oryzae

In Fig. 4, the prediction of solvent accessibility for the β-galactosidase from *A. oryzae* is reported. The solvent accessibility is a measure of the accessible area of a residue when a probe (a sphere of 0.14 nm which models a water molecule) is rolled around the Van der Waal's surface of the protein. The relative solvent accessibility of a residue is the ratio between the area of its exposed surface in the protein and its maximal accessibility. This parameter gives information about the exposure of each residue on the protein surface. In Table 1, the solvent accessibility prediction is listed with reference to the residues of the two amino acids, tyrosine or arginine, involved in the two different immobilization processes. The table shows that more than 93% of the 30 arginine residues are exposed to the solvent, while only 33% of the 45 tyrosine residues are exposed. If the accessibility is restricted to values higher than 40%, then the number of arginine residues is reduced to 9, while that of the tyrosine residues reduces to 2. This circumstance favors multipoint enzyme attachment to the activated support in the case of membrane M_2.

Fig. 5a shows the alignment between the active site of BGAL_ASPOR (residues 47–396) and the active site of BGAL_ECOLI (residues 334–626). Given the low sequence identity (about 20%), the alignment was adjusted to match motifs of secondary structures as predicted with the SecPred program [25] taking care of keeping aligned the position of functionally important residues. Glu 461 and 537, forming the catalytic site of BGAL_ECOLI, correspond to Glu 200 and Glu 298 in the target covalent structure.

Table 1
Number of arginine and tyrosine residues exposed under different solvent accessibility degree

	Total no.	Accessibility ≤ 15%	15% < accessibility ≤ 40%	Accessibility > 40%
Arg	30	2	19	9
Tyr	45	30	13	2

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        10         20         30         40         50         60
MKLLSVAAVALLAAQAAGASIKHRLNGFTILEHPDPAKRDLLQDIVTWDDKSLFINGERI
eeppppbbbbbbbppebpppbepebepbpbpppeepeepebpppbpbppebbbbpppepp

        70         80         90        100        110        120
MLFSGEVHPFRLPVPSLWLDIFHKIRALGFNCVSFYIDWALLEGKPGDYRAEGIFALEPF
bbbbbbbbbbpppbpebbeebbepbeeppbpbbbbbbbbbbpbpeeeebpbeppppbbpb

       130        140        150        160        170        180
FDAAKEAGIYLIARPGSYINAEVSGGGFPGWLQRVNGTLRSSDEPFLKATDNYIANAAAA
bebbpepbbbbbbbbbpepbbbbpppbbbbbbeebeppbppepeebpebbpebbepbbep

       190        200        210        220        230        240
VAKAQITNGGPVILYQPENEYSGGCCGVKYTDADYMQYVMDQARKADIVVPFISNDASPS
bpepppeepbbbbbbbbbbbeppppppbebeppbbpbbbebbeebebbbbbbbpepppe

       250        260        270        280        290        300
GHNAPGSGTGAVDIYGHDSYPLGFDCANPSVWPEGKLPDNFRTLHLEQSPSAPYSLLEFQ
pppppppbbbbbbbbbpppppppepebpebebbpeppbbepbppbppepeeepbppbbbbb

       310        320        330        340        350        360
AGAFDPWGGPGFEKCYALVNHEFSRVFYRNDLSFGVSTFNLYMTFGGTNWGNLGHPGGYT
bbbbppbpppppeppbepbppbbbbbbbppbbbbbbbbbbbbbbbbbbbbbppbepeppbb

       370        380        390        400        410        420
SYDYGSPITETRNVTREKYSDIKLLANFVKASPSYLTATPRNLTTGVYTDTSDLAVTPLI
bbbbbbpbpppppppepepbbbbepbpebbeppeepbppppeebppbpbpppeepppppbp

       430        440        450        460        470        480
GDSPGSFFVVRHTDYSSQESTSYKLKLPTSAGNLTIPQLEGTLSLNGRDSKIHVVDYNVS
pppebbbbbbpbbpbpbeeebpbppbebpepeepbpbppbpppppepepeepppbpbppbebp

       490        500        510        520        530        540
GTNIIYSTAEVFTWKKFDGNKVLVLYGGPKEHHELAIASKSNVTIIEGSDSGIVSTRKGS
epebppbpbpbbbppebeeepbbbbbbbpeepppbpbpbppepepbepeebbbbbeeepb

       550        560        570        580        590        600
SVIIGWDVSSTRRIVQVGDLRVFLLGKNSAYNYWVPELPTEGTSPGFSTSKTTASSIIVK
bbbbppeppbpppbpppppbpbbbbpepbpbpbbppepepeeepeeppebeebbbpbbbp

       610        620        630        640        650        660
AGYLLRGAHLDGADLHLTADFNATTPIEVIGAPTGAKNLFVNGEKASHTVDKNGIWSSEV
bbbbbpbbpbepbpbpbbbpbeppbpbpbbbbpppbpbbbbppeepppbpbeebbbpbeb

       670        680        690        700        710        720
KYAAPEIKLPGLKDLDWKYLDTLPEIKSSYDDSAWVSADLPKTKNTHRPLDTPTSLYSSD
epebpebebepbeebpbepbppbeebeppppeeepppbeebeepeeppeebeepppbpbbp

       730        740        750        760        770        780
YGFHTGYLIYRGHFVANGKESEFLIRTQGGSAFGSSVWLNETYLGSWTGADYTMDGNSTY
bbbbbbbbbbpbpbbbepepbpbbbpbpppbpbbbbbbbbpbpbbbbbbbbbpbpppbpb

       790        800        810        820        830        840
KLSQLESGNYHVITVVIDNLGLDENWTVGEETMKNPRGILSYKLSGQDASAITWKLTGNL
epepbpepebpbbbbbbbbbbbpepbebbpebbeppbbbbppbeeeebppbbbpbpppp

       850        860        870        880        890        900
GGEDYQDKVRGPLNEGGLYAERQGFHQPQPPSDSWESGSPLEGLSKPGIGFYTAQFDLDL
ppeppepebpppppeppbpbpppppppeepepeepeeepepepppeepbbbbbbpbppep

       910
PKRAEGPSSTS
peeeeeeeeee
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Fig. 4. Prediction of the solvent accessibility of the β-galactosidase from *A. oryzae*. Relative solvent accessability is classified by means of the symbols e, p, and b. For 'e': exposed residues (predicted solvent accessibility >40%); 'p': partially exposed residues (predicted solvent accessibility between 15% and 40%); 'b': buried residues (predicted solvent accessibility <15%).

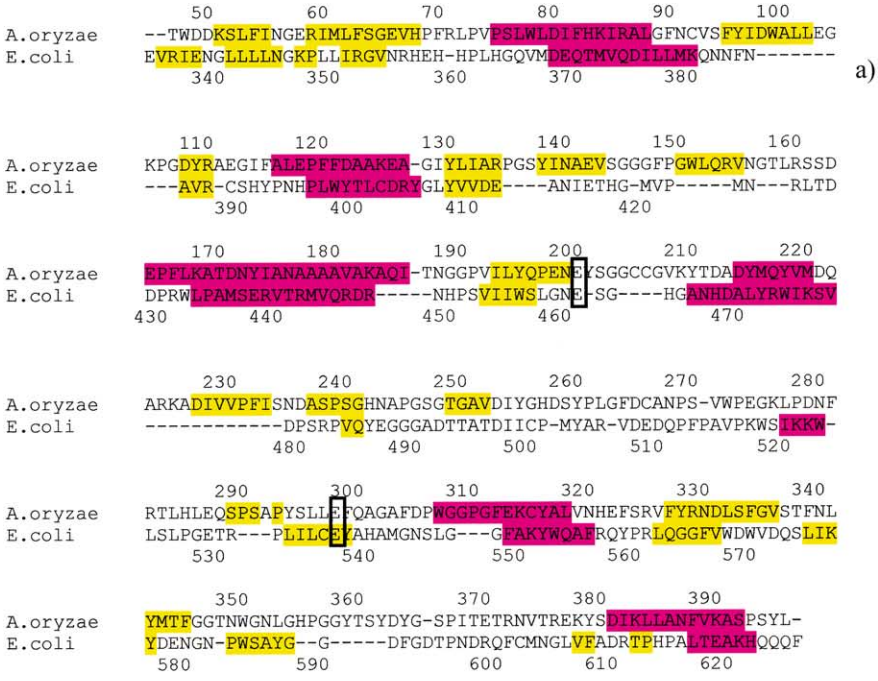

Fig. 5. (a) Alignment between the β-galactosidase from *A. oryzae* and from *Escherichia coli*. (b) Model of the active site of β-galactosidase from *A. oryzae*.

The model of the active site of the target sequence is visualized in Fig. 5b. The active site consists of two glutamic residues (highlighted with a spacefill representation and coloured in red) in a groove quite accessible to the solvent comprising a TIM-barrel structure. Three exposed tyrosine residues (Tyr 138, Tyr 201 and Tyr 260, highlighted in green) lie within 1 nm from the active glutamic acid. Within the same distance no arginine exposed residues can be found. Binding through diazotization of tyrosine residues, therefore, protect the active site more than immobilization through condensation.

3.2. Physical characterization of the membranes

In order to obtain catalytic membranes, prepared via diazotization or condensation, having the same grafting degree and mass transport properties, different lots of each membrane type were prepared. Membranes endowed with the same properties were chosen in each of the two lots and used in this research.

In Table 2, some of the properties of the membranes M_1 and M_2 are reported. Grafting degree (GD) was determined according to the expression

$$\mathrm{GD} = \frac{W_\mathrm{A} - W_\mathrm{B}}{W_\mathrm{B}} \times 100 \tag{1}$$

where W_B, and W_A are the dry membrane mass before and after the grafting process, respectively.

The coefficient A, measured in $\mathrm{m^4\,N^{-1}\,s^{-1}}$, is known as hydraulic permeability coefficient and is calculated through the equation

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

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

The coefficient B, measured in $\mathrm{m^2\,K^{-1}\,s^{-1}}$, is known as thermo-osmotic permeability coefficient, and is calculated through the equation

$$J_\mathrm{water}^\mathrm{therm.} = B\frac{\Delta T}{\Delta l} = B^*\frac{\Delta T^*}{\Delta x} \tag{3}$$

where $J_\mathrm{water}^\mathrm{therm.}$, expressed in $\mathrm{m\,s^{-1}}$, is the non-isothermal water flux produced by the temperature difference ΔT (K) measured at the position of the thermocouples that are Δl (m) apart. B^* is the thermo-osmotic permeability coefficient which accounts for the actual temperature difference ΔT^* across the membrane thickness Δx.

Isothermal hydraulic fluxes have been measured in the pipe connected to one half-cell when the other is over pressured by means of a gas tank, as in Fig. 1, under the conditions $\Delta P \neq 0$ and $\Delta T = 0$.

Non-isothermal thermo-osmotic fluxes have been measured in the pipe connected to the cold half-cell under the conditions $\Delta T \neq 0$ and $\Delta P = 0$.

During the measurement of hydraulic or thermo-osmotic permeabilities concentration polarization on/or fouling of the membranes were absent since the experiments were carried out with pure, double-distilled water.

The values of the coefficients A and B relative to M_1 and M_2 membranes are listed in Table 2. The amount of immobilized enzyme, as well as the absolute and relative enzyme activity for each of the two membrane types, are also reported in the same table. The amount of immobilized enzyme has been calculated as difference between the amount of protein present in the solution used for the immobilization and the amounts present in the solution after the immobilization process and in the membrane rinsing buffer solution. The absolute membrane activity has been defined as activity for total membrane surface (two surfaces of $35\,\mathrm{cm^2}$ each one), while the specific activity as activity for mg of immobilized protein.

Table 2
Physical and biochemical properties of membranes M_1 and M_2

Membrane	Grafting percentage (%)	A ($\mathrm{m^4\,N^{-1}\,s^{-1}}$)	B ($\mathrm{m^2\,K^{-1}\,s^{-1}}$)	Amount of immobilized enzyme (mg)	Absolute membrane activity ($\mathrm{U\,cm^{-2}}$)	Specific membrane activity ($\mathrm{U\,mg^{-1}}$)
M_1	15.9	3.05×10^{-14}	3.7×10^{-12}	1.89	0.021	0.78
M_2	15.3	2.95×10^{-14}	3.9×10^{-12}	1.73	0.015	0.64

Considering all the parameters listed in Table 2 some preliminary conclusions can be drawn about the two membrane types used in this research.

The two membranes have practically the same physical characteristics in respect to the grafting percentage, hydraulic and thermo-osmotic permeabilities, amount of immobilized enzyme, thus their catalytic behavior can be compared. The lower values of the absolute and relative activity of membrane M_2 in comparison to the ones of membrane M_1 may be attributed to the presence of multipoint attachment of the enzyme to membrane M_2 with consequent twisting of the native structure of the catalytic site and reduction of its catalytic power.

3.3. Biochemical characterization of the membranes

3.3.1. Temperature dependence

The relative activity of both membrane types are reported in Fig. 6 as a function of temperature. In the same figure, the relative activity of the free β-galactosidase has been added to allow comparison. The experimental conditions were 200 mM lactose concentration in 0.1 M phosphate buffer solution, pH 6.5. From the figure it results that the immobilized enzymes exhibit higher optimum temperatures in comparison to that of the free counterpart, the optimum temperature occurring at 50 °C for the free β-galactosidase, and at 60 and 66 °C for the enzyme immobilized on membranes M_1 and M_2, respectively.

The shift of the optimum temperature towards high temperatures when the biocatalyst is immobilized indicates that the enzyme structure is strengthened by the immobilization process. When immobilized, the enzyme maintains the structure of the catalytic site at temperatures higher than those disactivating the native form. The higher optimum temperature for membrane M_2 in comparison to that of membrane M_1 indicates that the condensation process, allowing multipoint attachment of the enzyme to the membrane, strengthens the enzyme structure more than the diazotization process.

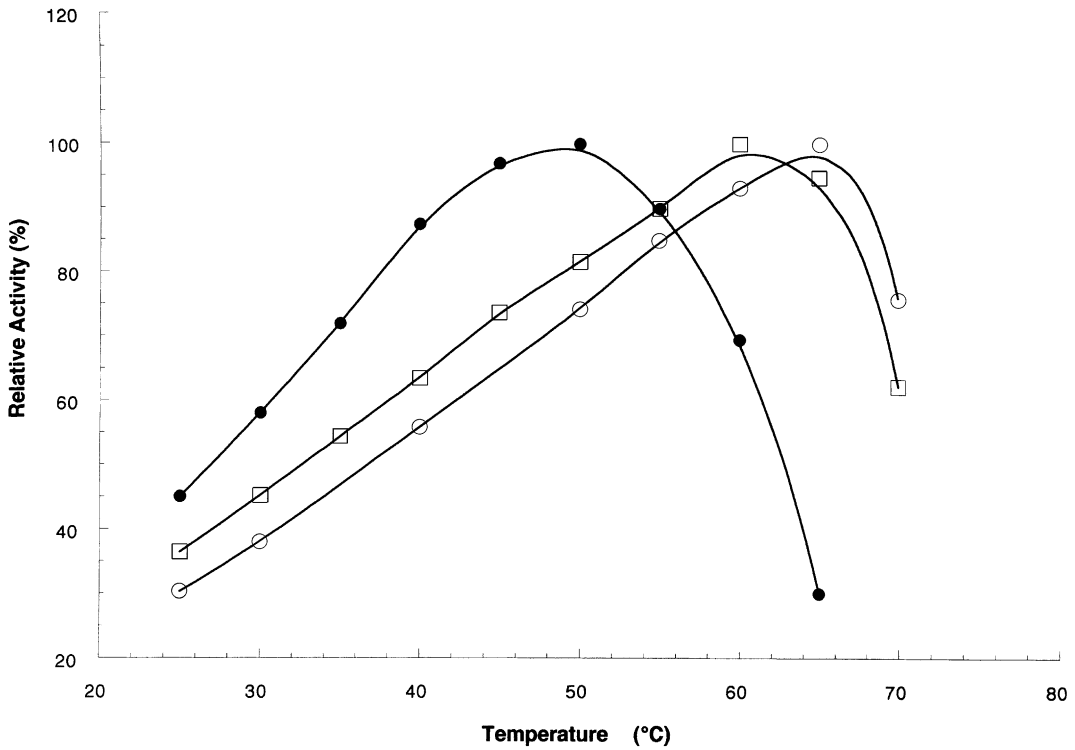

Fig. 6. Relative activity of free and immobilized β-galactosidase as a function of temperature. Symbols: (●) free enzyme; (□) membrane M_1; (○) membrane M_2.

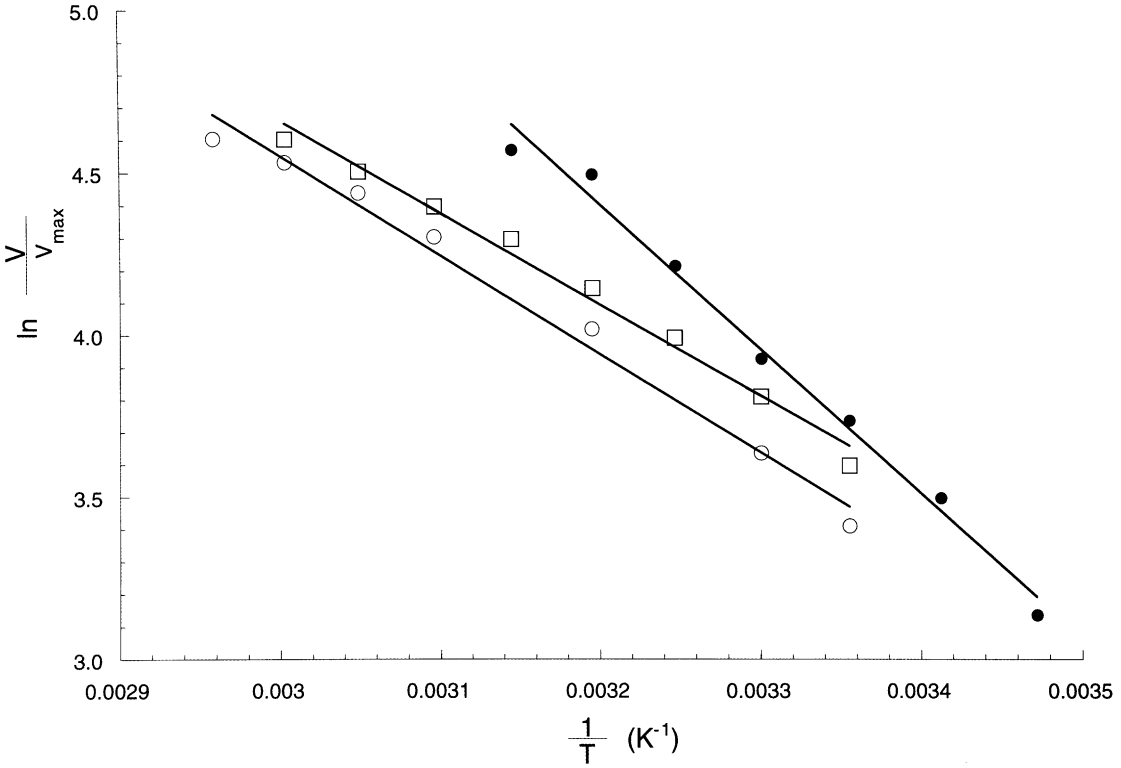

Fig. 7. Arrhenius plots of the points in Fig. 6. Symbols: (●) free enzyme; (□) membrane M_1; (○) membrane M_2.

Defining "optimum temperature range" the range in which the relative activity is comprised between 95 and 100%, it is possible to see that this range occurs between 44.2 and 52.8 °C for the free enzyme, between 57.2 and 64.1 °C for membrane M_1 and between 61.5 and 66.9 °C for membrane M_2. From these observations it is possible to deduce that membranes M_2 is more promising for industrial processes requiring high temperatures. Fig. 7 shows the Arrhenius plots relative to the results in Fig. 6. From Fig. 7, the activation energies of soluble and immobilized β-galactosidase were calculated. The results of this calculation are shown in Table 3. It clearly appears that the values of the activation energies for the β-galactosidase immobilized on both membrane types are lower than the value for the soluble enzyme. This is a clear indication that the enzyme reaction is diffusion limited for both catalytic membranes. The practically coincident values of the activation energy for both catalytic membrane types indicate that substrate and reaction products diffusion across the two membranes takes place with the same law of temperature dependence.

Table 3
Activation energies for the free and immobilized β-galactosidase

Enzyme form	Activation energy (kcal mol^{-1})
Free	8.8
Immobilized on M_1	5.6
Immobilized on M_2	6.0

3.3.2. *pH dependence*

The relative activity of both enzyme derivatives as a function of pH are reported in Fig. 8. The experimental conditions were: 200 mM lactose concentration in 0.1 M phosphate buffer, pH 6.5 and temperature 25 °C. The relative activity of the free enzyme is added, for comparison. From Fig. 8, a shift of the optimum pH position towards more acidic values is observed for immobilized enzymes in comparison to the position of the free enzyme. The optimum pH

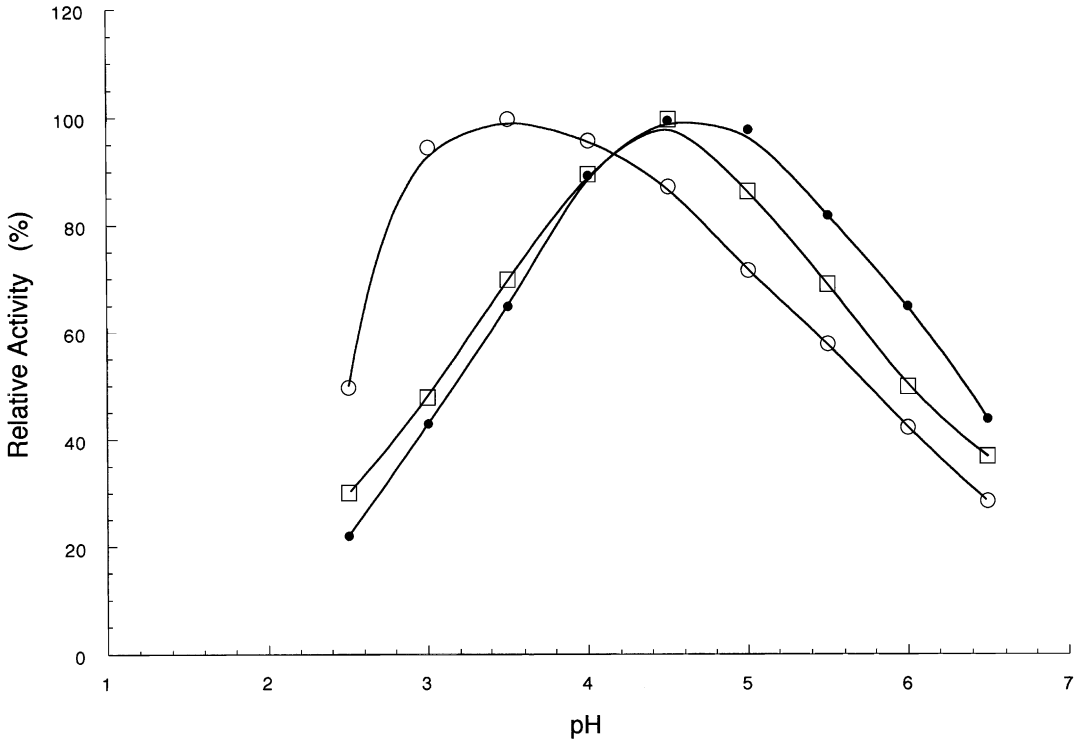

Fig. 8. Relative activity of free and immobilized β-galactosidase as a function of pH. Symbols: (●) free enzyme; (□) membrane M_1; (○) membrane M_2.

values occur at pH 4.7 for the free enzyme, at pH 4.5 for the enzyme immobilized on membrane M_1, and at pH 3.5 for the enzyme immobilized on membrane M_2. This means that the immobilized enzymes, at least in experiments carried out for short times, appear more resistant to extreme acidic solution than the free form. This behavior is explained in terms of the partitioning effect induced by the presence of a negative charge density on the Nylon membranes. This charge density increases the H^+ concentration in the microenvironment in which the enzyme is operating in respect to the H^+ concentration in bulk solution. On the basis of the partitioning effect we would expect a higher shift for membrane M_1 in respect to membrane M_2, since the presence of glutaraldehyde in M_2, keeping the enzyme further away from the support, should minimize the partitioning effect. This does not occur, probably because the great efficiency of the condensation process in strengthening the structure of the catalytic site overcomes the partitioning effect.

The lower optimum pH for membrane M_2 in respect to that of membrane M_1 indicates that membrane M_2 is more promising for industrial processes occurring in presence of extreme acidic solution.

Defining "optimum pH range" the range in which the relative activity is comprised between 95 and 100%, it is possible to see that this range occurs between 4.2 and 5.0 for the free enzyme, between 4.2 and 4.7 for membrane M_1 and between 3.0 and 4.7 for membrane M_2.

3.3.3. Concentration dependence

The catalytic activity of both membrane types as a function of substrate concentration up to 300 mM has been reported in Fig. 9a. Substrate concentrations were obtained by dissolving the appropriate lactose amount in 0.1 M phosphate buffer solution, pH 6.5. The experimental temperature was 25 °C. Fig. 9a shows that in all the concentration range explored the catalytic activity of M_1 membrane is higher than the

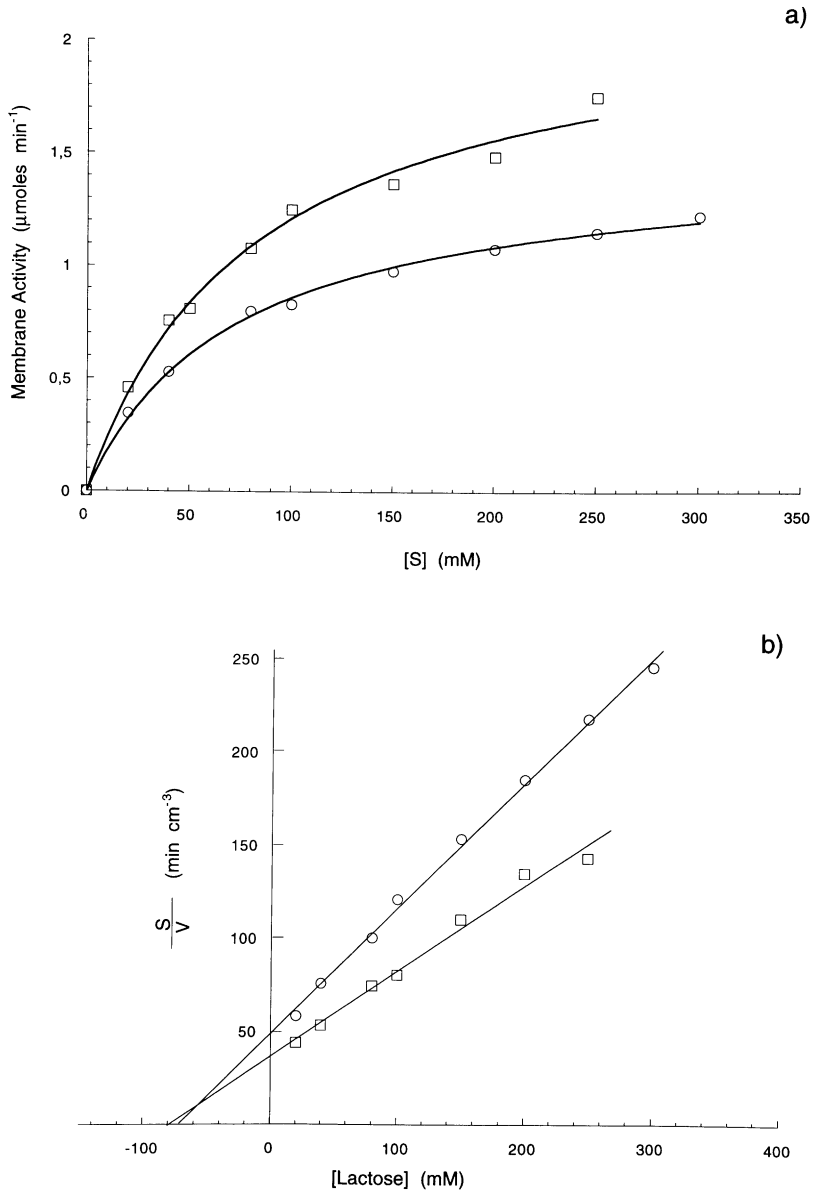

Fig. 9. (a) Catalytic membrane activity as a function of substrate concentration; (b) Hanes plots relative to the experimental points of (a). Symbols: (□) membrane M_1; (○) membrane M_2.

activity of M_2 membrane thus, confirming the values reported in Table 2. A Michaelis–Menten behavior is also evident. The Hanes plots relative to the experimental data in Fig. 9a are reported in Fig. 9b. From this figure the values of the kinetic parameters K_m and V_{max} of the enzyme reaction for the immobilized β-galactosidase have been calculated. These values are reported in Table 4, together with the analogous value for the free enzyme [12]. Inspection of this table shows that the value of the apparent K_m of the immobilized enzyme for both enzyme derivatives are higher than the corresponding value of the free

Table 4
Values of the apparent kinetic parameters for the free and immobilized β-galactosidase[a]

Enzyme form	K_m (mM)	V_{max} (μmol min^{-1})
Free	21.4	3.20
Immobilized on M_1	72.0	2.00
Immobilized on M_2	73.0	1.52

[a] The experimental temperature was 25 °C.

counterpart. The increase of the apparent K_m after immobilization clearly indicates an apparent lower affinity of the immobilized enzyme for its substrate compared to that of the free enzyme. This result may be attributed to changes in enzyme structure induced by the interaction of the macromolecule with the support. Alternatively, the increased diffusional resistance encountered by either the substrate in its approach to the catalytic site or by the reaction products in their removal from the same place could be responsible for the higher values of K_m. Another cause may be related to the changes induced by the immobilization process in the microenvironment in which the enzyme operates. These changes can induce nearby the catalytic site substrate concentrations different from those in the bulk. Similar results were obtained by us with β-galactosidase and urease immobilized on Teflon or Nylon membranes, grafted with different monomers [15,16,18].

The equal values of the apparent K_m's in the case of the two membranes indicate that the presence of the glutaraldehyde on M_2 does not modify the diffusive transport of substrate and products. This observation agrees with the macroscopic behavior evident in Table 2 through the equivalent values of hydraulic and thermo-osmotic permeability coefficients.

4. Conclusion

The influence of the immobilization method on the enzyme activity of a catalytic membrane has been characterized.

Results have demonstrated that in the case of β-galactosidase, its immobilization via condensation on Nylon graft membranes strengthens the enzyme structure in comparison to the immobilization via diazotization. For this reason the former membranes showed, at least in experiments carried out for short time, higher resistance to temperature and acidic solution than the latter. Immobilization via condensation involves multipoint attachment of the enzyme to the membrane through arginine residues, while immobilization via diazotization occurs through tyrosine residues.

Solvent accessibility to both residues as well as the 3D structure of the catalytic site, obtained by computer simulation using β-galactosidase from *E. coli* as template, supported the interpreted results.

The apparent K_m values of the immobilized β-galactosidase are higher than that of the free enzyme under the same experimental conditions. This circumstance reduces the advantages of employing these new catalytic membranes in traditional industrial processes. Nevertheless, the hydrophobic character of the membranes, obtained through experiments of thermo-osmotic permeability, suggests their use in bioreactors operating under non-isothermal conditions. Results obtained with these bioreactors are reported in a separate paper [23].

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