Enzyme immobilization on graft copolymers

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Contents

Chapter 1

SHORT INTRODUCTION

I. Enzyme immobilization

Enzymes, which are very efficient and advantageous catalysts, can catalyze specific reactions under mild conditions, i.e. in neutral aqueous solutions at room temperature and atmospheric pressure. However, when using organic solvents or high temperatures the above-mentioned advantages turn into disadvantages. One of the approaches of preparing more superior biocatalysts for these and other applications is enzyme immobilization. This answers part of the question of why enzymes are sometimes immobilized. The use of immobilized enzymes can lead to easier purification of the product. Another reason concerns the running and investment costs, which if lowered can provide the design of a more efficient process.

Immobilization is defined as 'physical confinement or localization of the enzyme molecule with retention of its catalytic activity, so that it can be used repeatedly or continuously'. The principal immobilization techniques (Figure 1) can be divided into the following groups:

1. ***Adsorption***

This immobilization procedure is very simple: an enzyme solution is added to the support, mixed, and then surplus enzyme is removed by washing [1-5].

2. ***Covalent binding***

Enzymes are usually immobilized through their amino or carboxylic groups. In most cases, the immobilization procedure is conducted in two steps: activation of the support and attachment of the enzyme [2, 6 -12].

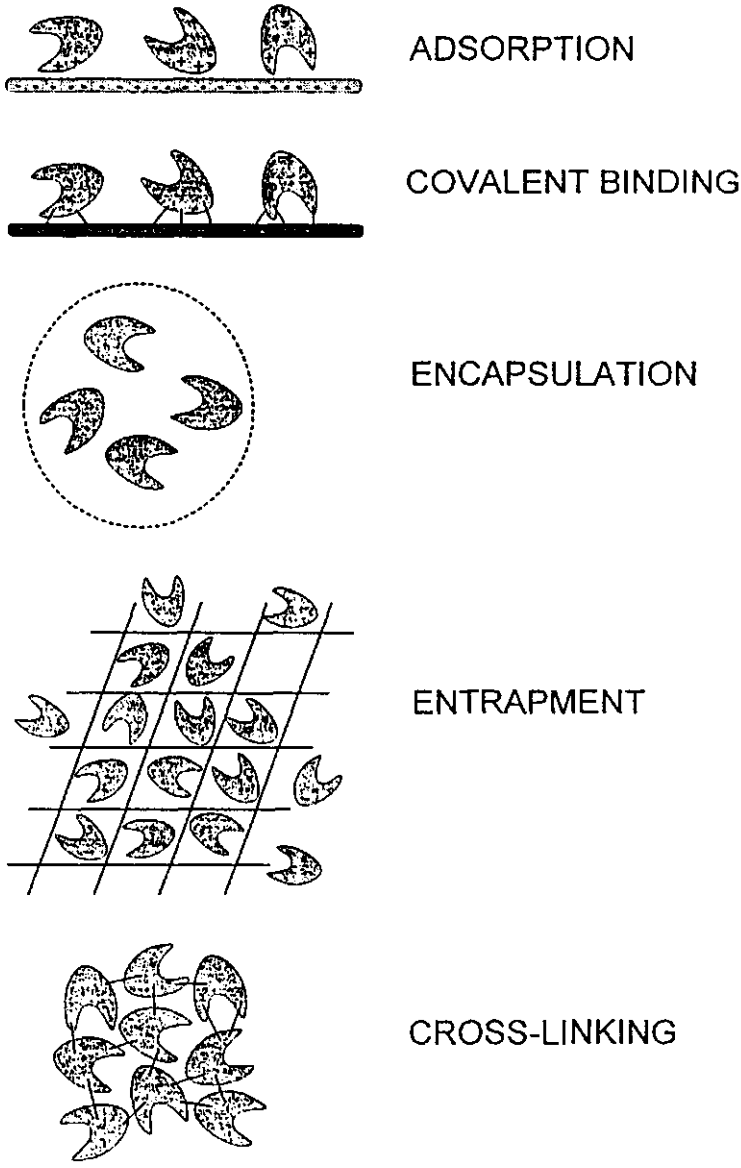

Figure 1. Principal methods of immobilization

3. *Encapsulation*

In this approach, enzymes are captured within various types of membranes that are impermeable to enzymes and other macromolecules but permeable to substrate and products of low molecular weight [16-18].

4. *Entrapment*

Here, the enzyme is added to a solution of synthetic monomers or natural polymers before the gel is formed. Gel formation is then initiated by changing the temperature, by adding a gel-inducing chemical or by using radiation of high or low energy [13-15].

5. *Crosslinking*

The enzymes crosslink through their amino groups or through their carboxylic groups using different crosslinkers [19].

II. Graft copolymers

The use of graft copolymers as carriers for enzyme immobilization has attracted much attention lately. Through this technique of support preparation the number of reactive groups can be considerably increased and controlled, and the microenvironment of the enzyme can also be altered. The unique value of using graft copolymers as carriers has been reported [20-22]. Figure 2 represents a schematic diagram of the grafting process.

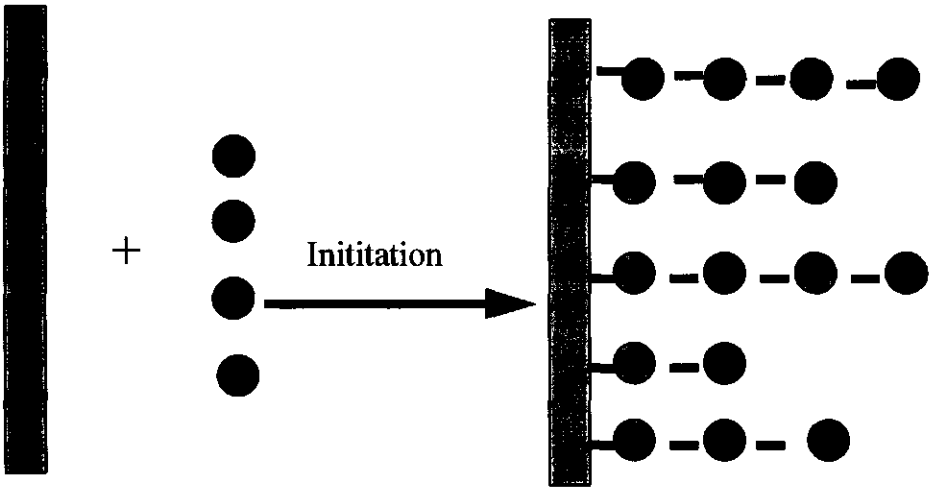

Figure 2. Schematic diagram of the grafting process

III. Non-isothermal bioreactors

A recent discovery [23-29] is the possibility of increasing the activity of membrane immobilized enzymes by operating under non-isothermal conditions. As a matter of fact, the activity of the catalytic membrane increases by 20 to 50% when a temperature difference of 1°C is applied across it. The increase in activity is dependent on the enzyme and immobilization methods used. Positive results have been obtained with purified enzymes as well as with immobilized cells. In the latter case the activity of both internal and membrane-wall enzymes was studied. A schematic diagram of the non-isothermal membrane bioreactor is shown in Figure 3.

IV. Outline of the thesis

This thesis is focused on the preparation of different catalytic membranes using two different hydrophobic materials, i.e. Teflon and nylon. β-Galactosidase was immobilized by entrapment and covalent binding onto grafted Teflon membranes, whereas penicillin G acylase was immobilized by covalent binding onto grafted nylon membranes and particles. The catalytic membranes prepared by the covalent immobilization method were tested in non-isothermal bioreactors. The flow diagram of the work is presented in Figure 4.

In Chapter 2 a literature review is presented covering different strategies to overcome diffusion limitation problems. It explaines how the design of the immobilization matrices and the controlling of the processing conditions can affect the diffusion process.

Chapter 3 shows the modification of the Teflon membrane. Here, γ-radiation was used for the grafting of acrylic acid monomer. In the second step 2-hydroxyethyl methacrylate-enzyme solution was grafted to the polyacrylic acid-grafted membranes. The effect of the grafting parameters on the activity of the immobilized β-galactosidase was investigated.

Characterization of this catalytic membrane from the physico-chemical point of view is presented in Chapter 4. For comparison, different catalytic membranes were prepared by grafting different monomers onto the Teflon membranes in the first grafting step, i.e. methacrylic acid and /or acrylamide. Testing the catalytic membranes in non-isothermal

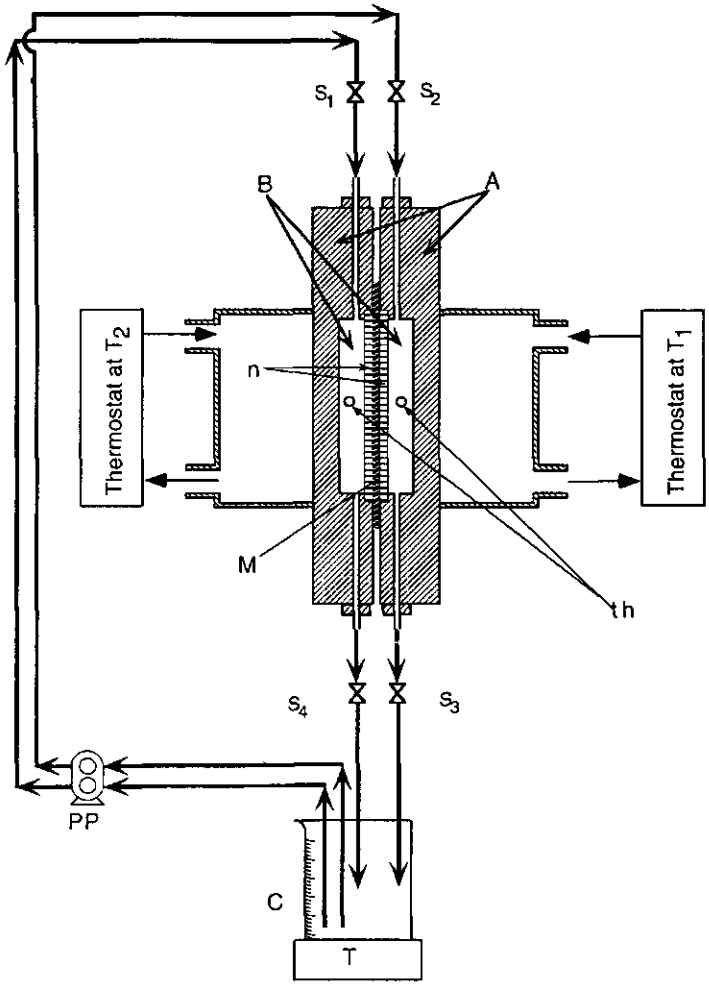

Figure 3. Schematic (not to scale) representation of the bioreactor.

(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.

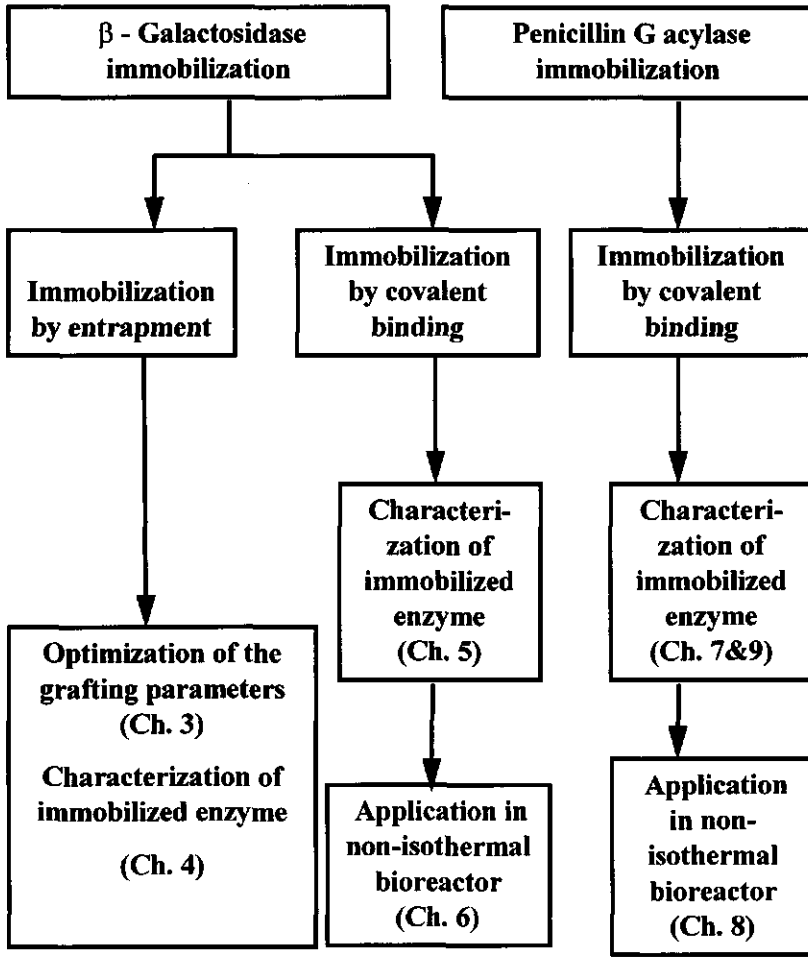

Figure 4. Flow diagram of the thesis.

bioreactors showed that all the immobilized enzyme had leaked from the membranes in the first experiment and no more activity was detected after that. This result led to the conclusion that the enzyme must be immobilized by covalent binding to the membrane to repeatedly serve the non-isothermal bioreactor. One of the membranes was first prepared without enzyme and then activated chemically by its -COOH groups to which the enzyme was immobilized later on. Both activity and permeability were tested under isothermal and non-isothermal conditions (Chapter 6). This membrane has two kinds of functional groups, i.e. -COOH and -OH. The effect on the properties and the kinetic parameters of immobilized enzymes was then investigated by immobilizing the enzyme either via the -COOH or via the -OH groups (Chapter 5).

In Chapter 7, a description is given of the immobilization of a different enzyme, penicillin G acylase, onto another hydrophobic membrane, i.e. nylon. First, two different monomers were grafted onto the membrane followed by covalent binding of the penicillin G acylase after activation of the membranes using a coupling agent. The properties of the immobilized enzyme were investigated. A noticable shift of the pH-optimum of the immobilized enzyme to the alkaline side was found. One of the catalytic membranes was applied in a non-isothermal bioreactor. Chapter 8 presents the effect of non-isothermal conditions on the activity of the immobilized enzyme.

All the polymeric supports (membranes) were grafted using γ-rays as initiation system for the grafting process. In Chapter 9 a different physical form of nylon particle was grafted using a chemical initiator (potasium persulphate). Penicillin G acylase was immobilized on the grafted nylon particles using the same coupling agent which bound the enzyme to the nylon membranes described in Chapter 7. The parameters of the particles activation and enzyme immobilization were studied.

Finally, a general discussion is presented in Chapter 10 on the potential of non-isothermal bioreactors and its applications in different areas.

REFERENCES

1. Messing, R.A. (1976). Adsorption and inorganic bridge formations. In: *Methods in Enzymology,* (ed.) K. Mosback, vol. **XLIV** , pp. 148-169. New York, Academic.

2. Woodward, J. (1985). Immobilized Enzymes: adsorption and covalent coupling. In: *Immobilized Cells and Enzymes: A Practical Approach,* (ed) J. Woodward, pp. 3-17. Oxford, IRL.

3. Goldstein, L. (1976). Kinetic behaviour of immobilized enzyme systems, In: *Methods in Enzymology,* (ed.) K. Mosback, vol. **XLIV**, pp. 397-443. New York, Academic.

4. Rudge, J. & G. F. Bickerstaff (1984). *Biochem. Soc. Trans.* **12:** 311-312.

5. Toher, J., A. M. Kelly & G. F. Bickerstaff (1990). *Biochem. Soc. Trans.* **18:** 313-314.

6. Porath, J. & R. Axen (1976). Immobilization of enzymes to agar, agarose and sephadex supports. In: *Methods in Enzymology,* (ed.) K. Mosback, vol. **XLIV**, pp. 19-45. New York, Academic.

7. Kabral, J. M. S. & J. F. Kennedy (1991). Covalent and coordination immobilization of proteins. In: *Protein Immobilization,* (ed.) R. F. Taylor, pp. 73-183. New York, Marcel Dekker.

8. Srere, P. A. & K. Uyeda (1976). Functional groupe on enzymes suitable for binding to matrices. In: *Methods in Enzymology,* (ed.) K. Mosback, vol. **XLIV**, pp. 11-19. New York, Academic.

9. White, C. A. & J. F. Kennedy (1980). *Enzyme Microb. Technol.* **2:** 82-90.

10. Taylor, R. F. (1991). Commercially available supports for protein immobilization. In: *Protein Immobilization,* (ed.) R. F. Taylor, pp. 139-160. New York, Marcel Dekker.

11. Gemeiner, P. (1992). Materials for enzyme engineering. In: *Enzyme Engineering,* (ed.,) P. Gemeiner, pp. 13-119. New York, Ellis Horwood.

12. Scouten, W. H. A (1987). survey of enzyme coupling techniques. In: *Methods in Enzymology,* (ed.) K. Mosbach, vol. **135** , pp. 11-19. New York,Academic.

13. O'Driscoll, K. F. (1976). Techniques of enzyme entrapment in gel. In: *Methods in Enzymology,*(ed.) K. Mosback, vol. **XLIV** , pp. 169-183. New York,Academic.

14. Brodelius, P. (1985). Immobilized plant cells. In: *Enzymes and immobilized cells in biotechnology,* (ed.) A. I. Laskin, pp. 109-148. London, Benjamin Cummings.

15. Bucke, C. (1983). Immobilized cells. *Phil. Trans. R. Soc.* **B 300:** 369-389.

16. Kierstan, M. P. J. & M. P. Coughlan (1991). Immobilization of proteins by noncovalent procedures: principles and applications. In: *Protein Immobilization,* (ed.) R. F. Taylor, pp. 13-71. New York, Marcel Dekker.

17. Nilsson, K. (1987). *Trends Biotechnol.* **5:** 73-78.

18. Groboillot, A., D. K. Boadi, D. Poncelot & R. J. Neufeld (1994). *Crit. Rev. Biotechnol.* **14:** 75-107.

19. Broun, G. B. (1976). Chemically aggregand enzymes. In: *Methods in Enzymology,* (ed.) K. Mosback, vol. **XLIV**, pp. 263-280. New York, Academic.

20. Beddows, C. G. & J. T. Guthrie (1982). *Biotechnol. Bioeng.* **24:** 1371.

21. Abdel-Hey, F. I. & C. G. Beddows (1983). *J. Polym. Sci. Polym. Chem. Ed.* **21:** 2463-2472.

22. Hsiue, G. H. & C. G. Wang (1990). *J. Appl. Polym. Sci.* **40:** 235.

23. Mita, D.G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69-81.

24. Mita, D.G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F.S. Gaeta (1995). *Biotechnol. Appl. Biochem.* **22:** 281-294.

25. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, F. Palumbo, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25-31.

26. Russo, P., A. Garofalo, U. Bencivenga, S. Rossi, D. Castagnolo, A. D'Acunto, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141-159.

27. Russo, P., De A. Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F.S. Gaeta & D.G. Mita (1997). *Research in Microbiology.* **148:** 271-281.

28. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D.G. Mita (1997), *J. Membrane Sci.* **129:** 175-184.

29. Febbraio, F., M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta & D.G. Mita (1998). *Biotechnol. Bioeng.* **59:** 108-115.

Chapter 2

IMMOBILIZED ENZYMES

STRATEGIES FOR OVERCOMING THE DIFFUSION-LIMITATION PROBLEM

Summary

One of the most serious drawbacks of immobilized enzymes can be diffusion limitation. A generic solution to this problem increases the range of applications for immobilized enzymes. Different strategies for overcoming this problem have been investigated. The advantages and disadvantages of each strategy are summarized and discussed.

This chapter has been submitted by the authors

M.S. Mohy Eldin, A.E. M.Janssen, D.G.Mita, and J. Tramper

Introduction

Immobilized enzymes using insoluble materials as supports offer several advantages over free enzymes, including easy recovery, the potential of continuous operation, simplified downstream processing, and sometimes enhanced stability. These techniques have found widespread applications in many industrial processes.

However, when the enzymes are used in an insoluble form, effective enzyme reaction can be hampered by solid-liquid heterogeneous reaction and diffusion limitation of substrate and/or product in carriers, especially when the product is an inhibitor of the enzyme reaction. Particularly in enzyme reactions using a solid substrate or solid product, it is impossible to use the insoluble enzyme. The different strategies that have been adapted to overcome the problem of an insoluble enzyme reaction system can be divided into the following groups:

1. ***Immobilization of the enzymes onto soluble-insoluble matrices***

These types of immobilized enzymes show a reversible soluble state and insoluble state. This means that the immobilized enzymes are in soluble form during the enzymatic reaction, and can be recovered in their insoluble form by changing the pH, ionic strength, temperature, and/or salt concentration of the reaction medium after completion of the reaction.

2. ***Immobilization of the enzymes in thermally reversible hydrogels***

Hydrogels exhibiting a lower critical solution temperature (LCST) shrink and deswell when warmed up to its LCST. Above the LCST the gel collapses. Reversibly, the gel expands and reswells when it is cooled below the LCST. The thermal cycling acts like a 'hydraulic pump' which enhances mass transfer of the substrate in and the product out of the gel, minimizing the diffusion limitation and product inhibition problems.

3. ***Immobilization of the enzymes in pressure-sensitive gels***

These gels swell or collapse sharply upon the rise or fall of environmental pressure. Under pressure cycling operation, the gel is analogous to a cylinder and a piston, such as in a 'micro-pump'. The piston pushes up and draws back, corresponding to the swelling and collapsing of the gel. In addition to the diffusive flow, a convective flow occurs in this way, which

enhances mass transfer within the gel and reduces diffusional limitation and product inhibition.

4. ***Processing the enzymatic reaction under non-isothermal conditions***

The reaction can be carried out under non-isothermal conditions in a membrane bioreactor. The temperature difference across the membrane causes thermal diffusion for both water and salts which is known as 'thermodialysis effect'. This effect brings the substrate in and the product out through the membrane, thus minimizing both the diffusion-limitation and the product-inhibition problems. The net result is enhancement of the reaction rate.

A discussion of these four strategies follows below accompanied by examples.

1. Immobilization of the enzymes onto soluble-insoluble matrices

pH-sensitive soluble-insoluble matrices

The great strength of enzymes immobilized onto soluble-insoluble carriers is apparent when dealing with soluble or poorly soluble substrate of high molecular weight. Here water-insoluble carriers suffer from poor contact between a high molecular weight or insoluble substrate and the immobilized enzyme. Furthermore separation of the immobilized enzyme from unreacted solid-substrate residues will be difficult. The hydrolysis of cellulose represents a good example of this problem. Taniguchi *et al.* [1] immobilized cellulase covalently to methacrylic acid/methylmethacrylate copolymer, which is reversibly soluble above pH 5.0 and insoluble below pH 3.9. The immobilized cellulase was repeatedly used to hydrolyze microcrystalline cellulose. Experiments confirmed that 100% of the immobilized enzyme activity can be recovered by precipitation and by dissolving it again by alternately changing the pH. Comparison of the specific activity of the immobilized cellulase for different substrates with that of native cellulase has revealed that 'immobilized' cellulase has very close values to the native cellulase, indicating the absence of diffusion limitation.

Margolin *et al.* [2] immobilized penicillin amidase on polyelectrolyte complexes. The enzyme was covalently attached to the polycation part of the complex by using cyanuric chloride as coupling agent. The enzyme-polyelectrolyte complex was found to revert to the insoluble state

either by a slight change in pH from 6.0 to 5.8 or by an increase of the ionic strength up to 0.3M NaCl. This concentration can be reduced to ≈ 0.01 M when using bivalent cations, because they effectively bind to carboxylate anions. The mechanism for transition from the soluble to the insoluble state and the reverse is shown in Figure 1.

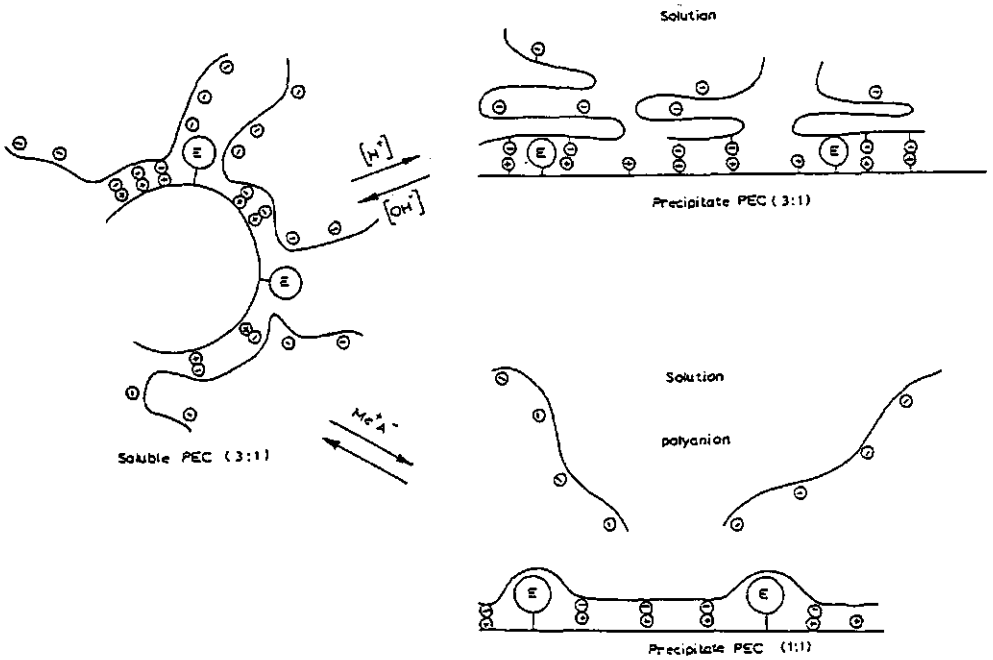

Figure 1. Influence of pH and ionic strength on the reversible transition between the soluble and insoluble forms of penicillin amidase immobilized in polyelectrolytes (adapted from reference 2).

The authors found that all activity is recovered during repeated precipitation and redissolution cycles. In comparison to the kinetic parameters of the native enzyme, the K_m value of the immobilized enzyme for benzyl penicillin was not increased showing the absence of diffusion limitation. Interestingly, the inhibition constant of the product, phenyl acetic acid, increased from $2*10^{-5}$ to $1*10^{-3}$ M. This result shows the remarkable effect of the negatively charged shell of the polymethacrylic acid which causes repulsion with the negative charges on phenyl acetic acid, removing it away from the enzyme active site.

A unique character of this enzyme derivative came from its high sensitivity to changes in ionic strength of the reaction medium. Since the reaction was carried out at a constant pH by adding KOH to neutralize the liberated phenyl acetic acid, the ionic strength increased in time. This leads to precipitation of the enzyme matrix thus stopping the reaction. By varying the initial ionic strength of the solution, the reaction can be stopped at any degree of conversion. Other examples of enzymes immobilized onto different soluble-insoluble pH sensitive supports are listed in Table 1 and supporting the conclusion of elimination of the diffusion-limitation problem.

Temperature-sensitive soluble-insoluble matrix

Hoshino *et al* [9] immobilized an amylase onto a novel thermo-responsive polymer. The polymer was prepared by copolymerization of methacrylic acid (MAA) and N-isopropyl acrylamide. The enzymes were covalently attached to the carboxylic groups of MAA by using soluble carbodiimide, producing a good solubility response of the immobilized enzyme to temperature soluble below 32^0C and insoluble above 42^0C. The response to temperature change was sharper in the presence of NaCl. The immobilized enzyme showed a high specific activity as compared to the free enzyme and superiority to other immobilized enzyme preparation especially when using uncooked starch as substrate. The K_m and the inhibition constant for glucose of the immobilized enzyme were found to be 50 and 65%, respectively, less than the values of the free enzyme showing no indications of the presence of diffusion limitation. Although after thirty batches of soluble starch hydrolysis the immobilized enzyme showed a stable specific activity with repeated soluble-insoluble cycles, 20% of its activity was lost. This decrease in activity was explained by the incomplete recovery of the immobilized enzyme after each reaction cycle and not to enzyme inactivation. Selection of the precipitation temperature of the support was done by copolymerization of the N-isopropyl acrylamide with a suitable hydrophilic or hydrophobic comonomer. Polymers with an adjustable precipitation temperature in the range of 25^oC - 53 ^{o}C were obtained [10-12], which is an added advantage for these kinds of supports. Table 2 represents different enzymes immobilized on different supports sensitive to temperature changes which confirm the

Table 1. Different enzymes immobilized on soluble-insoluble supports

Support name	Enzyme	Soluble pH	Insoluble pH	Reaction	Ref.
Methacrylic acid-methyl acrylate-methyl methacrylate (MPM-06)	Papain Chymotrypsin	> 5.8	< 4.8	Hydrolysis Synthesis	5
Methacrylic acid-methyl methacrylate (Eudragit L100)	Cellulase	> 5.0	< 3.7	Hydrolysis	1
Hydroxy propyl methyl cellulose acetate succinate (AS-L)	Cellulase	> 5.0	< 3.8	Hydrolysis	6
Hydroxy propyl methyl cellulose acetate succinate (AS-L)	Chitinase	> 5.2	< 4.5	Hydrolysis	7
Hydroxy propyl methyl cellulose acetate succinate (AS-L)	Lysozyme	> 6.2	< 4.6	Hydrolysis	8
Polyelectrolyte complex of poly(4-vinyl-N-ethylpyridinium bromide) and poly(methacrylic acid)	Penicillin amidase	> 6.0 < 0.2 M NaCl	< 5.8 > 0.2 - 0.4 M NaCl	Hydrolysis	2
Polyelectrolyte complex of poly(4-vinyl-N-ethylpyridinium bromide) and poly(methacrylic acid)	α-Chymotrypsin Urase	> 6.1	< 5.7	Hydrolysis	4
Polyelectrolyte complex of poly(4-vinyl-N-ethylpyridinium bromide) and poly(methacrylic acid)	Alcohol dehydrogenase	> 5.9	< 5.7	Hydrolysis	3

positive effect of using such supports on the elimination of the diffusion-limitation problem.

Table 2. Some thermally sensitive supports for enzyme immobilization

Support name	Enzyme	Soluble temp.	Insoluble temp.	Reference
N-isopropyl acrylamide-co-methacrylic acid	Amylase	< 32 ^{0}C	> 42 ^{0}C	9
N-isopropyl acrylamide-co-N-acryloxysuccinimide	Amylase	< 34 ^{0}C	> 34.7 ^{0}C	13
N-isopropyl acrylamide-co-glycidyl methacrylate	Alkaline phosphatase	< 34 ^{0}C	> 34 ^{0}C	14
N-isopropyl acrylamide-co-glycidyl methacrylate	Amylase	< 32 ^{0}C	> 44 ^{0}C	15
N-isopropyl acrylamide-co-2-hydroxyethyl methacylate	Trypsin	< 32 ^{0}C	> 34 ^{0}C	16

Salt- sensitive soluble-insoluble matrices

One of the main components of bovine milk protein, α_{s1}- casein, precipitates as a calcium caseinate in the presence of an appropriate concentration of calcium ions. The precipitated casein can be reversibly solubilized by trapping the calcium ions with a chelating agent such as EDTA. Therefore, it is worth attempting to use this property of α_{s1}- casein for the construction of soluble-insoluble interconvertible enzymes. Chiba and his collaborators immobilized different proteins on casein to derive soluble-insoluble protein preparations [17-18]. They first prepared an enzyme-α_{s1}-casein conjugate using a heterobifunctional crosslinking reagent. However, the enzyme-α_{s1}-casein conjugate did not show sufficient calcium-dependent precipitation. Modification of the method of immobilization by executing the polymerization of the enzymes-casein conjugate by transglutaminase enhanced the enzyme-preparation, response to the $CaCl_2$ concentration. Almost complete precipitation was

obtained in the presence of over 50 mM $CaCl_2$. Comparison of the activity of the immobilized enzymes with that of the free ones revealed that the overall reactions in both catalytic systems proceeded at exactly the same rate. This result indicates that the enzymes can act without diffusion limitations. Phosphoglyceromutase, enolase, and peroxidase were used in this study.

2. Immobilization of the enzymes in thermally reversible hydrogels

Thermally reversible hydrogels exhibiting a lower critical solution temperature (LCST) deswell and collapse when warmed up to and over its LCST. Reversibly, the gel expands and reswells when it is cooled below the LCST. A schematic diagram of the process is shown in Figure 2. The thermal cycling acts like a 'hydraulic pump' which enhances mass transfer of the substrate into and the product out of the gel, thereby increasing the conversion dramatically relative to isothermal operation at either the upper or lower temperature. The increased conversion can also be the result of reduced product inhibition. Park *et al.* [19] entrapped β-galactosidase in a thermally reversible hydrogel. This hydrogel was prepared from copolymers of N-isopropyl acrylamide (NISAAm) and acrylamide (AAm) crosslinked by N, N` methylene bis-acrylamide. The enzyme was entrapped in the copolymer beads formed during inverse suspension polymerization. In Figure 3 a comparison is given of the conversion of O-nitrophenol β-D-galactopyranoside (ONPG) by the immobilized β-galactosidase operated under thermal cycling between 30 and 35 ^{0}C and isothermal conversion at 30 and 35 ^{0}C. The figure clearly shows that the conversion increased by about 60% due to the effect of the temperature cycling. Other enzymes and cells entrapped in the same matrix are presented in Table 3 showing the same behavior with temperature cycling.

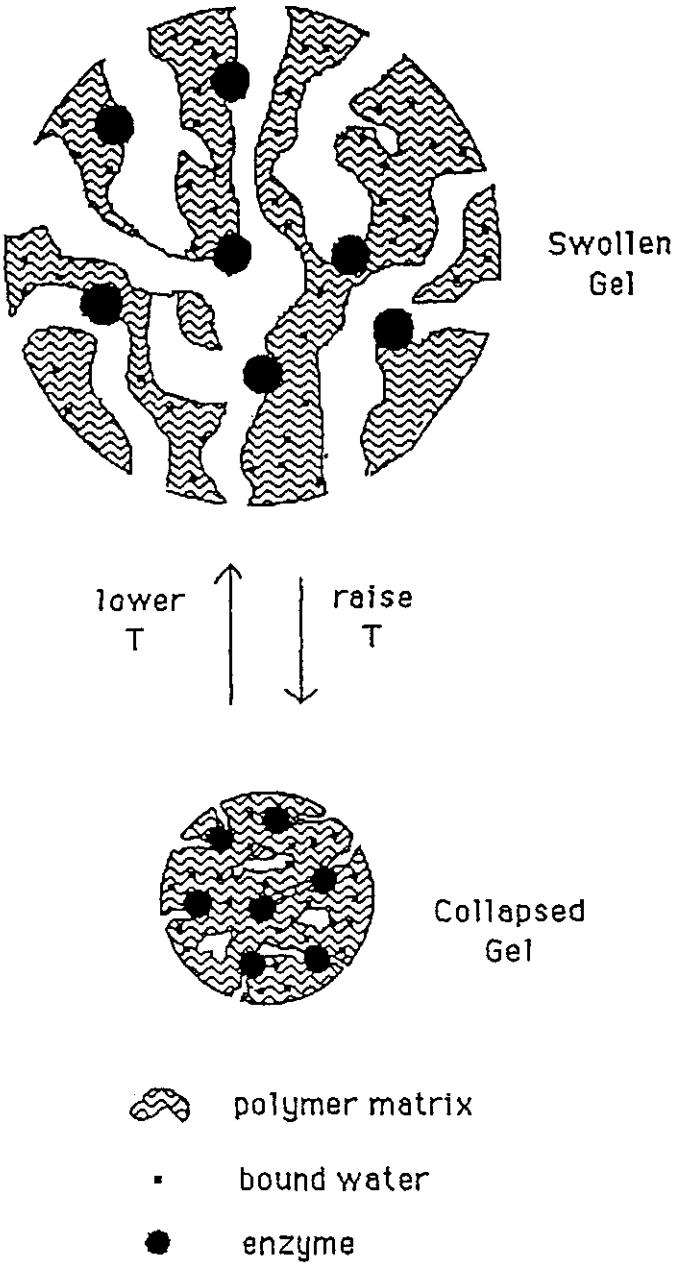

Figure 2. Schematic diagram of the water and pore structure in a swollen and collapsed thermally reversible (LCST) hydrogel containing an immobilized enzyme (adapted from reference 19).

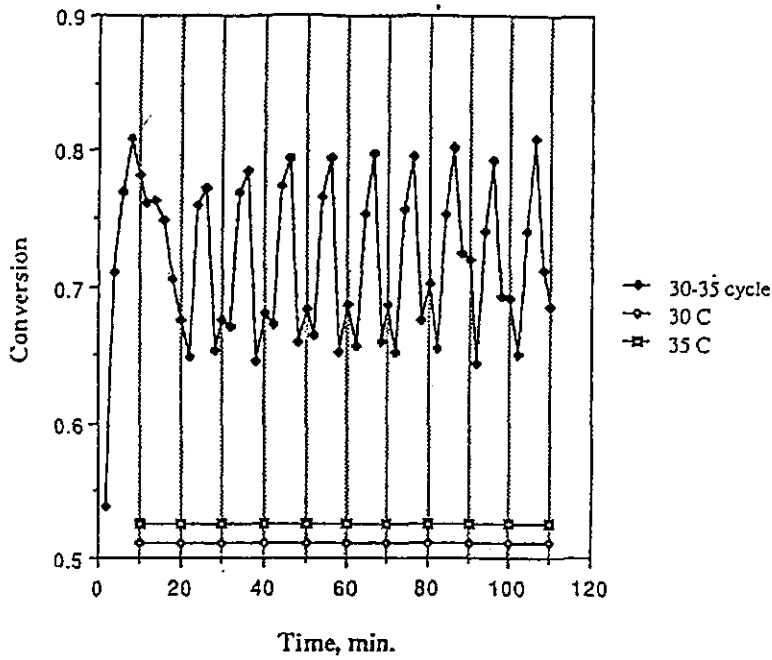

Figure 3. Conversion of O-nitrophenol β-D-galactopyranoside by β-galactosidase as a function of time in the packed bed reactor operated isothermally at 30 and 35 °C or cycled between 30 and 35 °C. Conversion is the ratio of outlet product (molar) concentration to inlet substrate (molar) concentration (adapted from reference 19).

Table 3. Biocatalysts entrapped in N-isopropyl acrylamide-co-acrylamide thermally reversible hydrogel.

Enzyme or cells	Reaction	Swell temp. ^{0}C	Deswell temp. ^{0}C	Reference
β-galactosidase	Hydrolysis of ONPG	< 32	> 33	19
Conjugate asparaginase	Hydrolysis of Aspargine	< 30-40	> 35-45	20
Arthrobacter simplex cells	Dehydrogenation of steroid	< 27	> 32	21-22

3. Immobilization of the enzymes in pressure sensitive gels

Wang *et al.* [23] entrapped β-galactosidase by inverse suspension polymerization in poly (N-isopropyl acrylamide) (NIPA) which is a pressure sensitive gel. In a normal reaction system under isobaric operation, the gel keeps the enzyme from leaking out and allows substrate and product to enter and exit by diffusion (Figure 4). A noticeable increase in the conversion rate was achieved by the pressure-cycling operation (Figure 4 & 5). The improvement in conversion rate ranged between 27 and 58%. The authors proved that pressure cycling and not the high pressure was responsible for the conversion-rate improvement, since pressure was found not to have effect on the activity of the free enzyme up to $120*10^5$ Pa. They optimized the operational conditions and found that increasing the pressure-cycling amplitude (pressure difference), increased the conversion, whereas the pressure-cycling range had no effect. The pressure- cycling period (time necessary for completion of one cycle) also strongly affected the conversion increment. The increase in the conversion could be explained by the enhancement of mass transfer inside the gel beads during the pressure-cycling operation in which the gel swelled or shrinked sharply upon the fall or rise of the operational pressure, respectively.

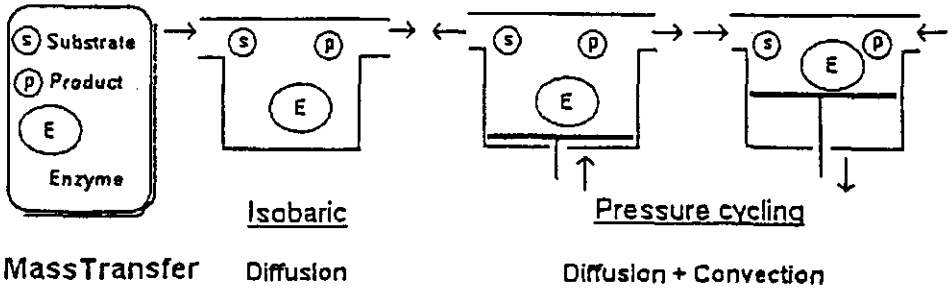

Figure 4. The micro-pump characteristics of pressure-cycling operation as compared to isobaric operation (adapted from reference 23)

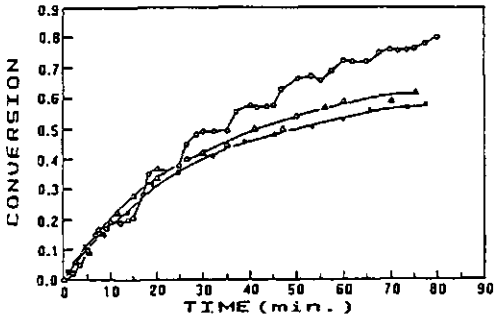

Figure 5. Conversion of O-nitrophenol β-D-galactopyranoside by immobilized β-galactosidase pressure-cycling and isobaric operations. (■) = 1 x 10^5 Pa, (Δ) = 60 x 10^5 Pa, (o) = 1 x 10^5 - 60 x 10^5 Pa (cycle) (adapted from reference 23).

4. Processing the enzymatic reactions under non-isothermal conditions

Recently, Mita and his collaborators discovered that the activity of immobilized enzymes increased significantly when the biocatalytic reaction proceeded under non-isothermal conditions in a membrane reactor. Their biocatalytic system normally consists of two components: an immobilization matrix in which the biocatalyst is entrapped or covalently attached to and an hydrophobic porous membrane to insure complete hydraulic separation between cold and warm substrate solutions. The membrane usually faces the cold side. The apparatus used for the experiments is shown in Figure 6.

In the first publication about this effect, the authors investigated the behavior of the invertase catalytic activity [24]. The enzyme was entrapped in a crosslinked gelatin membrane. A Teflon 200 membrane was used as hydrophobic membrane to induce the thermodialysis effect. They found that the activity under non-isothermal conditions was higher than under isothermal conditions (Figure 7). The temperature of the system under the non-isothermal conditions was taken as average temperature, assuming a uniform enzyme distribution and a linear temperature change over the membrane thickness.

The percentage of activity increment under non-isothermal conditions increased with the increase in temperature difference across the membrane. The percentage of increment ranged between 100 and 300 %, being proportional to temperature differences between 10 and 30°C, respectively.

To have real benefits from applying this technique, the activity of the system under non-isothermal conditions at a specific average temperature must be higher than the activity of the system in isothermal conditions at the higher temperature, i.e. the temperature of the warm side in the non-isothermal experiment. For example, the activity measured at average temperature (T_{av}) = 30°C with applied temperatures of 20°C and 40°C for the cold side and warm side, respectively ($A_{20:40}$), should be higher than the activity of the system at 40°C under isothermal conditions ($A_{40:40}$). Mita *et al* showed that this is indeed the case. β-Galactosidase immobilized by the same method showed the same behavior, but with a lower percentage of activity increment [25].

Covalently immobilizing the enzymes to a hydrophilic nylon membrane did not change the behavior of the enzyme activity compared to the entrapped enzyme [25-28]. The authors compared the kinetic parameters of β-galactosidase, immobilized covalently onto the nylon membrane, under isothermal and non-isothermal conditions. The results show that with lactose substrate the maximum activity under non-isothermal conditions is higher than under isothermal conditions. Noteworthy is the decrease of the K_m value when the reaction proceeded under non-isothermal conditions, suggesting a reduction of diffusion limitation.

Mita *et al* explained the effect of the non-isothermal conditions on the activity by two theories. First, the higher rate of product removal and substrate enrichment as a result of thermodialysis and second, the conformational changes of the enzyme structure. They believe that both effects occur as a result of the flux of thermal energy. To confirm the effect of temperature difference on the substrate enrichment and product removal, they measured the fluxes of substrate and products under non-isothermal conditions with an enzyme-free membrane system. What they found was that the flux of the products transported away from the membrane was higher than the flux of the substrate available. This indeed may be decisive for the increase of the apparent reaction rate of the enzyme.

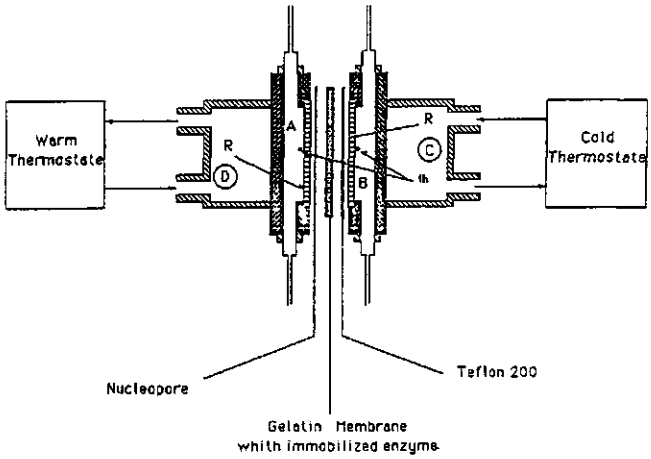

Figure 6. Schematic representation (not to scale) of a thermodialysis experimental unit: (A) warm working volume, (B) cold working volume, (C and D) thermostating jackets; (R) supporting nets, (th) thermocouples (adapted from reference 24).

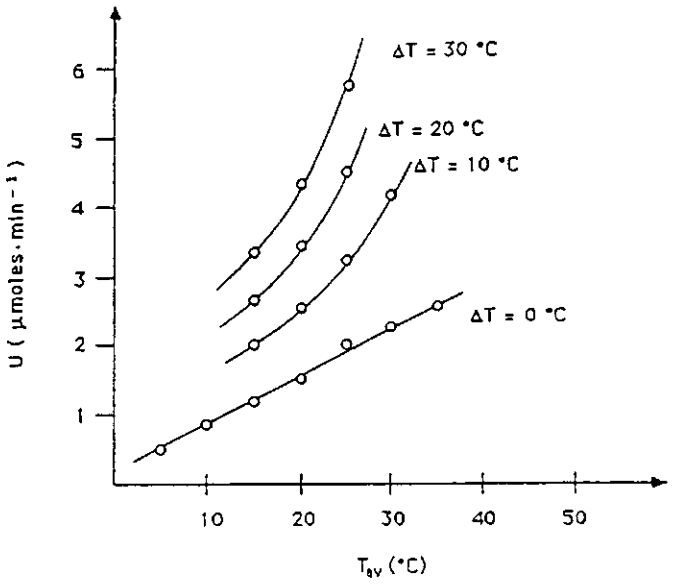

Figure 7. Isothermal and non-isothermal activity as a function of average temperature (adapted from reference 24).

A simple and interesting experiment was designed to make the case more clear. The cold half-cell of the apparatus was filled with a buffer solution containing lactose at a concentration of 300mM, and the warm half-cell was filled with a substrate-free buffer solution. In this way the immobilized enzyme was able to interact with the substrate by diffusion through the nylon membrane when the system was isothermal, or by thermal diffusion and thermodialysis when the system was non-isothermal. The results for the isothermal and non-isothermal conditions are shown in figure 8a and 8b, respectively. Comparison of the two figures shows that the substitution of purely diffusive for thermodiffusive transport increases the apparent velocity of the enzyme reaction by a full order of magnitude. More recently, the authors tried, by grafting technique, to simplify the biocatalytic membrane system by using a membrane that was both hydrophobic and catalytic. Table 4 presents the different membranes used, i.e. Teflon and nylon [24-26], with the maximum activity increment obtained.

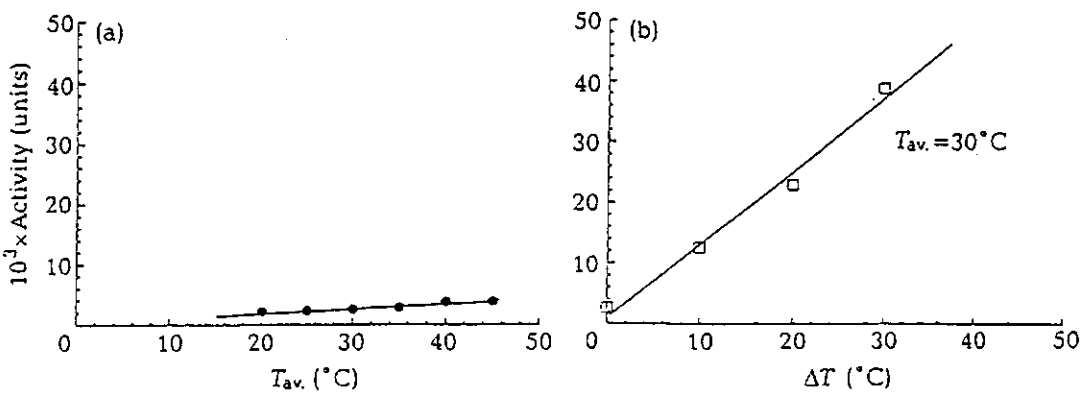

Figure 8. Apparent activity of the system β-galactosidase-nylon as a function of the average temperature, under the effect of substrate transport by diffusion (a) or by thermodialysis (b) (adapted from reference 25).

Table 4. Percentage activity increment of different enzymes in non-isothermal bioreactors.

Grafted support name	Enzyme	Grafting technique	Maximum activity increment %	Reference
Teflon-grafted methylmethacrylate	β-galactosidase	γ-radiation	47	27
[Teflon-grafted methacrylic acid]-grafted 2-hydroxy ethyl methacrylate	β-galactosidase	γ-radiation	70	28
Nylon-grafted methyl methacrylate	penicillin G acylase	γ-radiation	100	29

Concluding remarks

Different strategies for overcoming diffusion limitation in immobilized-enzyme systems have been devised. In the case of a high molecular weight, soluble or poorly soluble substrate, the solution was to immobilize the enzymes onto a support that has the ability to remain soluble under the reaction conditions and to precipitate by changing the pH, ionic strength, temperature, and/or salt concentration.

However, not all the supports are suitable for application on an industrial scale. For example, casein and polyelectrolyte complexes precipitate in the presence of calcium and /or monovalent cation such as sodium or potassium ions, which usually are part of enzymatic reaction mixture, limiting the use. Enzymes immobilized on N-isopropyl acrylamide polymer or its copolymers lost their activity due to incomplete recovery of all enzyme matrices when repeatedly going through precipitation and dissolution as result of temperature cycling. Also, the temperature-change cycles affected the stability of the immobilized enzyme.

More or less the same can be said about the polymers which are sensitive to pH changes of the solution. Furthermore, with enzyme soluble-insoluble carriers, there is the problem of product stability under conditions of insolubilization. In addition, most of these carriers need centrifugation to separate them from the reaction medium, which means additional energy costs. Indeed, some carriers have the properties of autoprecipitation, but what remains is the problem of incomplete recovery of the carriers. For instance, 80% of the carrier could be recovered after autoprecipitation [6].

Other solutions have been found for soluble substrates apart from enzymes immobilization onto soluble-insoluble carriers. Those solutions depend on finding ways of facilitating removal of the products from and the supply of the substrate into the carriers. This goal has been achieved by three techniques:

1- immobilization of the enzymes on thermal-sensitive hydrogels;

2- immobilization of the enzymes on pressure-sensitive hydrogels; and

3- carrying out the enzymatic reaction under non-isothermal conditions.

The main disadvantages of the first two techniques are the additional cost of cycling the temperature and the pressure of the temperature and pressure-sensitive hydrogel, respectively. The main disadvantage of non-isothermal bioreactors is the problem of keeping the temperature difference constant when using large volume bioreactors. The balance between the additional costs and the obtained benefits from applying those techniques is obviously the decisive factor.

Among the different techniques that have been found to overcome the diffusion limitation problem not one of them is suitable for all enzymes. It is essential, therefore, that a careful choice of strategy is made for each enzyme.

References

1. Taniguchi, M., M. Kobayashi & M. Fujii (1989). *Biotechnol. Bioeng.* **34:** 1092-1097.

2. Margolin, A.L., V.A. Izumrudov, V.K. Svedas, A.B. Zezin, V.A. Kabanov & I.V. Berezin (1981). *Biochimica et Biophysica Acta.* **660:** 359-365

3. Margolin, A.L., V.A. Izumrudov, V.K. Svedas & A.B. Zezin (1982). *Biotechnol. Bioeng.* **24:** 237-240

4. Margolin, A.L., S. F. Sherstyuk, V.A. Izumrudov, A.B. Zezin & V.A. Kabanov (1985). *Eur. J. Biochem.* **146:** 625-632

5. Fujimura, M., T. Mori & T. Tosa (1987). *Biotechnol. Bioeng.* **29:** 747-752

6. Taniguchi, M., K. Hoshino, K. Watanabe, K. Sugai & M. Fujii (1992). *Biotechnol. Bioeng.* **39:** 287-292

7. Chen, J-P. & K-C. Chang (1994). *J. Chem. Tech. Biotechnol.* **60:** 133-140

8. Chen, J-P. & Y-C. Chen (1996). *Biotechnol. Tech.* **10:** 749-754

9. Hoshino, K., M. Taniguchi, T. Sasakura, M. Katagiri & M. Fujii (1994). *J. Ferm. Bioeng.* **77:** 407-412

10. Steinke, K. & K. D. Vorlop (1987). *proc. 4th European Congress on Biotechnology*, vol **2**, pp. 185-188

11. Vorlop, K. D. & K. Steinke (1988). *Chemi. Ing. Tech.* **60:** 790-791

12. Auditore-Hargreaves, K., R. L. Houghton, N. Monji, J. H. Priest, A. S. Hoffman & R. C. Noviski (1987). *Clin. Chem.* **33:** 1509-1516

13. Chen, J-P., Chu, D-H. & Y-M. Sun (1997). *J. Chem. Tech. Biotechnol.* **69:** 421-428

14. Nguyen, A.L. & J. H.T. Loung (1989). *Biotechnol.Bioeng.* **34:** 1186-1190

15. Hoshino, K., M. Taniguchi, M. Katagiri & M. Fujii (1992). *J. Chem. Eng.* **25:** 569-574

16. Steinke, K. & K. D. Vorlop (1990). *DECHEMA Biotechnol. Conf.* **4:** 889-892

17. Yoshikawa, M., M. Goto, K. Ikura, R Sasaki & H. Chiba (1982). *Agri. Biol. Chem.* **46:** 207-213

18. Okumura, K., K. Ikura, M. Yoshikawa, R. Sasaki & H. Chiba (1984). *Agri. Biol. Chem.* **48:** 2435-2440

19. Park, T.G. & A.S. Hoffman (1988). *Appl. Biochem. Biotechnol.* **19:** 1-9

20. Dong, L.C. & A.S. Hoffman (1986). *J.Contr. Rel.* **4:** 223-227

21. Park, T.G. & A.S. Hoffman (1990). *Biotechnol.Bioeng.* **35:** 152-159

22. Park, T.G. & A.S. Hoffman (1989). *Biotechnol. Lett.* **11:** 17-22

23. Wang, Y., X. Zhong & S. Wang (1996). *J. Chem. Tech. Biotechnol.* **67:** 243-247

24. Mita, D. G., M. A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F. S. Gaeta (1993). *J. Membrane Sci.* **78:** 69-81

25. Mita, D. G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F. S. Gaeta (1995). *Biotechnol. Applied Biochem.* **22:** 281-294

26. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, P. Canciglia, F. Palumbo, F. S. Gaeta & D. G.Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25-31

27. Mohy Eldin, M.S., A. De Maio, S. De Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Membrane Sci.* **146:** 237-248

28. Mohy Eldin, M.S., A. De Maio, S. Di Martino, N. Diano, V. Grano, N. Pagliuca, S. Rossi, U. Bencivenga, P. Canciglia, F. S. Gaeta & D.G. Mita (1999). Isothermal and non-isothermal lactose hydrolysis by β-Galactosidase immobilized on double-grafted membrane. Sumitted

29. Mohy Eldin, M.S., M. Santucci, S. Rossi, U. Bencivenga, P. Canciglia, F. S. Gaeta & D.G. Mita (1999). Non-isothermal caphalexin hydrolysis by penicillin G acylase immobilized on grafted nylon membranes. *J. Molecular Cat. B.* In Press.

β-GALACTOSIDASE IMMOBILIZATION ON PRE-MODIFIED TEFLON MEMBRANES USING γ-RADIATION GRAFTING

ABSTRACT

Double grafting by γ-radiation was used to immobilize the β-galactosidase enzyme and monomers of 2-hydroxyethyl-methacrylate (HEMA) on Teflon (PTFE) membrane previously grafted with acrylic acid (AA) monomers. This technique was found to improve the catalytic activity of the membrane.

Membrane activity was studied as a function of some of the most relevant parameters affecting the grafting degree and the amount of enzyme used for immobilization. Experimental conditions producing the best membrane activity were characterized.

The advantages of using Teflon catalytic membranes in non-isothermal bioreactors, which are more efficient than the analogous isothermal bioreactors, are also discussed.

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INTRODUCTION

Polymer membranes are widely used as enzyme carriers. In many cases modifications have been introduced on the polymeric matrices to increase their catalytic activity. Graft copolymerization is a well-known method for introducing such modifications [1], the grafting being carried out chemically [2-4] or by irradiation [5-18].

When the grafted membranes are biocatalytic they are generally employed in the construction of biosensors. Other practical applications of grafted membranes are the immobilization of: drugs for controlled release and targeting; antigens and antibodies for immunodiagnostics; and micro-organisms for biomass conversion. All these applications are performed under isothermal conditions.

Grafted membranes free of biocatalysts have been employed in water desalination [8] and in azeotropic mixtures separation by pressure gradients under isothermal conditions [17-18] or by temperature gradients [19-20].

We recently focused our research activity on the employment of biocatalytic membranes in non-isothermal reactors where the presence of temperature gradients across membranes favourably affects the rate of the enzyme reaction [21-26]. It is proven that the bioreactor efficiency increases with increasing temperature gradient applied across the catalytic membrane. In all cases the system used was a biocatalytic membrane coupled with a Teflon (PTFE) membrane. The role of the hydrophobic Teflon membrane is to induce mass transport (including substrate and products) when it is interposed between two liquid solutions of equal or different composition, kept at different temperatures. This non-isothermal process of matter transport is known as thermodialysis [27-31].

The aim of this work was to immobilize an enzyme directly onto a suitably grafted Teflon membrane to simplify the system used in the non-isothermal bioreactor, making it easier to study the physical causes that affect the enzyme reaction in the presence of temperature gradients. β-Galactosidase was chosen as the enzyme model, also in view of its application in lactose hydrolysis in milk and whey to render these foods suitable for persons with lactose intolerance.

MATERIALS AND METHODS

Materials

Polytetrafluoroethylene (PTFE) membranes of the type TF-450, manufactured by the Gelman Instrument Company (Ann Arbor, Michigan 48106 USA), have been used as a solid support for grafting purposes. These membranes, consisting of a Teflon film supported by a polypropylene net, are 150 μm thick and are endowed with anastomizing pores of 450 μm in diameter.

The monomers used for the grafting were 2-hydroxyethyl-methacrylate (HEMA) and acrylic acid (AA). Ferrous ammonium sulphate (FAS) was used as inhibitor for the formation of AA homopolymers, since the radiation grafting is performed without oxygen using the mutual technique.

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 GmbH, D-68298 Mannheim, Germany) and expressed as $\mu moles \cdot min^{-1}$.

All chemical products, including the enzyme, were purchased from Sigma Chemical Company (St. Louis, Missouri 63178, USA) and used without further purification.

Methods

Catalytic membrane preparation

Membrane grafting took place by irradiation with γ-rays. The irradiation source was caesium 137 in a gamma cell 1000 Elite from Nordion International Inc., Canada. The average dose rate in the core of the radiation chamber (central dose rate) was 2.35×10^4 rad/hour. Since the direct grafting of HEMA in the presence of enzyme solution has produced membranes endowed with low catalytic activity we used a 'double grafting technique', which allowed us to obtain more active membranes with the same initial concentration of enzyme.

First grafting: modification of PTFE membrane by AA grafting

The first grafting was done by irradiation of the PTFE membranes in the gamma cell using a solution of acrylic acid monomers and FAS, thus obtaining a PTFE-AA membrane. The experimental conditions used are specified according to the following scheme: a = % AA concentration (v/v); b = % FAS concentration (w/v); c = t_1 = irradiation time used during the first grafting (h).

Second grafting: immobilization of β-galactosidase by HEMA grafting

The second grafting was performed by irradiation the previously grafted membranes in a solution of HEMA and enzyme. The experimental conditions used are specified according to the following scheme: d = % HEMA concentration (v/v); e = enzyme concentration (mg/ml); f = t_2 = irradiation time during the second grafting (h).

Determination of the grafting percentage

We adopted the classical definition used for the percentage of grafting. The degree of grafting (X, %) was determined by the difference between membrane masses before, G_B, and after, G_A, the grafting done according to the expression:

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

In the case of the AA grafting percentage G_B is the membrane mass before the first irradiation and G_A is the mass of the dry membrane after the irradiation. Similarly, in the case of the HEMA grafting percentage G_B is the dry membrane mass before the second irradiation and G_A is the mass of the dry membrane after the second irradiation.

Determination of the activity of the catalytic membrane

To determine the membrane activity the catalytic membranes were put in 35 ml of a well-stirred 200 mM lactose 0.1 M buffer phosphate solution of pH 6.5 maintained at 40°C. The

glucose production was followed in the course of time. Membrane activity is given by the angular coefficient of the linear plot of the glucose production as a function of time.

Determination of the time stability of the catalytic membrane

The time stability of the biocatalytic membranes was assessed by analyzing their activity daily under the same experimental conditions. After three days during which the membranes lost some activity, a stable condition was reached remaining unchanged for over two months. Only these stabilized membranes were used in the comparative experiments reported in the following. When not being used the membranes were stored at 4°C in 0.1 M buffer phosphate pH 6.5.

Treatment of the experimental data

Each experimental point reported in the figures represents the average of three experiments performed under the same conditions. The experimental errors never exceeded 6 %. For each of the three experiments the procedures in the various steps were performed according to the following methodology. Twenty-four Teflon membrane disks 2.5 cm in diameter were weighed and put in the solution for the first grafting. After irradiation the membrane disks were repeatedly washed under vigorous stirring in abundant double-distilled water to remove the adherent homopolymers. Then eight disks were taken for determining the AA grafting degree and the remainder used for the second grafting. At the end of this operation the disks were washed using 0.1 M buffer phosphate solution pH. 6.5, then separated into two groups of eight membranes each for determining the HEMA grafting degree and catalytic activity, respectively.

RESULTS AND DISCUSSION

Because the activity of the biocatalytic membranes was affected by the irradiation time during the first and the second grafting and the concentrations of enzyme, HEMA, FAS and AA, we studied the glucose production independence of each of these parameters.

Dependence on irradiation time during the first grafting

To optimize the performance and to evaluate the cost/benefit of the grafted membranes, the dependence on the irradiation time of their enzyme activity must be known. To this aim we performed the first grafting immersing the membrane disks in an aqueous solution of 15 % acrylic acid (v/v) and 2.5% FAS (w/v), putting the container in the γ-cell for the desired times. After washing the disks and following the previously described procedure, a new grafting was carried out for 16 hours with a solution of 10 % of HEMA (v/v) in 0.1 M phosphate buffer at pH 6.5 and 0.5 $mg \cdot ml^{-1}$ enzyme concentration. The results obtained, reported in Table I, indicate that the AA grafting degree was not dependent on the irradiation time. Since the catalytic activity of the membrane was practically constant we can conclude that the amount of the immobilized enzyme depends on the AA grafting degree, which controls also the second grafting. For irradiation times less than 6 hours during the first grafting we found scarcely reproducible smaller values of acrylic acid grafting.

Dependence on the enzyme concentration

Study of the dependence of the catalytic membrane activity on the enzyme concentration that revealed that six hours of irradiation time during the first grafting are sufficient to obtain the maximum and constant value of the AA grafting degree. The results of the series of experiments with varying enzyme concentration are reported in Table II, which shows how the glucose production increases with the amount of the enzyme used in the second grafting. When the membrane activity is reported in the graphical form of Figure 1 we obtain a linear dependence of the activity on the enzyme concentration.

Table I. Effect of irradiation time during the first grafting.

Irradiation time used during the 1st grafting (h)	AA grafting degree (%)	Irradiation time used during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}} \times 10^3$
6	20	116	51	7.5
12	22	116	49	8.2
18	21	116	48	7.0

We conducted another series of experiments identical to the ones just reported, but changing only the FAS concentration to 0.1 %. The results of this experiment are reported in Table III and Figure 1, which shows that the activities of the membranes prepared using 0.1% FAS are surprisingly higher than those prepared using 2.5% FAS. This suggests that at 2.5 % FAS concentration inactivation of the enzyme occures, which might be due to protein-protein interaction.

To optimize the performance of the biocatalytic membrane, having ascertained that 0.1 % FAS concentration gives better results, we have tested the dependence on the first irradiation time in the last mentioned conditions. We conducted a series of experiments at 45 mg/ml enzyme concentration and at different irradiation times, all other parameters being the same as those which led to the results reported in Figure 1. The new results are reported in graphical form in Figure 2, which shows how both the AA grafting degree and membrane activity linearly increase with the duration of the first irradiation time.

Table II. Effect of enzyme concentration used for preparing the catalytic membrane at fixed 2.5% FAS concentration.

Enzyme concentration (mg/ml)	Irradiation time used during the 1st grafting (h)	AA grafting degree (%)	Irradiation time used during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}} \times 10^2$
5	6	18.2	16	30.2	8.3
10	6	19.4	16	25.0	16.6
15	6	17.0	16	27.3	23.0
20	6	18.0	16	28.4	31.6
25	6	21.5	16	31.1	37.2
30	6	19.0	16	22.5	44.4
35	6	18.5	16	30.6	53.4
45	6	18.1	16	27.2	68.3

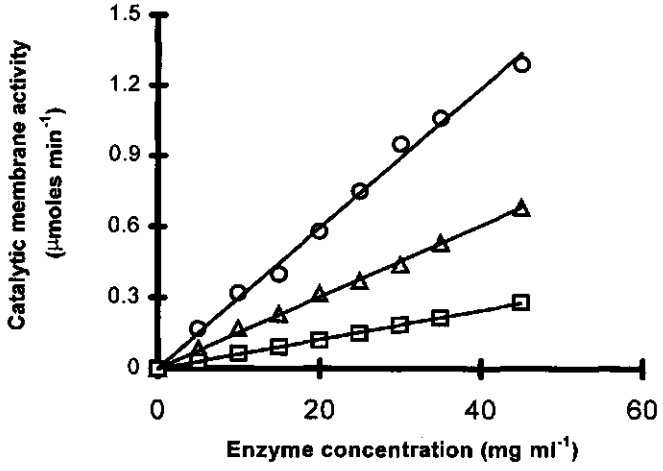

Figure 1: Membrane activity as a function of initial enzyme concentration used for the grafting. Symbols: (O) = 0.1 % FAS concentration; (Δ) = 2.5 % FAS concentration; (□) = HEMA directly grafted on the Teflon membrane.

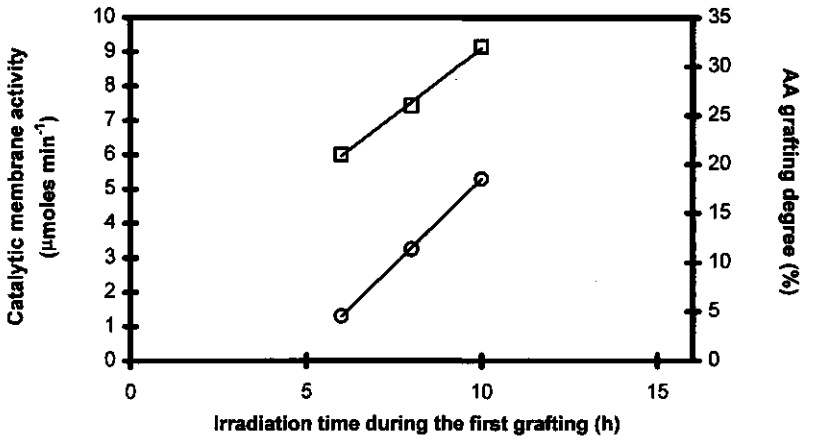

Figure 2: Catalytic membrane activity (O) and AA grafting degree (□) as a function of irradiation time used during the first grafting.

Table III. Effect of enzyme concentration used to prepare the catalytic membrane at fixed 0.1 % FAS concentration.

Enzyme concentration (mg/ml)	Irradiation time for the 1st grafting (h)	AA grafting degree (%)	Irradiation time for the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}}$ x 10
5	6	21.3	16	50.1	1.7
10	6	20.5	16	47.2	3.2
15	6	21.7	16	49.0	4.0
20	6	21.0	16	52.1	5.8
25	6	21.0	16	51.3	7.5
30	6	21.2	16	50.4	9.5
35	6	21.2	16	44.3	10.6
45	6	21.7	16	50.2	12.9

Dependence on the irradiation time for the second grafting

We then proceeded to test the dependence of membrane activity on the second irradiation time, the step during which the membranes, previously grafted with acrylic acid, become biocatalytic. We conducted a series of experiments under the best experimental conditions as described above and exposed the samples to different irradiation times during the second grafting. The results, reported in Table IV and in Figure 3, show that the membrane activity linearly increases with the second irradiation time, thus indicating that the amount of the immobilized enzyme depends at least in the time intervals used on the time employed for the immobilization.

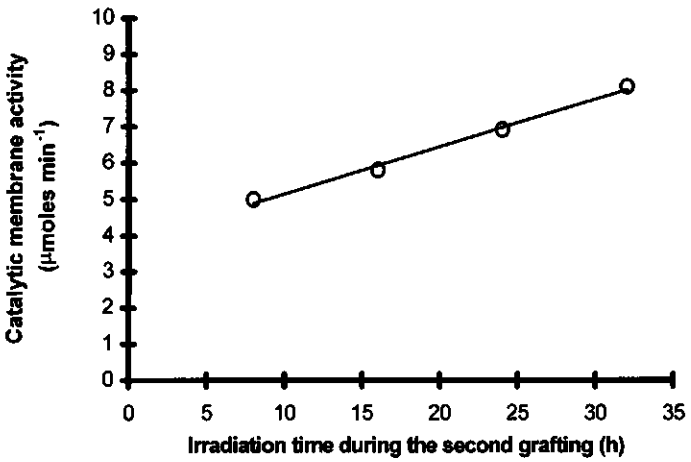

Figure 3: Catalytic membrane activity as a function of irradiation time during the second grafting.

Dependence on FAS concentration

From investigating the effect of the enzyme concentration on the membrane activity we concluded that the FAS concentration plays an important role. Next, studied this role in more detail using FAS concentrations ranging from 0.1 to 2.5%. The initial enzyme concentration was 45 mg/ml. The results reported in Table V and in Figure 4 show that the change in the FAS concentration did not have significant effects on both the AA and HEMA grafting degrees, like the results obtained by other authors [7]. The second grafting exhibited the same behaviour owing to its dependence on the first grafting. More different is the effect on

Table IV. Effect of irradiation time during the second grafting.

Irradiation time during the 1st grafting (h)	AA grafting degree (%)	Irradiation time during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}}$
10	31.6	8	41.2	5.1
10	32.5	16	43.0	5.8
10	30.7	24	44.5	6.9
10	32.0	32	49.1	8.1

membrane activity which decreased exponentially with the increase of FAS concentration. These results agree with the hypothesis of the occurrence of protein-protein interaction, which becomes relevant at FAS concentrations above 0.5%. It is well known that Fe^{2+} ions react with hydroxyl radicals produced by radiolysis of water, the process responsible for the initiation of homopolymerization, according to the reaction:

$$^{\cdot}OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^{-}$$

An increase of Fe^{2+} concentration thus reduces the homopolymer formation and therefore more of the free radical monomer units are available to react with the free radicals on the Teflon membrane. As a result the number of grafted branches, that is the density of AA branches on the membrane surface, is increased. Consequently, when the same AA grafting degree occurs at different FAS concentrations the length of the grafted branches must decrease there where the FAS concentration is higher. The synergetic occurrence of the increase of the branch density and of the decrease of the grafted branch length creates protein-protein interaction and formation of hydrogen bonds between the functional groups of the enzyme and the grafted branches. Both these two circumstances make the membrane less active, inactivating some enzyme and changing the micro-environment near the catalytic site.

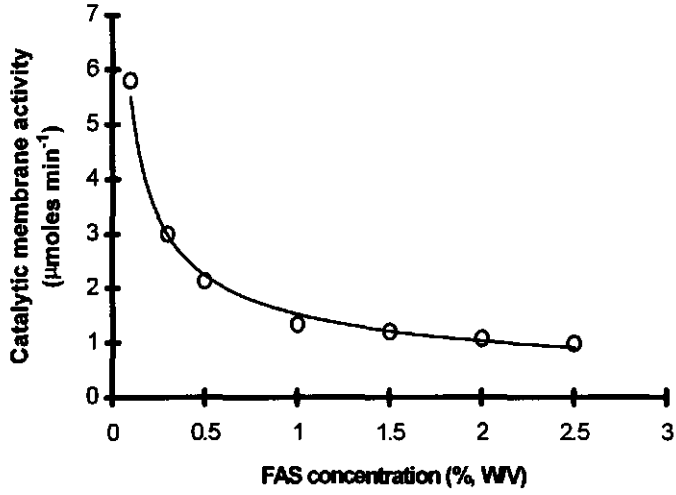

Figure 4: Catalytic membrane activity as a function of FAS concentration.

Table V. Effect of FAS concentration during the first grafting.

FAS concentration (%)	Irradiation time during the 1st grafting (h)	AA grafting degree (%)	Irradiation time during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}}$
0.1	10	32.0	16	43	5.20
0.3	10	30.0	16	43	3.00
0.5	10	31.0	16	41	2.13
1.0	10	30.0	16	42	1.33
1.5	10	29.5	16	41	1.20
2.0	10	28.0	16	40	1.07
2.5	10	20.0	16	47	0.98

Dependence on AA concentration

Next the dependence of the catalytic membrane activity on the AA concentration was examined, remembering that the AA chains constitute the most probable attachment sites for the HEMA grafting. To this end we carried out experiments under the same conditions described in the previous section with the exception that the FAS concentration was kept constant at 0.1%, while the AA concentration ranged between 5 and 25%. The results reported in Table VI and in Figures 5a and 5b show a maximum at 15% AA concentration. To understand these results it is sufficient to explain the behaviour of the first grafting degree, which controls the second grafting degree and the catalytic membrane activity. The FAS affects the production of the grafted branches on the surface of the Teflon membrane and inhibits the AA homopolymerization. These two processes are in competition and dependent on the AA and FAS concentrations. Keeping constant the FAS concentration, at low AA concentrations the rate of grafting is higher than the rate of homopolymeritation, while at high AA concentration the reverse occurs. In our experiments the AA concentrations giving the best conditions for obtaining the maximum of grafting degree occur in a range centred around the 15%. The same behaviour of Figure 5a was found by others with a styrene monomer [5,8].

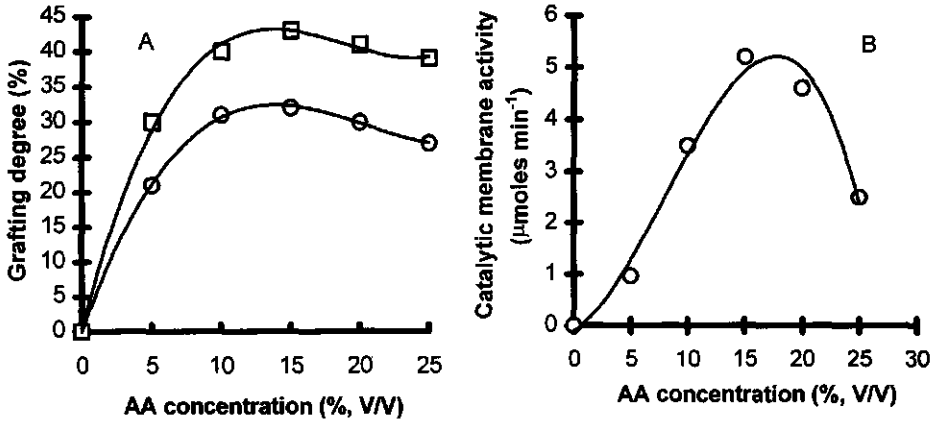

Figure 5: A) AA (O) and HEMA (□) grafting degree as a function of AA concentration. B) Catalytic membrane activity as a function of AA concentration.

Table VI. Effect of AA concentration.

AA concentration (%)	Irradiation time during the 1st grafting (h)	AA grafting degree (%)	Irradiation time during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}}$
5	10	21.5	16	13.2	0.97
10	10	31.5	16	30.1	3.50
15	10	32.5	16	43.0	5.80
20	10	30.0	16	41.2	4.60
25	10	27.0	16	39.4	2.51

Dependence on HEMA concentration

The experiment in which the effect of the HEMA concentration was studied, except that the AA concentration was kept constant at 15% and the HEMA concentration ranged between 2 and 15%. The results of these experiments are reported in Table VII showing how the AA grafting degree remains constant under the same conditions for the first grafting, while the HEMA enzyme grafting linearly increases with the HEMA concentration and the activity of the catalytic membrane has a maximum. This behaviour is more evident in Figure 6, where the relative activity of the catalytic membrane is reported as a function of the HEMA concentration.

CONCLUSIONS

Concluding this work we want to remark how the double grafting technique improved the activity of the biocatalytic membrane. The direct grafting of HEMA and enzyme was indeed some 5 times less active than the double grafting technique as shown in Figure 1. The conditions for obtaining the best activity of the double grafted membranes are: a = 15 % AA

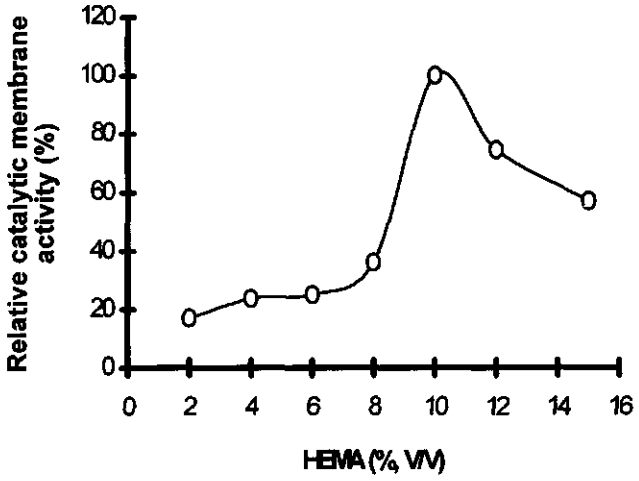

Figure 6: Relative catalytic membrane activity as a function of HEMA concentration

Table VII. Effect of HEMA concentration.

HEMA concentration (%)	Irradiation time used during the 1st grafting (h)	AA grafting degree (%)	Irradiation time used during the 2nd grafting (h)	HEMA grafting degree (%)	Catalytic membrane activity $\frac{\mu \text{ moles}}{\text{min}}$
2	10	30.0	16	11.0	1.00
4	10	30.6	16	13.0	1.38
6	10	31.0	16	24.0	1.46
8	10	29.7	16	46.5	2.10
10	10	32.0	16	43.0	5.20
12	10	30.5	16	72.0	4.32
15	10	30.5	16	79.0	3.32

concentration (v/v); b = 0.1 % FAS concentration (w/v); c = t_1 = 10 hours for the irradiation time during the first grafting; d = 10 % HEMA concentration (v/v); e = 45 mg/ml for enzyme concentration; f = t_2 = 16 hours for irradiation time during the second grafting.

One of the aims of this work was to obtain a Teflon catalytic membrane to be used in non-isothermal bioreactors, which have been found to provide higher efficiencies than bioreactors operating under comparable isothermal conditions [21-26]. Having obtained such a membrane by the double grafting technique, we are now able to improve the functioning of non-isothermal bioreactors. Experiments in this direction are currently being planned in our laboratory.

The aim of this work was the biochemical characterization of the biocatalytic membranes obtained and the study of the dependence of their activity on the substrate concentration, pH and temperature. These results are reported elsewhere [32] together with these of the same enzyme immobilized on Teflon membranes grafted with other monomers, such as acrylamide (AM) or methacrylic acid (MAA).

ACKNOWLEDGEMENTS

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REFERENCES

1 Box, G., A. Chapiro, M. Huglin, A. M. Jendrychowska Bonamour, & T. O'Neill (1968). *J. Polym. Sci.* **22:** 493

2. McCormick, C. & L. Park (1984). *J. Polym. Sci.* **22:** 49

3 Godjevargova,. T. S., A. R. Dimov, & N. Vasileva (1994). *J. Appl. Polym. Sci.* **54:** 355

4. Godjevargova T. S. & A. R. Dimov (1995). *J. Appl. Polym. Sci.* **57:** 487

5. Garnett, J. L., S. V. Jankiewicz, R. Levot, & D. F. Sangster (1985). *Rad. Phys. Chem.* **25:** 509

6. Kaetsu, I., M. Kumakura, T. Fujimura, M. Yoscida, M. Asano, N. Kasai, & M. Tamada (1986). *Rad. Phys. Chem.* **27:** 245

7. Hoffman, A. S., W. R. Gombotz, S. Uoenoyama, L. C. Dong, & G. Schmer (1986). *Rad. Phys. Chem.* **27:** 265

8. Garnett, J. L., S. V. Jankiewicz, R Levot, & D. F. Sangster (1986). *Rad. Phys. Chem.* **27:** 301

9. Arica, Y. & V. N. Hasirci (1987). *Biomaterials.* **8:** 489

10. Carenza, M. & G. Palma (1988). *Ann. N. Y. Acad. Sci.* **542:** 115

11 Alves da Silva,. M., C. G. Beddows, M. H. Gill, J. T. Guthrie, A. J. Guiomar, S. Kotov & A. P. Piedade (1990). *Rad. Phys. Chem.* **35:** 98

12. Docters, E. H., E. E. Smolko & C. E. Suarez (1990). *Rad. Phys. Chem.* **35:** 102

13. Hajizadhe, K., H.B. Halsall & W.R. Heinemann (1991). *Anal. Chim. Acta.* **243:** 23

14. Gursel, I. & V. N. Hasirci (1992). *Biomaterials.* **13:** 150

15. Doretti, L., D. Ferrara & S. Sora (1993). *Biosensors and Bioelectronics.* **8:** 443

16. Godjevargova, T. S. (1996). *J. Appl. Polym. Sci.* **61:** 334

17. Bentvelzen, J. H., F. Kimura-Yih, H. B. Hopfemberg & V. Stannet (1973). *J. Appl. Polym. Sci.* **17:** 809

18. Tealdo, G. C., P. Canepa & S. Munari (1981). *J. Membrane Sci.* **9:** 191

19. Aptel, P., N. Challard, J. Cuny & J. Neel (1976). *J. Membrane Sci.* **1:** 271

20. Morel, G., J. Jozefowicz & P. Aptel (1979). *J. Appl. Polym. Sci.* **23:** 2397

21. Mita, D. G., M. A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F. S. Gaeta (1993). *J. Membrane Sci.***78:** 69

22. Mita, D. G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F. S. Gaeta (1995). *Biotechnol. Applied Biochem.* **22:** 281

23. Russo, P., A. Garofalo, U. Bencivenga, R. Rossi, D. Castagnolo, A. D'Acunto, F. S. Gaeta & D. G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141

24. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, P. Canciglia, F. Palumbo, F. S. Gaeta & D. G. Mita (1996) . *Biotechnol. Appl. Biochem.* **24:** 25

25. Russo, P., A. De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F. S. Gaeta & D. G. Mita (1997). *Research in Microbiology*. **148:** 271

26. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D. G. Mita (1997). *J. Membrane Sci.* **129:** 175

27. Gaeta, F. S. & D. G. Mita (1978). *J. Membrane Sci.* **3:** 191

28. Bellucci, F., E. Drioli, F. S. Gaeta, D. G. Mita, N. Pagliuca & F. G.Summa (1979). *Trans. Farad. Soc. II.* **75:** 247

29. Pagliuca, N., G. Perna, D. G. Mita, F. S. Gaeta, B. Karamanlis & F. Bellucci (1983). *J. Membrane Sci.* **16:** 91

30. Mita, D. G., U. Bencivenga, A. D'Acunto, N. Pagliuca, S. Rossi & F. S. Gaeta (1988). *Gazzetta Chimica Italiana.* **118:** 79

31. Gaeta, F. S., E. Ascolese, U. Bencivenga, J. M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi & D. G. Mita (1992). *J. Phys. Chem.* **96:** 6342

32. Mohy Eldin, M. S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D. G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 613

CHARACTERIZATION OF THE ACTIVITY OF β-GALACTOSIDASE IMMOBILIZED ON TEFLON MEMBRANES PREACTIVATED WITH DIFFERENT MONOMERS BY γ-IRRADIATION

ABSTRACT

Characterization from a biochemical and biophysical point of view was done of the activity of β-galactosidase, immobilized by grafting on TEFLON membranes pre-activated with four different monomers. The monomers used were acrylic acid (AA), acrylamide (AM), methacrylic acid (MAA), or 2-hydroxyethyl-methacrylate (HEMA). When HEMA was used in the second grafting, one of the first three monomers were used in the first modification step.

The behaviour of the free and immobilized enzyme was analyzed as a function of temperature and pH. We have found general equations relating the absolute enzyme activity to pH and temperature for each catalytic membrane, and was able to calculate the experimental conditions giving the best yield of each catalytic membrane from these expressions. We were also able to determine the kinetic parameters for the four membranes and indicate the advantages of using these membranes in non-isothermal bioreactors.

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INTRODUCTION

Immobilized enzymes are widely used in biotechnological processes spanning from food industry to ecology, from fine chemical production to the construction of biosensors.

Besides the enzyme, the other most important component contributing to the performance of a biocatalytic membrane is the carrier. At present synthetic carriers constitute the largest number of supports available for catalyst immobilization, owing to their resistance against microbial attack and their ability to be copolymerized with different monomers. Modifications of pre-formed polymers are easily done chemically [1-3] or by irradiation [4-17] using grafting techniques.

In a previous article [18] we reported a technique by which we successfully immobilized β-galactosidase on a pre-modified Teflon membrane using γ-irradiation grafting. What we obtained was a Teflon (PTFE) membrane on which acrylic acid (AA), 2-hydroxyethyl-methacrylate (HEMA) and the enzyme were attached in sequence by γ-irradiation. The best performance of this catalytic membrane was found as a function of the most relevant experimental parameters affecting its behaviour.

We now wanted to characterize, from a biochemical and biophysical point of view, the behaviour of the β-galactosidase immobilized on Teflon membranes, differently pre-modified. The activity of an immobilized enzyme is affected by the immobilization process which induces changes in the spatial structure of the protein or in the microenvironment in which the immobilized enzyme operates. To understand how the microenvironment affects the enzyme activity we chose four different monomers with different chemical and physical properties, as spacer arms. Additional factors affecting enzyme activity are the pH and the temperature, therefore the dependence of the enzyme performance on these factors was also studied and is discussed here covering each of the catalytic membranes, pre-modified with different monomers.

MATERIALS AND METHODS

Materials

The membranes used as solid support for the grafting process were polytetrafluoroethylene (PTFE) membranes of the type TF-450 manufactured by the Gelman Instrument Company (Ann Arbor, Michigan 48106 USA). The characteristics of the membranes are reported elsewhere [18].

The monomers used for the grafting were 2-hydroxyethyl-methacrylate (HEMA) and acrylic acid (AA), acrylamide (AM), or methacrylic acid (MAA). Ferrous ammonium sulphate (FAS) was used during the pre-modification step as inhibitor of the formation of homopolymers.
The enzyme employed was β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*. β-Galactosidase hydrolyzes lactose in glucose and galactose. The methodology used for determining the enzyme activity has been described elsewhere [18].
All chemical products, including the enzyme, were purchased from Sigma Chemical Company (St. Louis, Missouri 63178, USA) and used without any further purification.

METHODS

Catalytic membrane preparation

Membrane grafting was performed by irradiation with γ-rays, the irradiation source being caesium 137 in a gamma cell 1000 Elite from Nordion International Inc., Canada. The average dose rate in the core of the radiation chamber (central dose rate) was of 2.35×10^4 rad/hour.

Double grafted membranes

First grafting: modification of PTFE membrane by AA or AM or MAA grafting

The first grafting by irradiation of the gamma cell of the PTFE membranes was done using a FAS solution of acrylic acid, acrylamide or methacrylic acid monomers, thus obtaining a PTFE-AA, a PTFE-AM or a PTFE-MAA membrane. The experimental conditions used are specified according to the following scheme: a = 10% of monomer concentration (v/v); b = 0.1 % FAS concentration (w/v); c = t_1 = 10 hours of irradiation time . Only in the case of the PTFE-AM membrane the b parameter was b = 5 % FAS concentration (w/v).

Second grafting: immobilization of β-galactosidase by HEMA radiation grafting

The second grafting was performed by irradiation in the gamma cell the previously grafted membranes immersed in a solution of HEMA and enzyme. The experimental conditions used are specified according to the following scheme: d = 10 % HEMA concentration (v/v); e = 45 mg/ml enzyme concentration; f = t_2 = 16 hours of irradiation time.

Mono grafted membranes

These membranes were directly obtained following the procedure used for the second grafting, i. e. under the conditions defined by: d = 10 % HEMA concentration (v/v); e = 45 mg/ml enzyme concentration; f = t = 16 hours of irradiation time.

Determination of the grafting degree

As for the 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, as expressed by the formula:

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

Determination of the swelling degree

The swelling degree, i.e. the membrane hydrophilicity, was calculated as the weight difference between the water-swollen and dry membrane divided by the weight of the dry membrane.

Determination of the activity of the catalytic membranes

To determine the activity of the catalytic membranes, they were placed in 20 ml of a well-stirred 200 mM lactose in 0.1 M buffer solution at the desired pH and temperature and the glucose production measured in the course of time; from that the activity of the catalytic membrane was calculated as previously described [18]. To study the activity as a function of the pH we used 0.1 M NaCl-HCL buffer solution for the pH 2, 0.1 M citrate buffer solution for 3-5 pH range, and 0.1 M phosphate buffer solution for the pH range 6-8.

Determination of the time stability of the catalytic membranes

The time stability of the biocatalytic membranes was assessed by daily analysis of their activity under the same experimental conditions. After three days, during which the membranes lost some activity, a stable condition was reached which remained unchanged for over two months. Only these stabilized membranes have been used in the comparative experiments reported below. When not utilized the membranes were stored at 4°C in 0.1 M buffer phosphate of pH 6.5.

Treatment of the experimental data

Each experimental point reported in the figures represents the average of three experiments performed under the same conditions. The experimental errors did not exceed 6 %. For each of the three experiments the procedures in the various steps were carried out according to the following methodology. Thirty-two Teflon membrane disks 2.5 cm in diameter were weighed and put in the solution for the first grafting. After irradiation the membrane disks were repeatedly washed under vigorous stirring in abundant double distilled water to remove the adherent homopolymers. Then eight disks were taken for determining the first degree of grafting and the remaining were used for the second grafting. At the end of this operation the disks were washed in 0.1 M phosphate buffer solution of pH 6.5 and then separated into three groups of eight membranes each for the determining of the second grafting degree, the swelling degree and the catalytic activity, respectively.

RESULTS AND DISCUSSION

Effect of temperature

A bell-shaped curve of relative activity emerged from the study of the dependence of enzyme activity on the temperature (Figure 1). The curve of the immobilized enzyme was broader or narrower than or equal to the curve of the free enzyme, while the optimum activity tended to shift towards higher temperatures upon immobilization. This means a higher resistance to enzyme thermal deactivation, probably because the immobilization strengthened the structure of the catalytic site due to strong bonds between the enzyme molecule and the carrier. On achieving a unaltered maximum position we can conclude that the structure of the active site and the microenvironment in which the enzyme is operating are probably the same in the free

and bound form. Figure 1 presents the temperature dependence of the β-galactosidase activity for the four types of catalytic membranes employed .

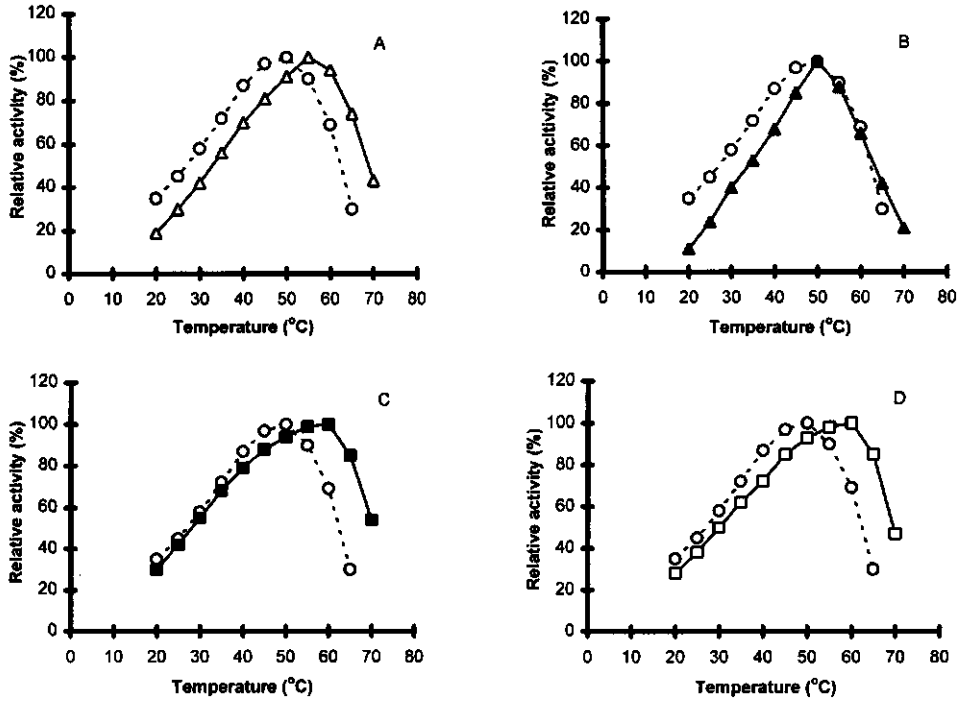

Figure 1. Relative activity of the free enzyme and catalytic membrane as a function of temperature for the systems: Teflon-MAA (A) ; Teflon-AA (B); Teflon-AM (C) and HEMA directly grafted on Teflon (D). The pH of the solution was 6.5.

The temperature dependence of the activity of free enzyme is also reported as reference curve. In all the experiments an approximatively bell-shaped curve was derived, the temperature profile remained virtually unchanged. What did change was the optimum activity position, which in the case of immobilized enzyme shifted toward higher temperatures. The only exception occurred with the Teflon-AA membrane (Figure 1 b) where the optimum activity temperature coincided with that of the free enzyme. All these experiments were performed at pH 6.5, which we chose because it corresponds to the pH of milk and we wanted to employ these new catalytic membranes in processes dealing with lactose hydrolysis of milk.

Effect of pH

It is well known that pH plays a relevant role on enzyme activity, a role that is clearly evidenced when the activities of soluble and immobilized enzymes are studied. 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. Of course if the results obtained for the two forms of the enzyme coincide, the conclusion can be drawn that the catalyst operates under the same environmental conditions.

We investigated the activity of the β-galactosidase in the free and immobilized states as a function of pH ranging between 2 and 8. The results of this investigation are shown in Figure 2, where the relative activities of each of the four catalytic membranes are reported as a function of pH. The figure shows the relative activity of the free enzyme also as reference curve. The temperature was kept constant at 30 °C in all the experiments. The optimal activity was obtained at pH 4.5 for the free enzyme and for the system Teflon-MAA. A shift of about half a pH unit toward more alkaline pH values was found with the Teflon-AA and Teflon-AM systems. On the contrary, a shift of about half a pH unit toward more acid pH values was found with the HEMA directly grafted on Teflon. The latter result is unexpected considering the neutral nature of HEMA. This behaviour can be attributed to secondary interactions between the enzyme and the modified polymeric matrix. Next, the causes of the observed shifts are discussed. As already stated, the most relevant factor influencing enzyme activity is the partitioning effect, directly related to the chemical nature of the support material (in this case the grafted monomers), which induces electrostatic or hydrophobic interactions between the matrix and the low-molecular weight species present in the solution. These interactions lead to alterations in the microenvironment in which the enzyme actually operates. In particular, the partitioning effects cause different concentrations of charged species, as hydrogen ions or hydroxyl groups, in the microenvironment of the immobilized enzyme. The pH profile is generally displaced toward more alkaline or acid pH values for negatively or positively charged matrices [19], respectively in our case the AM, AA and MAA branches behaved as if negatively charged in solution, thus inducing a shift of pH activity toward more alkaline pH values. The negative charge is determined by the two lone pair electrons on the nitrogen atom of the amino group in the case of AM or by the loss of H^+ ions from the functional carboxylic groups in the case of AA and MAA. The pH shift and the value of this shift is dependent on the nature of the charge and on the density of the charges, which in our case is directly proportional to the density of the grafted monomers.

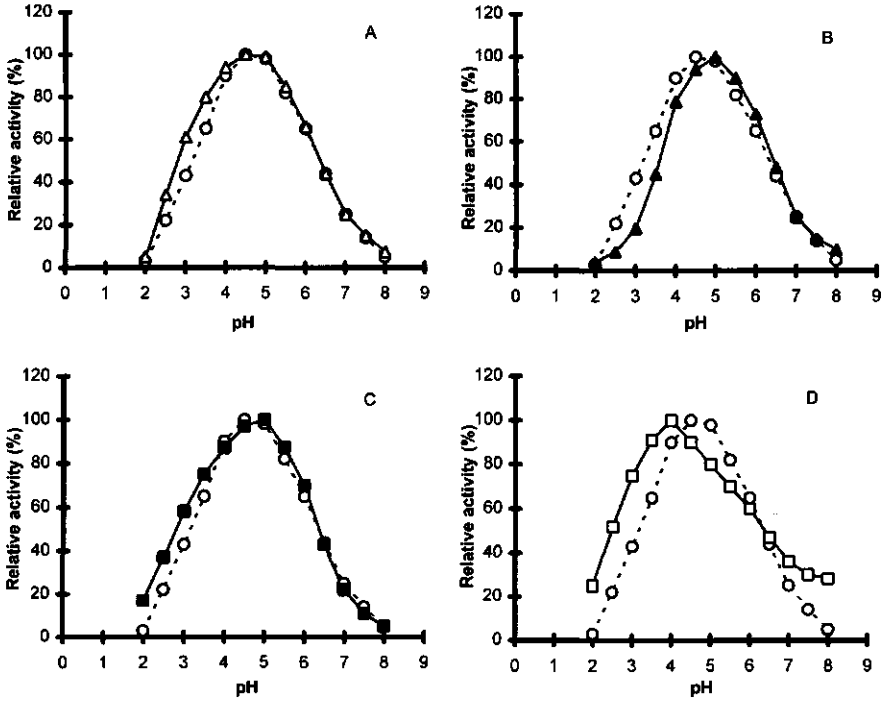

Figure 2. Relative activity of the free enzyme and catalytic membrane as a function of pH for the systems: Teflon-MAA (A); Teflon-AA (B); Teflon-AM (C) and HEMA directly grafted on Teflon (D). The temperature of the solutions was 30°C.

Since the grafting percentage is different for the various monomers (see Table I) the shift will also be different. The grafting degree of MAA is so low that practically no significant changes in the enzymatic micro environment are induced. For this reason the optimum pH is coincident for the free and the immobilized enzyme.

As for the change of pH activity profile, the broadening of the pH activity curve can be explained by the increased resistance of the enzyme to pH changes upon immobilization, the reverse holds when the curve profile becomes narrower.

Table I: Grafting and swelling degree.
The experimental conditions used are reported in the text.

Membrane system	Degree of the first grafting (%)	Degree of the second grafting (%)	Degree of the swelling (%)
Teflon-MAA	8	26	50
Teflon-AA	30	83	300
Teflon-AM	41	69	160
HEMA directly grafted on Teflon	none	10	10

Synergetic dependence on temperature and pH

Above, we found that the maximum relative activity of the catalytic membrane is a function of temperature and pH. Next, we wanted to see if there is some graphical form to show a simultaneous relation of the relative maximum activity of each catalytic membrane to the temperature and pH values at which this maximum occurs.
To this end we added two others pH values to our experiment, studying the catalytic behaviour of the grafted membranes as a function of temperature. Figure 3a presents the results of these experiments at pH 3.5 with the four membrane systems. Membrane activity has been expressed as relative activity. Comparison between these results and those reported in Figure 1 shows that the maximum activity is shifted towards lower temperatures when the solution pH is 3.5.

Figure 3b presents the results of experiments with the four membrane systems at pH 5.0. The membrane activity has been expressed as relative activity. Here, the comparison between

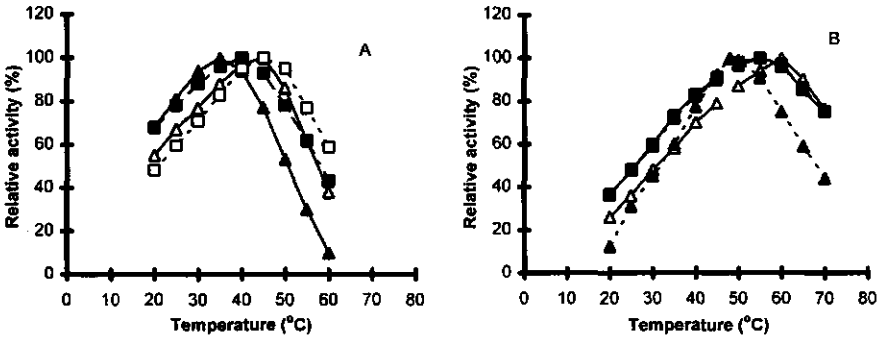

Figure 3. Relative activity of the catalytic membrane as a function of temperature for the systems: Teflon-MAA (△ ; Teflon-AA (▲ ; Teflon-AM (■) and HEMA directly grafted on Teflon (□ . The pH of the solution was 3.5 in the experiments reported in (A) and 5.0 in the experiments reported in (B).

these results and the ones reported in Figures 1and 3a shows that the maximum of the relative activity shifts when the solution pH is changed. Also the curve profiles are altered when the pH changes.

All the above results clearly indicate that there is a correlation between the position of the maximal relative activity and the corresponding values of the pH and temperature. To identify this correlation in Figure 4 we report the values of the temperatures at which the maximal relative activities occur as a function of corresponding pH values. Points are deduced from the experiments reported in Figures 1 and 3. We are aware that the three experimental points are too few to draw a curve, for this reason the curves in the figures were drawn by the computer imposing the best fit between the experimental data.The quadratic correlation coefficient between the three experimental points and the fitted curve is 0.95 ± 0.02 for each membrane system. Notwithstanding the limited experimental data, it is possible to write a mathematical equation satisfying the experimental points reported in the figures. This expression is a parabolic equation of the type:

$$1) \qquad T = M_2 (pH)^2 + M_1 (pH) + M_0$$

The parabolic profile is clearly evident in Figure 4a. Obviously any membrane system is characterized by its own M_j values. The M_i values for the four membranes systems are reported in Table II. The use of this equation allows to plan the best experimental conditions for obtaining the higher yield of the enzyme reaction when one of the two parameters,

temperature or pH are fixed. This means that if we use a Teflon-MAA membrane, for example, and the solution to be treated has a pH value of 6.0, the temperature for obtaining

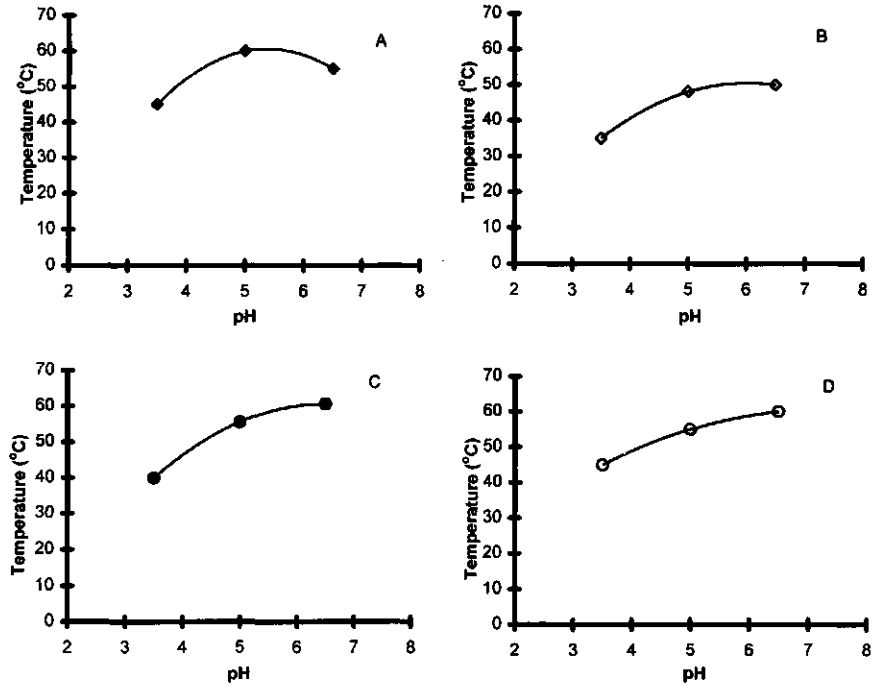

Figure 4. Temperature of the relative maximum of the catalytic membrane activity as a function of the corresponding pH value for the systems: Teflon-MAA (A); Teflon-AA (B); Teflon-AM (C) and HEMA directly grafted on Teflon (D).

the best yield of the process must be 59 °C. This value can be deduced by Figure 4 (a) as well as by solving the parabolic equation with the appropriate values for the M_i coefficients. Conversely, if we are compelled to work at a temperature of 48 °C to obtain the best yield we must use a solution of pH 3.7 with a Teflon-MAA membrane, a solution of pH 3.9 with a Teflon-AA membrane, a solution of pH 4.5 with a Teflon-AM membrane, and a solution of pH 5.0 with a Teflon membrane directly grafted by HEMA. A larger number of experimental results than available at present would be desirable to pinpoint the position of the absolute maximal membrane activity in our systems. However, on the basis of the data at hand, a function A(T, pH) of the absolute activity may be constructed for each immobilized enzyme system. To this end polynomial fitting is done for each group of measurements carried out, keeping constant one of the two variables, while the other varies within the adopted range. The A(pH) and A(T) curves obtained in this way can be fitted by a polynomial form which, in

each case, cannot be of higher degree than the total number of the corresponding independent experimental data, minus one.

Table II : Numerical values of the coefficients of the equation 1.
Coefficient of the parabolic equation relating the temperature of the maximum of the relative activity to the corresponding pH value.

Membrane system	M_0 (°C)	M_1 (°C)	M_2 (°C)
Teflon-MAA	- 67.77	47.77	- 4.44
Teflon-AA	- 38.11	29.44	- 2.44
Teflon-AM	- 37.00	30.17	- 2.33
HEMA directly grafted on Teflon	2.22	16.11	- 1.11

Thus fitting the pH-dependence with a second order and the T-dependence with a higher best polynomial equation, these curves could be visualized in a three-dimensional plane in a cartesian axis system (A, pH, T) (Figure 5). Each of the planes can be expressed by an equation of the general form:

$$2) \qquad A(T, pH) = [a + b(pH) + c(pH)^2] (d + eT + fT^2 + gT^3$$

from which, by a double partial differentiation of pH or T, the couple of values of the independent variables pH and T yielding the maximum membrane activity in the range of experimental conditions used, can be obtained. These values can be found in Table III.

Kinetic parameters

When using immobilized enzymes in bioreactors or in biosensors knowing the apparent kinetic parameters is essential. These parameters generally undergo variations indicating an affinity change of the substrate; the variations can be attributed to protein conformational changes, to steric hindrances and to partitioning and diffusion effects.

Table III : $T_{Optimum}$ and $pH_{Optimum}$ for the four catalytic membranes.

pH and temperature couples giving the best efficiency for each catalytic membrane. The values have been calculated by means of equation 2.

Membrane system	$T_{Optimum}$ (°C)	$pH_{Optimum}$
Teflon-MAA	58.6	5.2
Teflon-AA	52.2	5.2
Teflon-AM	56.1	4.9
HEMA directly grafted on Teflon	56.4	5.0

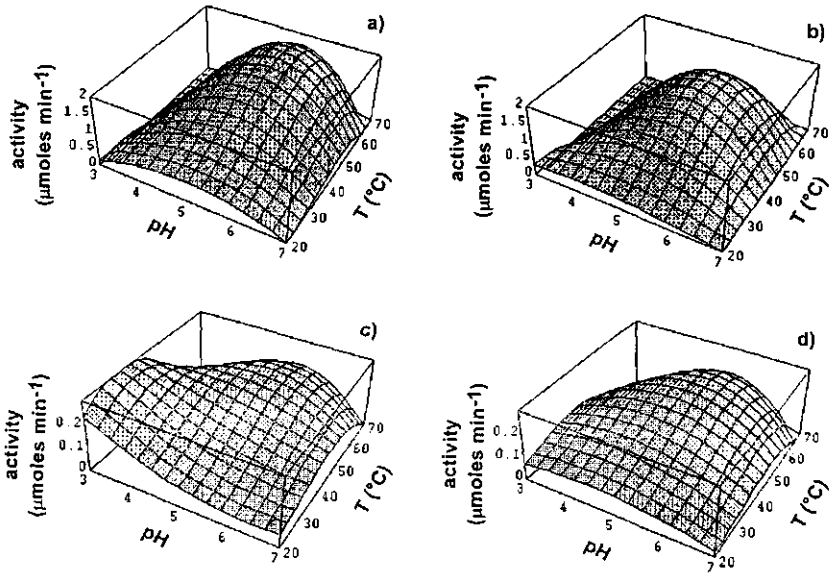

Figure 5. Catalytic membrane activity as a function of temperature and pH for the systems: Teflon-MAA (a); Teflon-AA (b); Teflon-AM (c) and HEMA directly grafted on Teflon (d).

All these factors may occur simultaneously or separately, consequently the affinity between enzyme and substrate may be modified by the immobilization. This modification could result in a decrease [20-21] or in an increase [22-23] of the apparent K_m value. A decrease in the K_m value of an immobilized enzyme leads to a faster reaction rate as compared to its free counterpart, while an increase of K_m implies the use of a higher substrate concentration to achieve the same rate of the reaction obtained with the free enzyme. The apparent K_m certainly decreases if the charges on the support and the substrate are opposite. The conformational changes of the protein molecule and steric hindrances usually lead to an increase in the K_m values caused by a decrease in the affinity between the enzyme and the substrate. Also the V_m values are affected by the immobilization procedure. The values of V_m obtained for the immobilized enzymes might be increased [24] or decreased [25].

To obtain the apparent values of K_m and V_m of the β-galactosidase immobilized on the four types of membranes used, we studied the activity of each catalytic membrane as a function of substrate concentration ranging from 25 to 500 mM, the pH and the temperature being 6.5 and 30 °C, respectively. The results of this investigation are illustrated in Figure 6. The figure clearly shows how each membrane exhibits different values of enzyme activity at the same substrate concentration. Having used the same initial enzyme concentration to load the membranes, the results indicate that the yield of the immobilization is different for the four membrane types. In particular, we observed that the absolute activity of the membranes follows the order AA >> MAA > AM > HEMA. None of the parameters in Table I seems to justify this behaviour. From the results in Figure 6 we calculated the K_m and V_m values for the β-galactosidase immobilized on each of the four membrane types. These values are in Table IV illustrating how the apparent values of K_m of the immobilized enzymes are higher than that of the free enzyme for all the membrane systems used. The remarkable decrease of the enzyme affinity for the substrate when the β-galactosidase is immobilized on our modified Teflon membranes points to some limitations of using these membranes in industrial processes. In these processes, anyway, the reduction of the affinity of an immobilized enzyme is compensated for by the possibility of reuse of the membrane. This is an added advantage in using immobilized enzymes in bioreactors. We have demonstrated [26] that the value of the apparent K_m of an immobilized enzyme operating under non-isothermal conditions is lower than that of the enzyme immobilized under isothermal conditions. This results in an efficiency increase of the yield of the enzyme reaction making available for industrial processes membranes endowed with low isothermal activity.

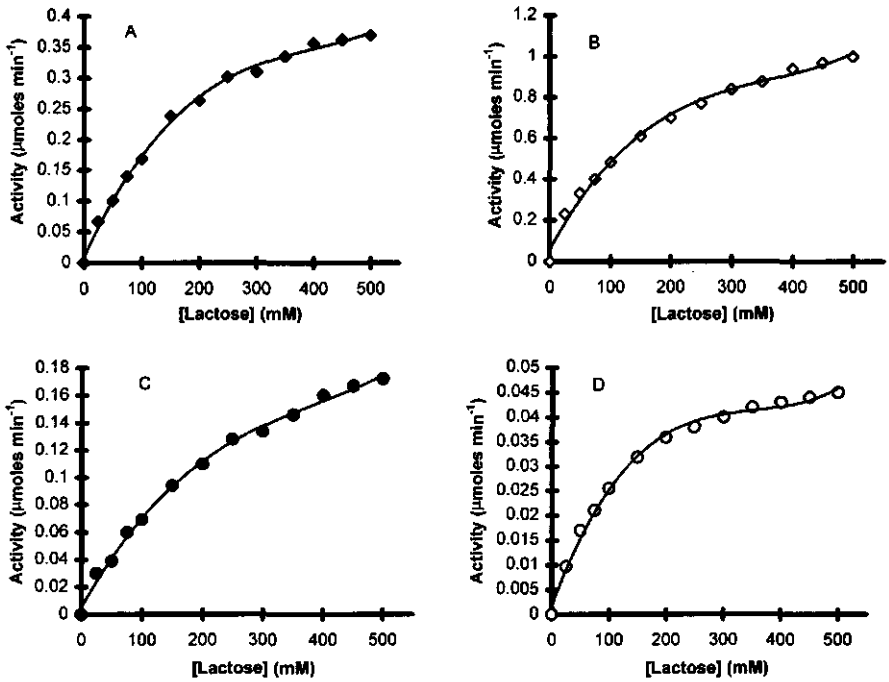

Figure 6. Catalytic membrane activity as a function of initial substrate concentration for the systems: Teflon-MAA (A); Teflon-AA (B); Teflon-AM (D) and HEMA directly grafted on Teflon (D).

CONCLUSIONS

The aim of characterizing each membrane system biochemically and biophysically has been achieved. Also the operational properties of the membranes have been defined. The time stability of each membrane proved to be good, since after an initial phase in which a small loss of enzyme activity was observed, the catalytic activity of the membranes remained unchanged for over two months.

More interesting for the practical use of our membranes are equations 1 and 2. Equation 1 allows a choice of the correlated pH and temperature parameters giving the highest membrane efficiency when the other parameter is imposed. Equation 2, instead, allows us to define for each membrane both the pH and temperature giving the best yield of each catalytic membrane.

Table IV: Kinetic parameters

System	K_M (mM)	V_M (μmoles min^{-1})
Free enzyme	21.4	3.2
Teflon-MAA	190.5	0.53
Teflon-AA	145.8	1.20
Teflon-AM	236.9	0.27
HEMA directly grafted on Teflon	120.0	0.06

The potential of using these catalytic membranes in bioreactors operating under non-isothermal conditions [26-31] by simplifying the membrane system used until now seems clear. In our previous work, indeed, the membrane system employed in non-isothermal bioreactors was a catalytic membrane of natural or synthetic origin, coupled with a Teflon membrane. The advantage of using non-isothermal bioreactors is that they have a greater efficiency than that of the same bioreactors operating under comparable isothermal conditions. The catalytic Teflon membrane obtained in this research must be tested in the bioreactors before deciding if they are useful or not for use under non-isothermal conditions. On the basis of the swelling degree, the following order is expected to be found in the performance of the non-isothermal bioreactor: HEMA >> MAA > AM > AA. This indication follows from the consideration that for thermodialysis [32-36], which is primarily responsible for the efficiency increase of a non- isothermal bioreactor, hydrophobic membranes must be employed. Experiments in this direction are currently underway in our laboratory.

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REFERENCES

1. McCormick, C. & L. Park (1984). *J. Polym. Sci.* **22:** 49
2. Godjevargova, T. S., A. R. Dimov & N. Vasileva (1994). *J. Appl. Polym. Sci.* **54:** 355
3. Godjevargova, T. S. & A. R. Dimov (1995). *J. Appl. Polym. Sci.* **57:** 487
4. Garnett, J. L., S. V. Jankiewicz, R Levot, & D. F. Sangster (1985). *Rad. Phys. Chem.* **25:** 509
5. Kaetsu, I., M. Kumakura, T. Fujimura, M. Yoscida, M. Asano, N. Kasai & M. Tamada (1986). *Rad. Phys. Chem.* **27:** 245
6. Hoffman, A. S., W. R. Gombotz, S. Uoenoyama, L. C. Dong & G. Schmer (1986). *Rad. Phys. Chem.* **27:** 265
7. Garnett, J. L., S. V. Jankiewicz, R. Levot & D. F. Sangster (1986). *Rad. Phys. Chem.* **27:** 301
8. Arica, Y. & V.N. Hasirci (1987). *Biomaterials.* **8:** 489
9. Carenza, M. & G.Palma (1988). *Ann. N.Y. Acad. Sci.* **542:** 115
10. Alves da Silva, M., C. G. Beddows, M. H. Gill, J. T. Guthrie, A. J. Guiomar, S. Kotov & A. P. Piedade (1990). *Rad. Phys. Chem.* **35:** 98
11. Docters, E. H., E. E. Smolko & C. E. Suarez (1990). *Rad. Phys. Chem.* **35:** 102
12. Hajizadhe, K., H.B. Halsall & W.R. Heinemann (1991). *Anal. Chim. Acta.* **243:** 23
14. Doretti, L., D. Ferrara & S. Sora (1993). *Biosensors and Bioelectronics.* **8:** 443
15. Godjevargova, T.S. (1996). *J. Appl. Polym. Sci.* **61:** 334
16. Bentvelzen, J. H., F. Kimura-Yih, H. B. Hopfemberg & V. Stannet (1973). *J. Appl. Polym. Sci.* **17:** 809
17. Tealdo, G.C., P. Canepa & S. Munari (1981). *J. Membrane Sci.* **9:** 191
18. Mohy Eldin, M. S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D. G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 613
19. Goldstein, L. & E. Z. Katchalsky (1968). *Anal. Chem.* **243:** 375
20. May, S. W. & N. N. Li (1972). *Biochem. Biophys. Res. Commun.* **47:** 1178
21. Horndy, W. E., M. D. Lilly & E. M. Crock (1968). *Biochem. J.* **107:** 668
22. Kleine, R., P. Spangerberg & C. Fleming (1976). *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 629
23. Sato, T., T. Mori & I. Chibata (1971). *Arch. Biochem. Biophys.* **147:** 788

24. Cabral, J. S. M., J. F. Kennedy & A. Novais (1982). *Enzyme Microb. Techn.* **4:** 343
25. Usami, S., E. Hasegarva & M. Karasawa (1975). *Hakko Kyokaishi* **33**:152
26. Mita, D. G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F. S. Gaeta (1995). *Biotechnol. Applied Biochem.* **22:** 281
27. Mita, D. G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69
28. Russo, P., A. Garofalo, U. Bencivenga, R. Rossi, D. Castagnolo, A. D'Acunto, F. S. Gaeta & D. G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141
29. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, P. Canciglia, F. Palumbo, F. S. Gaeta & D. G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25
30. Russo, P., A.De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F. S. Gaeta & D. G. Mita (1997). *Research in Microbiology.* **148:** 271
31. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D. G. Mita (1997). *J. Membrane Sci.* **129:** 175
32. Gaeta, F.S. & D.G. Mita (1978). *J. Membrane Sci.* **3:** 191
33. Bellucci, F., E. Drioli, F. S. Gaeta, D. G. Mita, N. Pagliuca & F. G. Summa (1979). *Trans. Farad. Soc. II.* **75:** 247
34. Pagliuca, N., G. Perna, D. G. Mita, F. S. Gaeta, B. Karamanlis & F. Bellucci (1983). *J. Membrane Sci.* **16:** 91
35. Mita, D. G., U. Bencivenga, A. D'Acunto, N. Pagliuca, G. Perna, S. Rossi & F. S. Gaeta (1988). *Gazzetta Chimica Italiana.* **118:** 79
36. Gaeta, F.S., E. Ascolese, U. Bencivenga, J. M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi & D. G. Mita (1992). *J. Phys. Chem.* **96:** 6342

Chapter 5

INFLUENCE OF THE MICROENVIRONMENT ON THE ACTIVITY OF ENZYMES IMMOBILIZED ON TEFLON MEMBRANES GRAFTED BY γ-RADIATION

ABSTRACT

The effect of the microenvironment and immobilization method on the activity of β-galactosidase, immobilized to various Teflon membranes, was investigated. Teflon membranes were grafted with different acrylic monomers by γ-radiation and activated by two different coupling agents through the functional groups of the grafted monomers. 2-Hydroxyethylmethacrylate (HEMA) and methacrylic acid (MAA) were grafted on the membrane, and 1-6 hexamethylendiamine (HMDA) was used as a spacer. Glutaraldehyde or cyanuric chloride were used as coupling agents to bind the enzyme to the membrane.

Four different catalytic membranes were obtained using the same solid support. Direct comparison between the isothermal behaviour of the biocatalyst in its free and immobilized form was carried out. The dependence of the isothermal activity on the temperature and pH was studied in particular and the kinetic parameters determined. The influence of the microenvironment on the observed activity of the four membranes was evidenced and discussed. The way of improving the yield of these catalytic membranes is also discussed.

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INTRODUCTION

Immobilized enzymes offer considerable advantages such as facility of removal and reuse, increased shelf life and thermal stability. For these reasons studies on immobilization techniques as well as on employment of catalytic membranes in industrial processes have been rapidly increasing in recent years [1-3].

Polymeric materials are generally used as support matrices for immobilization since they have various functional groups and can be easily modified chemically [4-6] or by irradiation [7-17]. The physico-chemical properties of the polymeric support, therefore, directly affect the choice of a suitable coupling procedure for immobilization and, consequently, the biochemical and biophysical behaviour of the immobilized enzymes. Among the various methods available for enzyme immobilization, is covalent binding, which is particularly important because it leads to preparation of stable enzyme derivatives [18-21].

In previous studies [22-23] we reported on a double grafting technique by which β-galactosidase was successfully immobilized by entrapment on Teflon membranes first grafted with methacrylic acid (MAA), and then with 2-hydroxyethyl methacrylate (HEMA).

Here we describe the changes produced by the immobilization method on the microenvironment in which the immobilized biocatalyst operates and, hence, the effect on the enzyme catalytic activity. The catalytic behaviour of four different membranes will be discussed. They were prepared using the same polymeric matrix but with different grafted monomers, coupling agents, immobilization methods, and in the presence and absence of spacers. The enzyme is covalently bound on three of the membranes and entrapped on the fourth. A direct comparison will also be made between the activity of the free and the immobilized form of the biocatalyst. The catalytic membranes will be characterized under isothermal conditions in order to obtain indications for the construction of new catalytic membranes that could used in non-isothermal bioreactors. In these bioreactors the enzyme reaction rate was found to increase proportionally to the temperature difference applied across the membrane. This increase depends on the enzyme and immobilization method and was found to be 20 to 50% when a temperature difference of 1°C was applied across the catalytic membrane [24-30].

MATERIALS AND METHODS

Teflon [polytetrafluoroethylene (PTFE)] membranes of the 450 type manufactured by the Gelman Instrument Company (Ann Arbor , MI, USA) were used as solid support for the grafting process. TheTeflon film membranes supported on one side by a polypropylene net, had a thickness of 150 mm and were endowed with anastomizing irregular channels of 0.450 mm in nominal diameter. The nominal pore diameter is the one of the smallest particles which the membrane is able to retain.

HEMA and MAA monomers were used for grafting. Ferrous ammonium sulphate (FAS) was used as inhibitor for the formation of MAA homopolymers, since the radiation grafting was performed without oxygen using the mutual technique.

1,6-Hexamethylendiamine (HMDA) was used as a spacer, and glutaraldehyde (GLU) or cyanuric chloride were used as coupling agents to bind the enzyme to the activated membranes.

β-Galactosidase (EC. 3.2.1.23) from *Aspergillus Oryzae* was used as a catalyst, chosen in view of the use of these catalytic membranes in the process of lactose hydrolysis in milk. The β-galactosidase activity was assessed by the GOD-Perid method for the glucose determination (Boehringher GmbH, Mannheim, Germany). All chemical products, including the enzyme, were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification.

Catalytic membrane preparation

Membrane grafting was performed by irradiation with γ-rays. The irradiation source was caesium 137 in a γ-cell 1000 Elite by Nordion International Inc. (Kanata, ON, Canada). The average dose rate in the core of the radiation chamber (central dose rate) was of 2.35×10^4 rad h^{-1}. Four membrane types were prepared, each one with a different grafting technique, monomer and coupling agent used. For this reason, each membrane type will be identified by a number, and the grafting procedure as well as the methodology for membrane activation clearly indicated. A 10 mg/mL β-galactosidase solution was used for the immobilization process. The solution was prepared dissolving the enzyme in 0.1 M phosphate buffer solution, pH 6.5 .

Membrane no.1

These membranes were obtained by grafting MAA on the Teflon and using HMDA as a spacer. β-Galactosidase was coupled to the activated membrane by means of glutaraldehyde. The resulting catalytic grafted membranes were Teflon/MAA/HMDA/GLU/enzyme (Figure1a). The experimental conditions used during grafting were: 10% (v/v) MAA concentration; 0,1 % (w/v) FAS concentration; and 10 hours of irradiation time. These conditions were found to be optimal in a preliminary work.

At the end of the grafting procedure the membranes were washed under running tap water to remove the unbound monomers and the homopolymer produced. The grafted membranes thus obtained were divided into two lots, the first to estimate the grafting percent and the second used to bind the enzyme. The latter was immersed in a 10% (w/v) HMDA aqueous solution, then washed with water before a further treatment with 2.5% (v/v) glutaraldehyde aqueous solution. Both processes were carried out at room temperature for one hour. The enzyme immobilization was done by immersing the preactivated membrane in the enzyme solution at 4 °C for 16 hours. Next, the enzyme was bound to the carboxylic group of the Poly-MAA (PMAA) branches via glutaraldehyde and HMDA.

Membrane no.2

These membranes were prepared by performing a second grafting before the attachment of the spacer and subsequent coupling of the enzyme. The resulting catalytic grafted membranes (Figure 1b) were Teflon/MAA/HEMA/HMDA/GLU/enzyme.

The first grafting was performed in the same way as that of membrane no. 1, while the second grafting was done by subsequent irradiation for 16 hours in the gamma cell and a 10% (v/v) HEMA concentration. Enzyme immobilization was carried out according to the procedure described for membrane no. 1, i.e. to the carboxylic group of the PMAA branches via HMDA and glutaraldehyde.

Membrane no.3

These membranes were prepared by the double grafting technique (first grafting with MAA and second grafting with HEMA), but using cyanuric chloride as coupling agent. After the

two grafting procedures were performed under the conditions described above, the membranes were immersed for 10 minutes in a 0.2 N NaOH aqueous solution. After this step the membranes were interposed between two paper filters to remove the NaOH excess from the membrane surfaces. At this point the membranes were immersed in 2.5% (w/v) cyanuric chloride/acetone solution for 20 minutes at room temperature. The cyanuric chloride excess was then removed by washing the membranes first with an acetone/water solution and subsequently with pure water. Enzyme immobilization was performed following the procedure described above, so that the enzyme was bound to the hydroxyl group of the HEMA branches via cyanuric chloride. The resulting catalytic grafted membranes were Teflon/MAA/HEMA/ /cyanuric chloride/enzyme (Figure 1c).

Membrane no.4

These membranes were prepared using only the double grafting technique, with the enzyme dissolved directly in the HEMA solution used during the second grafting. The experimental conditions were the same as for membrane no. 2. The resulting catalytic grafted membranes (Figure 1d) thus were Teflon/MAA/HEMA/enzyme, with the enzyme entrapped between PMAA and poly-HEMA (PHEMA) grafted branched chains.

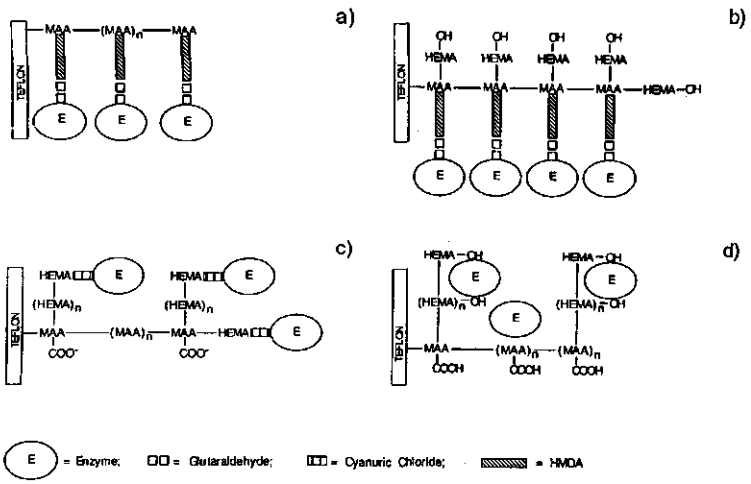

Figure 1. Hypothetical picture of the location of the immobilized β-galactosidase in the four membrane types: a) membrane no. 1; b) membrane no. 2; c) membrane no. 3; d) membrane no. 4.

We adopted the classical definition for the grafting degree. The degree of grafting (X, %) was determined by the difference between membrane masses before (G_B) and after (G_A), the grafting thus expressed by the formula:

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

Determination of catalytic membrane activity

For the determination of the activity of the catalytic membranes they were put in 20 ml of a well-stirred 0.1 M buffer phosphate solution, at the predetermined pH and temperature, containing lactose at a 200 mM concentration. Enzyme activity was determined by sampling the solution in contact with the membrane at regular time intervals and measuring the glucose concentration by the GOD-Perid test. The test uses a coupled enzyme reaction by which a coloured solution is obtained according to the scheme:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} \text{coloured complex} + H_2O$$

where GOD is glucose oxidase enzyme, POD peroxidase enzyme, and ABTS substrate for POD. The glucose concentration is proportional with the intensity of the solution colour, which is spectrophotometrically determined. Membrane activity, expressed as μmoles min^{-1}, is represented by the slope of a linear plot of the glucose production as a function of time. In the study of the activity as a function of the pH, 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 6-8 pH range.

Determination of the time stability of the catalytic membranes

The time stability of the biocatalytic membranes was assessed by analyzing their activity under the same experimental conditions every day. After three days, during which the

membranes lost some activity, a stable condition was reached remaining unchanged for over two months. Only these stabilized membranes were used in the comparative experiments reported on below. When not in use the membranes were stored at 4°C in 0.1 M buffer phosphate solution, pH 6.5.

Treatment of experimental data

Each experimental point reported in the figures represents the average value of four experiments performed under the same conditions. The experimental errors did not exceed 6%. The duration of each experiment was 30 minutes, but only the initial reaction rates were accounted for in the construction of the figures.

RESULTS AND DISCUSSION

Before discussing the results, it is relevant to know the microenvironment in which the biocatalyst operates, since it directly affects the enzyme activity. Two different immobilization methods were used: covalent binding for membranes no. 1, 2 and 3, and entrapment for membrane no. 4. The enzyme was bound to membranes no. 1 through the carboxylic groups of PMAA branches, making the microenvironment around the enzyme neutral. The biocatalyst was bound to membranes no. 2 through the carboxylic groups of the PMAA branches in the presence of the hydroxylic groups of the PHEMA branches, making the enzyme microenvironment alkaline. In the case of the membranes no. 3 the enzyme was attached to the hydroxyl groups of the PHEMA branches in the presence of the -COOH groups of the PHEMA branches, making the microenvironment in which the enzyme is operating acid. In the case of membranes no. 4 the enzyme was entrapped within the -OH groups of the PHEMA branches and the -COOH groups of the PMAA.

These considerations must be taken in account while discussing the results, especially when it causes the pH dependence of the catalytic membrane activity. This is only one of the aspects of the influence of the microenvironment on the enzyme activity. Immobilization can change the kinetics and other properties of the enzyme. Changes of the enzymatic properties are

thought to be caused by several factors, indicating conformational, steric, partitioning and mass transfer effects.

Grafting values

The average values of the MAA grafting percentage were (8 ± 2)% for all membrane types; those average values of the HEMA grafting percentage resulted (26 ± 3)%.

Temperature dependence of catalytic membrane activity

The temperature dependence of enzyme activity is represented by a bell-shaped curve with an activity optimum. The curve profile for the immobilized enzyme can be broader, narrower or equal to the one of the free enzyme, while the activity optimum generally shifts toward higher temperatures upon immobilization. This means a higher resistance to thermal inactivation of the protein because the structure of the catalytic site is strengthened by the immobilization procedure which creates strong bonds between the macromolecule and the carrier. If the position of the maximum remains unchanged, one might conclude that the structure of the active site and the microenvironment in which it is operating are the same in the free and bound forms. Figure 2 reports the temperature dependence of β-galactosidase activity of the four types of catalytic membranes, prepared using covalent (Figure 2a, b and c) or entrapment immobilization (Figure 2d). The temperature profile remains almost unchanged, except for the optimum activity position that is shifted towards higher temperatures for all the membranes. This shift is more evident in the case of enzyme bound to the carboxylic groups of PMAA grafted branches on membranes no. 1 and no. 2. The same behaviour is observed for the enzyme entrapped in membrane no. 4. The enzyme immobilized through the hydroxyl groups of the PHEMA branches on membrane no. 3 shows a less appreciable shift of the optimum temperature. Defining 'optimal temperature range' the range in which the relative activity is comprised between 95 and 100%, we see that this range occurs between 45 and 51 °C for the free enzyme, between 50 and 62 °C for membranes no. 1 and no. 2, between 50.5 and 53.5 °C

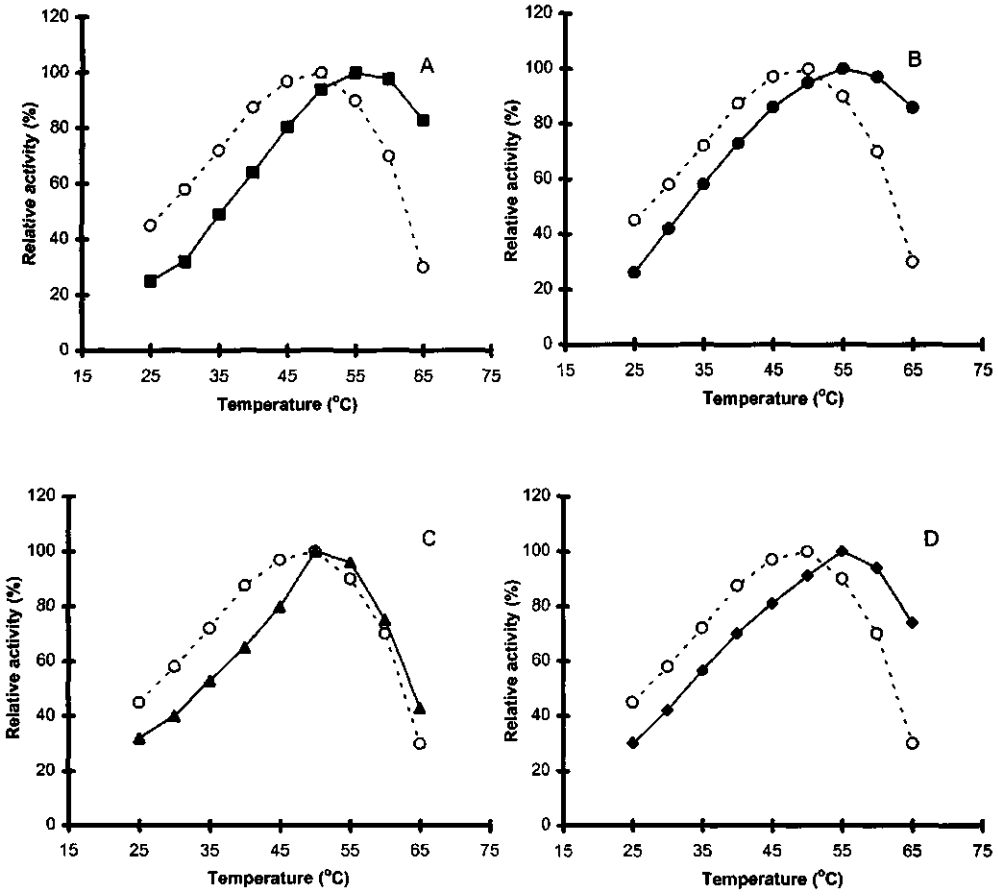

Figure 2. Relative activity of the free and immobilized form of the β-galactosidase in the four membrane types as a function of temperature: (A) membrane no. 1: (■)= immobilized form, (○)= free enzyme;; (B) membrane no. 2: (●)= immobilized form, (○)= free enzyme; (C) membrane no. 3: (▲)= immobilized form, (○)= free enzyme; (D) membrane no. 4: (◆)= immobilized form, (○)= free enzyme.

for membrane no. 3, and between 54 and 58 °C for membrane no. 4. From these observations it is can be deduced that membranes no. 1 and no. 2 are more promising for industrial processes requiring high temperatures, then subsequently membranes no. 4 and no. 3.

Similar conclusions can be drawn from the results of Figure 3 where the percentage of the enzyme inactivation is reported as a function of the temperature. Again, it is clear that at high temperatures the catalytic membrane activity follows the order: membrane no. 2 > membrane no. 1 > membrane no. 4 > membrane no. 3 > free enzyme. For example, at 65 °C, the catalytic power of the membrane is 12% less for membrane no. 2, 15% for membrane no. 1, 28% for membrane no. 4, 57% for membrane no. 3 and 70% for the free form of the enzyme. These data clearly indicate the influence of the microenvironment on the enzyme activity, membranes no. 1 and no. 2 ensuring more protection to the enzyme structure and, hence, to the enzyme activity.

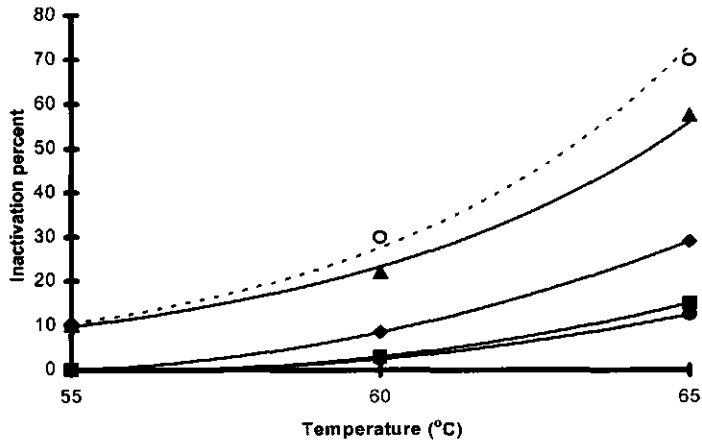

Figure 3. Percentage of enzyme inactivation as a function of temperature of the free and immobilized form of β-galactosidase: free form, (○); membrane no. 1; membrane no. 2: (●); membrane no. 3: (▲); membrane no. 4: (◆).

pH dependence of catalytic membrane activity

It is well known that the pH of the aqueous medium in which the biocatalyst operates plays a relevant role in enzyme activity. This role is more evident when the enzyme is immobilized, since the support itself may change the pH value around the catalytic site, thus determining differences in the behaviour between the free and bound states of the catalyst. This effect is known as the partitioning effect, directly related to the chemical nature of the supporting

material which induces electrostatic or hydrophobic interactions between the matrix and the molecular species dissolved in the solutions. These interactions alter the microenvironment in which the enzyme actually operates. Because of this effect, different concentrations of charged species, as H^+ or OH^-, exist in the microenvironment of the immobilized enzyme. Consequently, the local pH around the catalytic site differs from that of the bulk solution. Thus the pH profile of the activity of the immobilized enzyme compared to that of the free form is displaced toward more alkaline or acid pH values for negatively or positively charged matrices[31], respectively. Bearing these considerations in mind we investigated the activity of free and immobilized β-galactosidase in the pH range between 2 and 7. The results are reported in Figure 4 where the relative activity of each of the four catalytic membranes is reported as a function of the pH of the bulk solution. For comparison, the relative activity of the free enzyme is also shown. The temperature was kept constant at 30°C. Optimal activity of the free enzyme was found at pH 4.5, while a displacement towards a more acid pH value was observed with three membranes in which the catalyst was covalently bound to the solid support. The entrapped enzyme, however, did not exhibit relevant changes during maximal activity. The behaviour of membranes no. 1 and no. 2 may be attributed to the formation of Schiff's bases. We have no explanation for the behaviour of membrane no. 3 which we expected to shift towards the alkaline side owing to the absence of the formation of Schiff's bases and the concomitant presence of the negatively charged carboxylic groups of the PMAA branches. The behaviour of membrane no. 4 appears reasonable since the simultaneous presence of carboxylic groups of the PMAA and hydroxyl groups of PHEMA made the microenvironment around the enzyme practically neutral. The result was an almost similar profile for the free and immobilized form of the enzyme. The definition of the 'optimal pH range' the range of relative activity between 95 and 100% is between 4.5 and 4.9 for the free enzymes; between 3.9 and 4.1 for membrane no. 1; between 3.7 and 4.3 for membrane no. 2; between 3.95 and 4.65 for membrane no. 3; and between 4.45 and 4.95 for membrane no. 4. From the results of Figure 4 other considerations regarding the role of the microenvironment on enzyme activity can be deduced. While at high pH values the immobilized and free forms of the enzyme do not exhibit appreciable differences in the values of the relative activity,

marked differences exist at low pH values where the relative activity of the immobilized form always higher than that of the free form. This means that the immobilized form is more

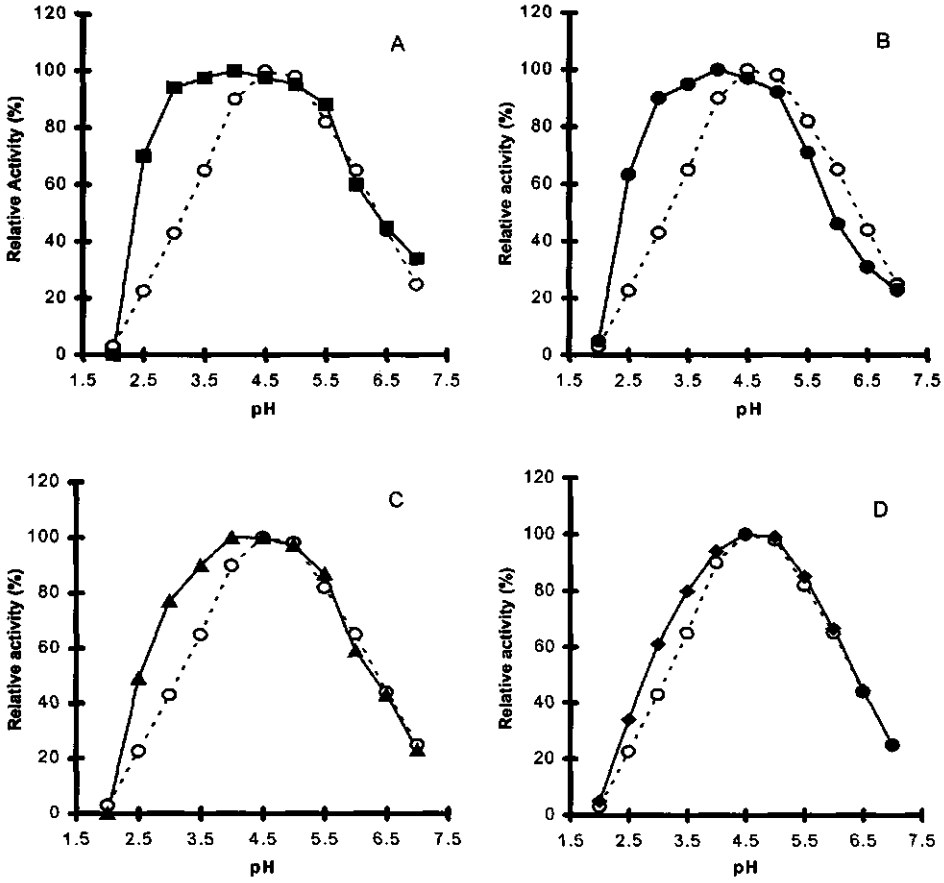

Figure 4. Relative activity as a function of pH of the free and immobilized form of the β-galactosidase in the four membrane types: (A) membrane no. 1: (■)= immobilized form, (○)= free enzyme; (B) membrane no. 2: (●)= immobilized form, (○)= free enzyme; (C) membrane no. 3: (▲)= immobilized form, (○)= free enzyme; (D) membrane no. 4: (◆)= immobilized form, (○)= free enzyme.

protected than the free form in more acidic solutions. For example: at pH 2.5 the relative activity of the free form is 20%; 75% for membrane no. 1; 65% for membrane no. 2; 50% for membrane no. 3; and 35% for membrane no. 4. This is a useful indication for the preparation of catalytic membranes to use in industrial biotechnological processes.

Kinetic parameters

When a biocatalyst is immobilized the kinetic parameters K_m and V_{max} undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change of the substrate. These variations can be attributed to several factors such as protein conformational changes induced by the attachment to the support, steric hindrances and diffusional effects. These factors, which may operate simultaneously or separately, alter the microenvironment around the enzyme. This means that the apparent K_m value of the immobilized form may decrease [32-33] or increase [34-35]. 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 certainly decreases if the electrical charge on the support and substrate are opposite. The reverse occurs if the support and the substrate have the same electrical charge. This is a classical example of how the microenvironment affects the reaction rate of an enzymatic process. Furthermore, the immobilization process also affects the V_{max} values. Similar values of V_{max} have been found for the free and the immobilized form of the enzyme, but also increases [36] or decreases [37] have also been reported. To determine the

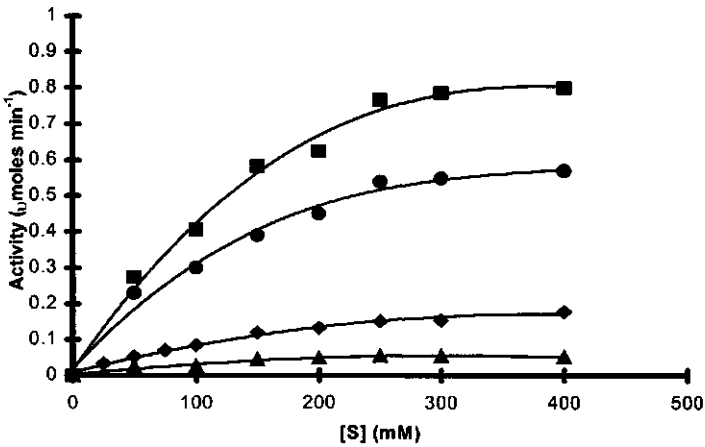

Figure 5. Absolute activity as a function of the initial substrate concentration for β-galactosidase in the four membrane types. Symbols: (■) = membrane no. 1; (●) = membrane no. 2; (▲) = membrane no. 3; (◆) = membrane no. 4.

microenvironmental influences on the kinetic parameters of β-galactosidase immobilized on the four types of membranes the activity of each catalytic membrane was studied as a function of substrate concentration. The pH and temperature of the solutions were 6.5 and 30 °C, respectively. The results can be found in Figure 5. Since the same initial enzyme concentration was used to load the four types of membranes, the differences shown in the figure indicate that the yield of immobilization is different. The absolute activity follows the order: membrane no. 1 > membrane no. 2 > membrane no. 4 > membrane no. 3. Membrane no. 1 and membrane no. 2 should theoretically have the same number of initial free radicals to which the MAA attachment occurs and, hence, the same number of MAA chains. In this respect, their activity should have been the same. However, the presence of branches due to PHEMA creates steric hindrances to the enzyme immobilization process (thus limiting the amount of the immobilized enzyme) and to the diffusive approach (or removal) of substrate (or of products) towards (or from) the catalytic site. This justifies the higher activity of membrane no. 1. Membrane no. 2 and membrane no. 3, on the other hand, have the same grafting history, thus their activity should be the same. On the contrary, the results in the figure show an activity of membrane no. 3 to be one order lower than that of membrane no. 2. This discrepanary in behaviour can be attributed to the presence of the HDMA as spacer on membrane no. 2. Indeed, the spacer keeps the enzyme further away from the membrane thus reducing the interactions with the support. The behaviour of membrane no. 4, can be explained by the presence of the dense polymeric net constituted by PHEMA and PMAA branches, resulting in a close barrier entrapping the enzyme and restricting the diffusional rates of substrate and products. A Hanes plot of the results in Figure 5 allows the calculation of the apparent kinetic parameters of the β-galactosidase immobilized on the four membrane types. This was done (Figure 6) with the apparent values of K_m and V_{max} were reported in Table I, together with those of the free enzyme. Remarkably, the most active membranes exhibit a lower affinity for the substrate. This is a further clear indication of the fact that: **I)** the immobilization technique affects the amount of immobilized enzyme; **II)** the microenvironment influences the rate of the enzyme reaction and the affinity for the substrate.

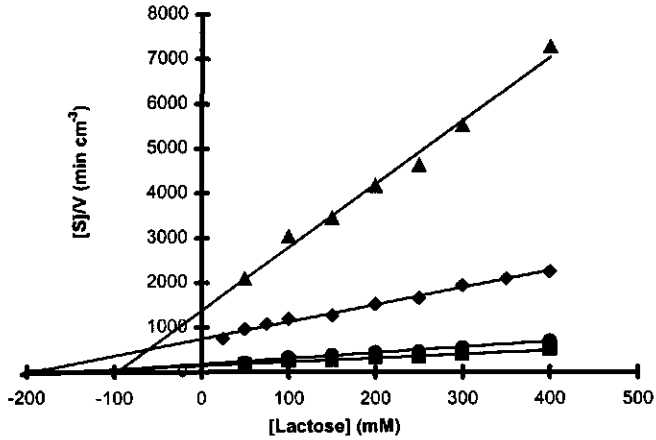

Figure 6. Hanes plot for β-galactosidase. Symbols: (■) = membrane no. 1; (●) = membrane no. 2; (▲) = membrane no. 3; (◆) = membrane no. 4.

CONCLUSION

This study, focused on the influence of the microenvironment on the activity of immobilized enzymes, achieved its objective. The pH and temperature profiles of the relative activity of the four membranes were observed to be different from each other and from the free form of the enzyme. Membranes no. 1 and no. 2 proved to be more suitable for practical applications because they offer more protection to the enzyme activity at high temperatures and at alkaline pH values. It has also been shown that the kinetic parameters are influenced by the microenvironmental properties in which the enzyme operates, while the absolute activity depends on the immobilization method. The axiom 'more activity, more affinity' in our case does not hold very well. Here it was, the more active the membranes, the less affinity for the substrate. The lower affinity of the substrate shown by the immobilized form as compared to the free enzyme can be overcome by employing the catalytic membranes in bioreactors

Table I. Apparent kinetic parameters K_m and V_{max} for the free and immobilized form of the β-galactosidase in the four membrane types. The values for the free enzyme were calculated by us in a previous work and are real values.

Enzyme system	K_m^{app} (m M)	v_{max}^{app} (μmoles min^{-1})
Free enzyme	21.4	3.2
Membrane no. 1	171.2	1. 20
Membrane no. 2	140.0	0.79
Membrane no. 3	97.6	0.074
Membrane no. 4	191.3	0.526

operating under non-isothermal conditions. Under these conditions we earlier observed not only an enzyme activity increase, but also an increase in affinity for the substrate [35-30]. Indeed, the K_m values of the catalytic membranes operating under non-isothermal conditions were lower than the values of the same membranes operating in isothermal bioreactors. These results were obtained with catalytic hydrophilic membrane coupled to a hydrophobic Teflon membrane. The same results together with an efficiency increase were recently obtained with a non-isothermal bioreactor employing a Teflon grafted membrane [38], not only hydrophobic but also catalytic. If the technology of the non-isothermal bioreactors is applied to the catalytic membranes prepared for this research, we expect an improvement of their enzymatic yield and an increase of the bioreactor efficiency. Experiments to this end are now in progress in our laboratory.

ACKNOWLEDGEMENTS

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REFERENCES

1. Taylor, R.F. (1991). *Protein Immobilization: Fundamentals and Applications*. New.York, Marcel Decker Inc.

2. Tanaka, A., T. Tosa, & T. Kobayashi (1993). *Industrial Application of Immobilized Biocatalysts*. New.York, Marcel Decker Inc.

3. Bickerstall, G.F. (1997). *Immobilization of enzyme and cells*. New Jersey,Humana Press.

4. McCormick, C. & L. Park (1984). *J. Polym. Sci.* **22:** 49

5. Godjevargova, T. S., A. R. Dimov, & N. Vasileva (1994). *J. Appl. Polym. Sci.* **54:** 355

6. Godjevargova, T. S. & A. R. Dimov (1995). *J. Appl. Polym. Sci.* **57:** 487

7. Garnett, J. L., S. V. Jankiewicz, R. Levot, & D. F. Sangster (1985). *Rad. Phys. Chem.* **25:** 509

8. Kaetsu, I., M. Kumakura, T. Fujimura, M. Yoscida, M. Asano, N. Kasai, & M. Tamada (1986). *Rad. Phys. Chem.* **27:** 245

9. Hoffman, A. S., W. R. Gombotz, S. Uoenoyama, L. C. Dong & G. Schmer (1986). *Rad. Phys. Chem.* **27:** 265

10. J. L. Garnett, S. V. Jankiewicz, R. Levot & D. F. Sangster (1986). *Rad. Phys. Chem.* **27:** 301

11. Arica, Y. & V.N. Hasirci (1987). *Biomaterials*. **8:** 489

12. Carenza, M. & G. Palma (1988). *Ann. N.Y. Acad. Sci.* **542:** 115

13. Alves da Silva, M., C. G. Beddows, M. H. Gill, J. T. Guthrie, A. J. Guiomar, S. Kotov & A. P. Piedade (1990). *Rad. Phys. Chem.* **35:** 98

14. Docters, E. H., E. E. Smolko & C. E. Suarez (1990). *Rad. Phys. Chem.* **35:** 102

15. Hajizadhe, K., H.B. Halsall & W.R. Heinemann (1991). *Anal. Chim. Acta.* **243:** 23

16. Hegazy, E.S.A., A.M. Dessouki, M. El-Sawy & M.A. El-Ghafar (1993). *J. Polym. Sci.* **A31:** 527

17. Doretti, L., D. Ferrara & S. Sora (1993). *Biosensors and Bioelectronics.* **8:** 443

18. Tarhan, L. & B. Perkin (1983). *Biotechnol. Bioeng.* **25:** 2773

19 Halcock, H.R. & S. Kwon (1986). *Macromolecules.* **8:** 1502

20. Kotorman, M., M.L. Simon, B. Szajani & L. Boross (1986). *Biotechnol. Appl. Biochem.* **8:** 53

21. Findlay, G.J., K.L. Parkin & R.Y. Yada (1986). *Biotechnol. Lett.* **8:** 649

22. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 613

23. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 625

24. Mita, D. G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69

25. Mita, D. G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F. S. Gaeta (1995). *Biotechnol. Applied Biochem.* **22:** 281

26. Russo, P., A. Garofalo, U. Bencivenga, R. Rossi, D. Castagnolo, A. D'Acunto, F. S. Gaeta & D. G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141

27. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, P. Canciglia, F. Palumbo, F. S. Gaeta & D. G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25

28. Russo, P., A. De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F. S. Gaeta & D. G. Mita (1997). *Research in Microbiology.* **148:** 271

29. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D. G. Mita (1997). *J. Membrane Sci.* **129:** 175

30. Febbraio, F., M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F. S. Gaeta & D.G. Mita (1998). *Biotech. Bioeng.* **59:** 108

31. Goldstein, L. & E. Z. Katchalcki (1968). *Anal. Chem.* **243:** 375

32. May, S. W. & N. N. Li (1972). *Biochem. Biophys. Res. Commun.* **47:** 1178

33. Horndy, W. E., M. D. Lilly & E. M. Crock (1968). *Biochem. J.* **107:** 668

34. Kleine, R., P. Spangerberg & C. Fleming (1976). *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 629

35. Sato, T., T. Mori & I. Chibata (1971). *Arch. Biochem. Biophys.* **147:** 788

36. Cabral, J. S. M., J. F. Kennedy & A. Novais (1982). *Enzyme Microb. Technol.* **4:** 343

37. Usami, S., E. Hasegarva & M. Karasawa (1975). *Hakko Kyokaishi.* **33:** 152

38. Mohy Eldin, M. S., A. De Maio, S. Di Martino, M. Portaccio, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F.S. Gaeta & D. G. Mita (1998). *J. Membrane Sci.* **146:** 237

Chapter 6

ISOTHERMAL AND NON-ISOTHERMAL LACTOSE HYDROLYSIS BY β-GALACTOSIDASE IMMOBILIZED ON DOUBLE-GRAFTED TEFLON MEMBRANES

ABSTRACT

A new hydrophobic and catalytic membrane was prepared by immobilizing β-galactosidase on a polytetrafluoroethylene (PTFE) membrane, double grafted by means of γ-radiation. Methacrylic acid (MAA) and 2-hydroxyethyl methacrylate (HEMA) were used as monomers to be grafted. 1,6-Hexamethylendiamine (HMDA) and glutaraldehyde (GA) were used as spacer and coupling agent, respectively.

The membranes were characterized physically and biochemically. The values of the hydraulic and thermoosmotic permeability coefficients were calculated. The dependence of catalytic membrane activity on the temperature, pH and substrate concentration was studied.

Lactose hydrolysis, investigated under isothermal and non-isothermal conditions, confirmed the usefulness of employing non-isothermal bioreactors in industrial processes. In particular, characteristic parameters were calculated of the non-isothermal process, such as α and β, indicative of the efficiency increase of the yield of enzymatic reaction when a difference of temperature of 1°C is applied across the membrane. These values were compared with those obtained so far with other membrane systems.

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INTRODUCTION

The binding of proteins to carriers in order to obtain insoluble systems has attracted the attention of scientists working in the field of the biotechnologythe last decades. Of great importance among the carriers used to immobilize enzymes are natural polymers and their derivatives. Particular attention has recently been foucused on carriers constituted by synthetic polymers [1-3] that may be treated to vary the character and amount of introduced functional groups and their physical characteristics, for instance, hydrophobicity. One of the prerequisites required from a carrier for the immobilization of enzymes operating in aqueous media is, indeed, its hydrophilicity, since this property facilitates access of the enzymes to the binding sites and ensures a microenvironment around the catalytic site not too different from that in which the free enzyme normally works. For this reason practically all catalytic membranes used in industrial processes or in analytical apparatuses, such as biosensors, have a hydrophilic character [4-7].

The physical process controlling the reaction rate is the diffusion of substrate and products toward and away from the catalytic site. This is the picture coming out when catalytic membranes are used in bioreactors or in biosensors operating under isothermal conditions.

More recently this picture has been subjected to some modifications since the advantage of employing catalytic membranes in non-isothermal bioreactors has been demonstrated [8-14]. Under these conditions it was found that the enzyme reaction proceeds at rates higher than those occurring in bioreactors operating under comparable isothermal conditions. The amount of reaction efficiency increase was dependent on the nature of the enzyme and on the immobilization method. This effect was observed with purified enzymes and with microorganisms. The membrane system consisted of a catalytic hydrophilic membrane coupled to a hydrophobic membrane, the latter being important because it induces the process of thermodialysis [15-19], which is necessary for the increase of enzyme activity under non-isothermal conditions. The term 'thermodialysis' indicates non-isothermal selective mass transport of water and solutes across an unselective hydrophobic membrane separating two solutions maintained at different temperatures. The force driving this transport is the temperature gradient.

With the aim of simplifying the double membrane system employed so far in non-isothermal bioreactors, we recently focused our attention on preparing single membranes that were both

hydrophobic and catalytic. This was achieved by grafting suitable monomers on hydrophobic commercial membranes by means of γ-radiation [20-23]. These membranes were biochemicaly and biophysicaly characterized under isothermal conditions. Only one of these membranes was successfully studied in bioreactors operating under non-isothermal conditions [24].

The aims of this paper are: I) to prepare a new grafted type of membrane, both catalytic and hydrophobic in nature; II) to characterize under isothermal conditions its behaviour with respect to its physical and its biochemical properties; III) to evaluate the advantage of using non-isothermal bioreactors; IV) to compare the present results with the ones obtained with other membrane systems.

MATERIALS AND METHODS

Materials

Teflon [polytetrafluoroethylene [PTFE)] membranes of the 450 type manufactured by the Gelman Instrument Company (Ann Arbor, MI, USA) were used as solid support for the grafting process. The membranes, consisting of a Teflon film supported on one side by a polypropylene net, were 150 mm thick and endowed with anastomizing irregular channels of 0.450 mm in nominal diameter. The nominal pore diameter of the membrane is one that is able to retain the smallest particles.

Methacrylic acid (MAA) and 2-hydroxyethyl methacrylate (HEMA) monomers were used for the grafting. Ferrous ammonium sulphate (FAS) was used as inhibitor for the formation of MAA homopolymers, as the radiation grafting was performed without oxygen, using the mutual technique. 1,6-Hexamethylene diamine (HMDA) and glutaraldehyde (GA) were used as spacer and coupling agents, respectively.

β-Galactosidase (EC. 3.2.1.23) from *Aspergillus Oryzae* was used as a catalyst. This enzyme was chosen in view of the employment of these catalytic membranes in the process of lactose hydrolysis in milk. The β-galactosidase activity was assessed by the GOD-Perid method for glucose determination (Boehringher GmbH, Mannheim, Germany).

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

Methods

Catalytic membrane preparation

Membrane grafting was performed by γ-irradiation. The irradiation source was caesium 137 in a γ-cell 1000 Elite by Nordion International Inc. (Kanata, ON, Canada). The average dose rate in the core of the radiation chamber (central dose rate) was 2.35 x 10^4 rad h^{-1}. A first grafting was performed by irradiating the PTFE membranes with a solution of methacrylic acid monomer and FAS in the gamma cell, thus obtaining a PTFE-grafted-polyMAA membrane. The experimental conditions used during the grafting were: 10% (v/v) MAA concentration; 0.1 % (w/v) FAS concentration; and 10 hours of irradiation time. At the end of the grafting procedure the membranes were washed under running tap water to remove the unbound monomer and the formed homopolymer. The grafted membranes thus obtained were divided into two lots, the first one employed to estimate the grafting degree, and the second one for the second grafting by subsequent irradiation for 16 hours with a 10% (v/v) HEMA concentration in the γ-cell. At the end of this step the membranes were again divided into two lots. The first lot was employed to estimate the grafting degree, the second was used for enzyme immobilization. The latter was first soaked in a 10% (w/v) HMDA aqueous solution and then immersed in a 2.5% (w/v) glutaraldehyde aqueous solution. Both processes were carried out at room temperature for one hour. Enzyme immobilization was done by immersing the preactivated membrane in the enzyme solution, obtained by dissolving the enzyme at a concentration of 10 mg/mL in a 0.1 M phosphate buffer solution pH 6.5, at 4 °C for 16 hours. The overall process of grafting, membrane activation and enzyme immobilization is schematically illustrated in Figure 1.

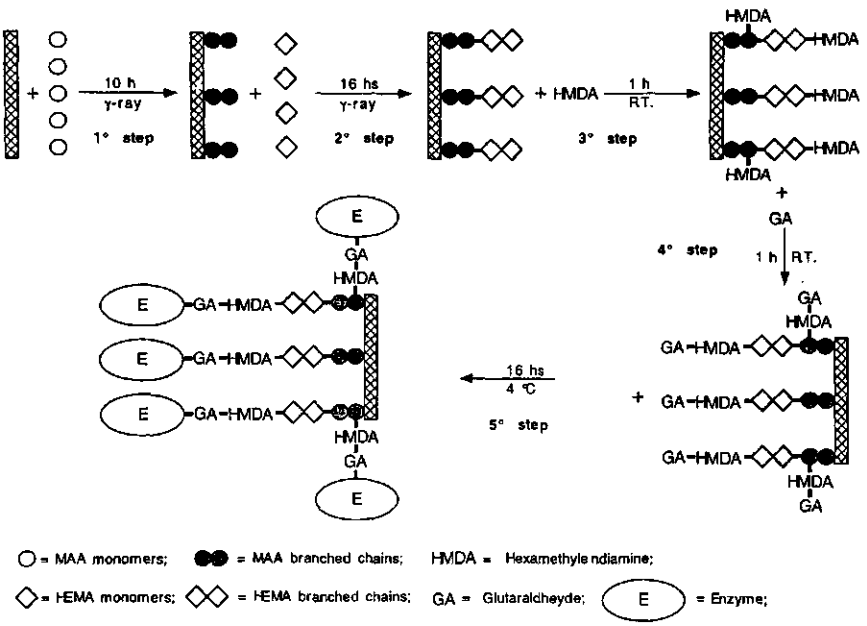

Figure 1. Schematic picture of the experimental steps followed during the processes of grafting and activation of the membrane and enzyme immobilization.

Determination of the grafting degree

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

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

Determination of catalytic membrane activity

Enzyme activity was determined from sampling, at regular time intervals, the solution in contact with the membrane and measuring the glucose concentration by the GOD-Perid test. The test consisted of a coupled enzyme reaction expressed as follows:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} \text{coloured complex} + H_2O$$

where GOD is glucose oxidase enzyme, POD peroxidase enzyme, and ABTS substrate for POD. The coloured solution was obtained was spectrophotometrically quantified. Glucose concentration, proportional to the colour intensity, were calculated from the readings. Membrane activity expressed as μmoles min^{-1} was obtained from the slope of a linear plot of the glucose production as a function of time.

Determination of the time stability of the catalytic membrane

The time stability of the biocatalytic membranes was assessed by analyzing their activity daily under the same experimental conditions. After three or four days, during which the membranes lost some activity, a stable condition was reached, remaining unchanged for over two months. Only these stabilized membranes were used in the comparative experiments reported below. When not in use the membranes were stored at 4°C in 0.1 M buffer phosphate solution, pH 6.5.

Treatment of experimental data

Each experimental point reported in the figures represents the average value of four experiments performed under the same conditions. The experimental errors did not exceed 6%. Each experiment lasted 30 minutes, but only the initial reaction rates were used in the construction of the figures.

The bioreactor

The apparatus consisted of two cylindrical half-cells (Figure 2), filled with the working solution containing the substrate separated by the catalytic membrane. In these experiments both solutions were recirculated in each half-cell by means of two peristaltic pumps through hydraulic circuits starting and ending in the common cylinder C. Each half-cell was controlled at a temperature T_i (i=1,2). When the apparatus worked under isothermal conditions T_1 was equal to T_2. Thermocouples, placed 1.5 mm from each of the membrane surfaces enabled measurement of the temperatures inside each half-cell and calculation of the temperature profile across the catalytic membrane when the apparatus is kept under non-isothermal conditions. The temperatures read by the thermocouples are indicated by T, and the temperatures calculated at the membrane surfaces by the symbol T*. The values related to warm and cold sides are indicated by the subscripts w and c, respectively. Under these assumptions $\Delta T = T_w - T_c$ and $\Delta T^* = T_w^* - T_c^*$, as well as $T_{av} = \frac{(T_w + T_c)}{2}$ and $T_{av}^* = \frac{(T_w^* + T_c^*)}{2}$. In non-isothermal experiments $T_w^* < T_w$, $T_c^* > T_c$ and $\Delta T^* < \Delta T$.
When the apparatus functioned as permeability cell or thermodialysis cell to measure hydraulic or thermo-osmotic water fluxes alternative hydraulic circuits and devices were used. The 'modus operandi' of the apparatus during these experiments will be described in the appropriate section.

RESULTS AND DISCUSSIONS

Before illustrating the results of lactose hydrolysis under isothermal and non-isothermal conditions, we will explaine how we characterized the catalytic membrane from the physical and biochemical points of view. Instrumental in understanding the results is the knowledge of the values of the grafting degree. For the first grafting we have found an MAA grafting degree equal to (8.5 ± 2.1)%; for the second grafting a HEMA grafting degree equal to (26.0 ± 5.5)%.

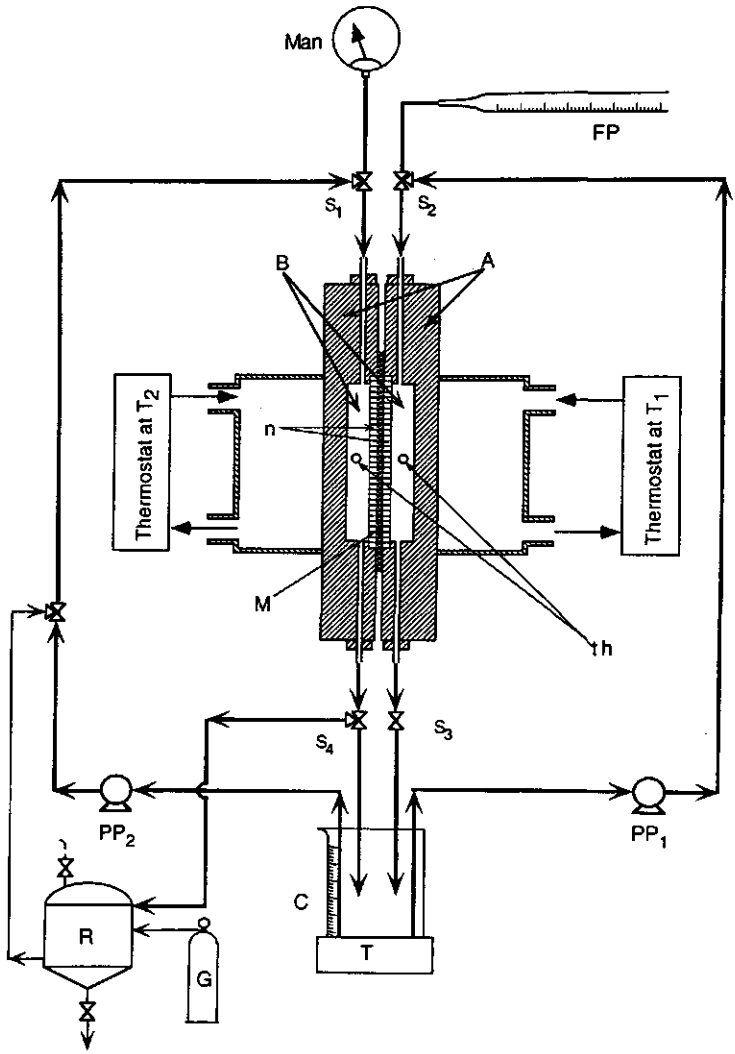

Figure 2. Schematic (not to scale) representation of the bioreactor. (A)=half-cells; (B)=internal working volumes; (C)=external working volume; (M)=membrane; (n)=supporting nets; (th)=thermocouples; (S_i)=stopcocks; (T)=thermostatic magnetic stirrer; (PP_i)=peristaltic pumps; (Man)= manometer; FP(flow-pipe); R=(reservoir of work solution); G=(pressurizing air tank).

Membrane characterization

Physical characterization

Since the grafted membranes are physically different from the untreated ones, it is interesting to study how the grafting process affects the transport parameters of the membrane. The hydraulic permeability coefficient A and the thermo-osmotic coefficient B are two parameters controlling the thermodialysis process. These coefficients are defined by the equations:

1) $$J_{volume} = \frac{m^3}{m^2\ sec} = A\frac{\Delta P}{\Delta x}$$

2) $$J_{volume} = \frac{m^3}{m^2\ sec} = B\frac{\Delta T}{\Delta x}$$

describing the volume flow (expressed in $m^3\ m^{-2}\ s^{-1}$) across a membrane, catalytic or not, produced under isothermal conditions by a hydraulic pressure gradient $\Delta P/\Delta x$ measured in $(N \cdot m^{-3})$ or under non-isothermal conditions by a temperature gradient $\Delta T/\Delta x$ measured in $(K \cdot m^{-1})$. A is expressed in $m^4\ s^{-1}\ N^{-1}$ and B in $m^2\ s^{-1}\ K^{-1}$.

Hydraulic fluxes were determined by pressurizing one half-cell by a gas cylinder and measuring the water volume transported to the other half cell by means of a graduated pipe. The temperature of the apparatus in these measurements was kept constant. Thermo-osmotic fluxes were determined by measuring in the graduated pipe the water volume transported from the warm half-cell to the cold half-cell, in the absence of a pressure gradient.

Figures 3A and 3B give some water fluxes measured under pressure gradients or temperature gradients, respectively. In Figure 3A, the hydraulic permeability coefficients A are represented by the angular coefficient of lines best fitting the experimental points. In the same manner in figure 3B, the thermo-osmotic permeability coefficients B are represented by the angular coefficients of the lines best fitting the experimental points.

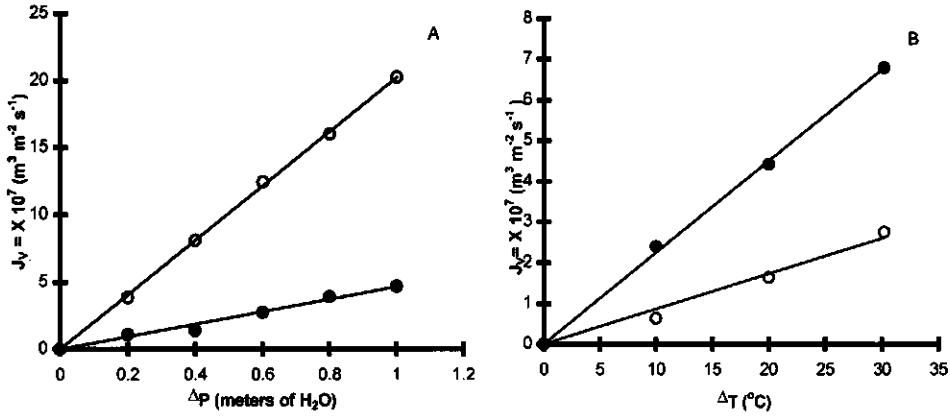

Figure 3. (A) Hydraulic water fluxes across the treated (o) and untreated (•) membrane as a function of pressure difference, temperature is 30 °C. (B) Thermo-osmotic water fluxes across the treated (o) and untreated (•) membrane as a function of temperature difference; T_{av} is 30 °C.

The values of the coefficient A and B as a function of temperature are respectively reported in Figures 4A and 4B. In figure 4B the temperature value is that of the average temperature, i.e. $T_{av} = \frac{T_W + T_C}{2}$ where T_W and T_C are the temperatures of the warm and cold half-cells, respectively. From the figure it is possible to observe how the grafted membranes have a greater value of A and smaller value of B as compared to the corresponding values of the untreated membrane. This means that somehow the treatment increases the hydraulic permeability and reduces the thermo-osmotic permeability of the membrane, which however remains hydrophobic in as much as it still provides thermodialysis. From equations 1 and 2 one can calculate the values of the steady-state pressure, $\Delta P_{s\text{-}s}$. This is the pressure able to stop the water flux from the warm to the cold half-cell produced across the membrane by the applied temperature difference. From the same equations one can also calculate the γ coefficient, i.e. the pressure produced by a unit temperature difference across the membrane. In this way we get:

$$3) \qquad \Delta P_{s-s} = \frac{B}{A} \Delta T$$

$$4) \qquad \gamma = \frac{\Delta P_{s-s}}{\Delta T} = \frac{B}{A}$$

The values of γ are given in Table I. For $\gamma>0$ thermo-osmotic flow is observed, for $\gamma=0$ no thermo-osmotic flow occurs. This means that it is possible to foresee the yield of a separation process by thermodialysis once the γ value for a given membrane has been measured. For example, starting with an equal initial lactose solution (0.2 M) in the two half-cells kept at

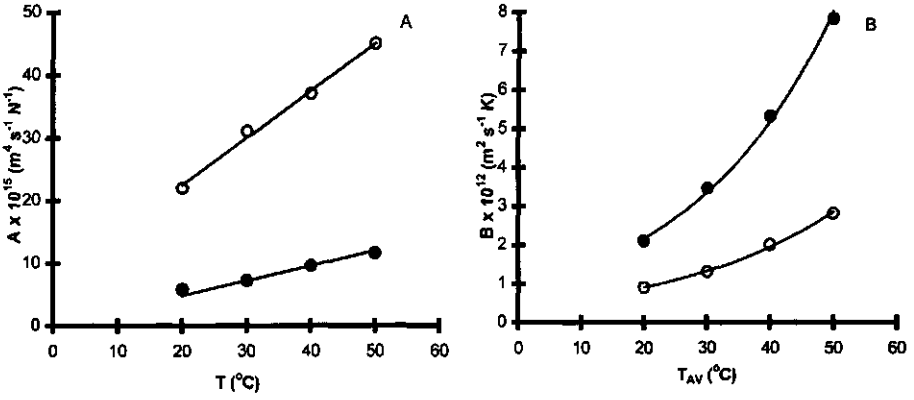

Figure 4. (A) Hydraulic permeability coefficient across the treated (o) and untreated (•) membrane as a function of temperature. (B) Thermo-osmotic permeability coefficient across the treated (o) and untreated (•) membrane as a function of average temperature.

T_W=60°C and T_C=20°C, respectively, and allowing water transport by thermodialysis, we have found after one hour of experimentation a concentration ratio (C_W/C_C) for the treated membrane equal to 1.3 and 2.5 for the untreated membrane. C_W and C_C are the lactose concentrations after 60 minutes in the warm and cold half-cells, respectively.

Table I. Values of the γ coefficient defined by equation 4).

Membrane system	$T_{average}$ (°C)	γ (N. m^{-2} . K^{-1})
Untreated membrane	20	370
	30	484
	40	556
	50	674
Treated membrane	20	40
	30	42
	40	54
	50	62

Biochemical characterization

For the biochemical characterization of the membrane we studied the dependence of the catalytic activity on temperature, pH and substrate concentration. The results of this study are reported in Figure 5. Figure 5A shows the relative membrane activity as a function of temperature. The relative activity of the free enzyme is also shown for comparison. The substrate solutions used for these experiments were 0.2 M lactose in a 0.1 M phosphate buffer solution, pH 6.5. Examination of the figure shows a shift of the optimum temperature toward higher temperatures in the case of the immobilized form. This means that immobilized β-galactosidase exhibits a higher resistance to thermal inactivation, probably since the structure of the catalytic site is strengthened by the immobilization process which creates strong bonds between the macromolecule and the modified carrier. Two other observations can also be made from the figure. In the first place, defining 'optimal temperature range' the range in which the relative activity is comprised between 95 and 100%, the figure shows that this range occurs between 45 and 51°C for the free enzyme and between 50 and 62 °C for the immobilized one. The second observation concerns the number of catalytic molecules still

active at high temperatures. For example, at 65 °C the catalytic power of the free enzyme is reduced by 70% and that of the immobilized enzyme by only 12%. From this observation we can conclude that the prepared catalytic membrane is promising for use in non-isothermal

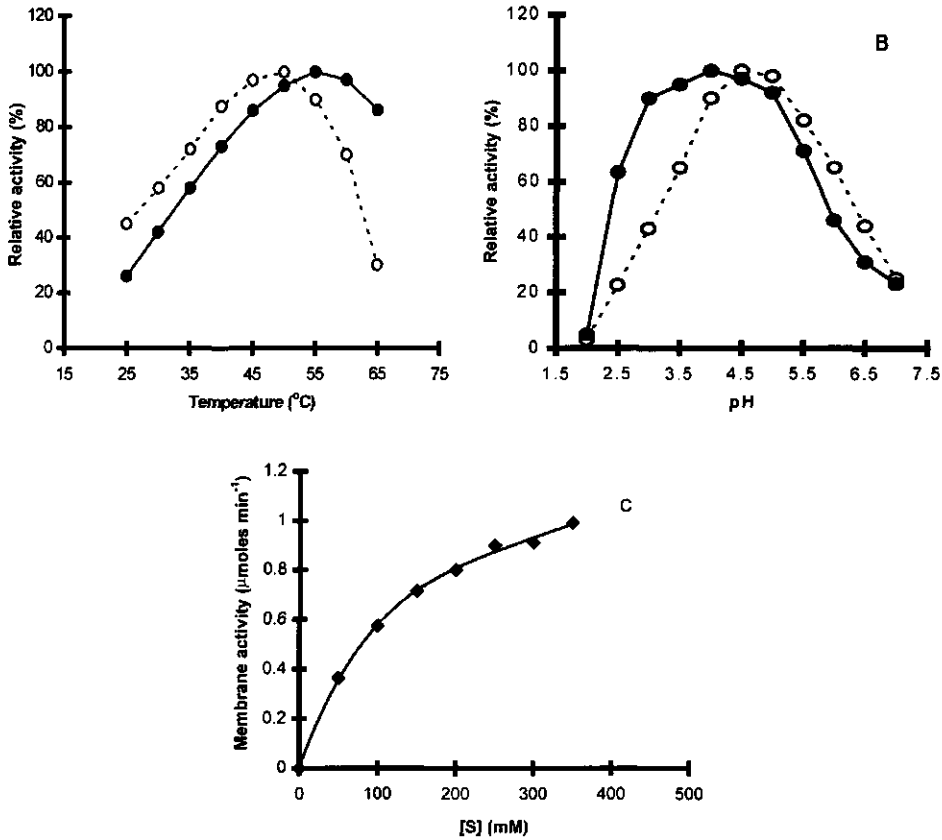

Figure 5. (A) β-Galactosidase relative activity as a function of temperature at pH 6.5. Symbols: (o) free enzyme; (•) immobilized enzyme. (B) β-Galactosidase relative activity as a function of pH at temperaturet 30 °C. Symbols: (o) free enzyme; (•) immobilized enzyme. (C) Membrane activity as a function of substrate concentration. Experimental conditions : T = 30 °C; pH = 6.5.

bioreactors, where higher temperatures are required for the creation of higher transmembrane temperature gradients. Figure 5B shows the membrane activity as a function of the pH. The relative activity of the free enzyme is also rgiven for comparison. The substrate solution used for these experiments were 0.2 M lactose in 0.1 M NaCl-HCl buffer solution at pH 2, in 0.1 M citrate buffer solution for the pH range between 3 and 5, and 0.1 M phosphate buffer solution

at pH ranges between 6 and 8. Temperature was kept constant at 30 °C. Optimal activity of the free enzyme was found at pH 4.5, while a displacement toward a more acid pH value of 3.8 was observed for the immobilized β-galactosidase. This shift is attributed to the partitioning effect directly related to the chemical nature of the supporting carrier, which generally induces electrostatic or hydrophobic interactions between the matrix and the molecular species dissolved in the solutions. Owing to these interactions, the local pH around the catalytic site can differ from the bulk solution. In our case this membrane behaviour can be attributed to the formation of Schiff's bases. As for the temperature, defining 'optimal pH range' the range at which the relative activity is between 95% and 100%, the figure shows that this range occurs between 4.5 and 4.8 for the free enzyme and between 3.7 and 4.5 for the immobilized β-galactosidase. This means that the immobilized form is better protected than the free form in more acidic solutions. For example, at pH 2.5 the relative activity of the free form is 20% and 65% for the immobilized form.

Figure 5C shows the absolute membrane activity as a function of substrate concentration. The pH and temperature of the substrate solutions were 6.5 and 30 °C, respectively. The Michaelis-Menten behaviour enabled us to calculate the apparent kinetic parameters for the immobilized β-galactosidase. The apparent values of K_m and V_{max} are reported in Table II, together with the values of the free enzyme, calculated by us in our previous work. Here too, as before, we found the immobilized form to have a lower affinity for the substrate than the free enzyme. This finding ought to discourage the use of this membrane in a biotechnological process, but the possibility of restoring ing the affinity for the substrate by operating the membrane in a non-isothermal bioreactor, encouraged us to continue with the experimentation.

Effect of the non-isothermal conditions

Figure 6A gives the lactose hydrolysis as a function of average temperature. The curve parameter is the temperature difference ΔT read by the thermocouples under non-isothermal conditions.

Table II. Values of apparent kinetic parameters for β-galactosidase immobilized on different carrier. The real value of the kinetic parameters for the free enzyme are also reported for comparison.

Enzyme system	T (°C)	K_m^{app} (mM)	V_{max}^{app} (μmoles. min^{-1})	Reference number
Free	30	21.4	3.2	20
Immobilized on TF-450/MAA/HEMA/HMDA	30	135	1.35	This work
Immobilized on TF-450/AA/HEMA	30	145	1.2	20
Immobilized on TF-450/AM/HEMA	30	237	.27	20
Immobilized on TF-450/HEMA	30	120	0.06	20
Immobilized on TF-450/MAA/HMDA	30	171.2	1.2	22
Immobilized on TF-450/MAA/HEMA/CCL	30	97.6	0.074	22
Immunodyne catalytic membrane + TF-450	20 30 40	150 125 100	5.2 7.5 9.3	9

The figure shows that under constant T_{av} the membrane activity increases with the temperature difference and, hence, with the temperature gradient applied across the membrane. This means that the same lactose hydrolysis rate can be obtained either at low T_{av} and high ΔT, or at high T_{av} and low ΔT.

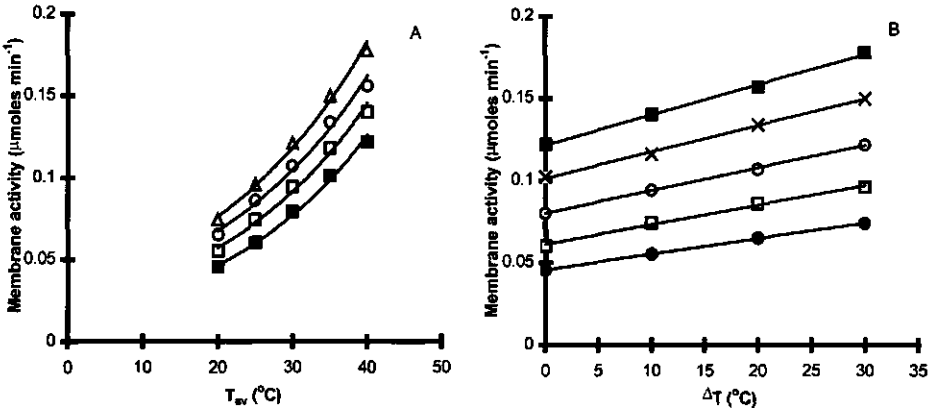

Figure 6. (A) Membrane activity as a function of T_{av}. Curve parameter is ΔT. Symbols: ΔT=0 °C (■); ΔT=10 °C (□); ΔT=20 °C (o); ΔT=30 °C (Δ);

(B) Membrane activity as a function of ΔT. Curve parameter is T_{av}. Symbols: T_{av}=20 °C (•); T_{av}=25 °C (□); T_{av}=30 °C (o); T_{av}=35 °C (x); T_{av}=40 °C (■).

Owing to the thickness of the membrane and the temperature range used, the activity profile inside the membrane can be considered to be linear. In this way the activity corresponding to point A (obtained at T_{av} = 30 °C, T_W = 45 °C, and T_C = 15 °C) must be compared with that corresponding to point B (obtained at T = T_{av} = T_W = T_C = 30 °C). There is no doubt that the membrane activity working at T_{av} = 30°C is increased in the presence of a temperature gradient when compared with the corresponding isothermal conditions.

A simple means of measuring the size of the effect produced on enzyme activity by the presence of a temperature gradient is to replot the experimental points reported in figure 6 A as done in Figure 6 B. In the latter, membrane activity is reported as a function of the ΔT read by the thermocouples. The curve parameter is T_{av}.

From the linear increase of the activity with the temperature difference for each value of T_{av} follows the equation:

$$5)\ \left[A(T)\right]_{\Delta T\neq 0}^{T=T_{av}} = \left[A(T)\right]_{\Delta T=0}^{T=T_{av}} \{1 + \alpha\Delta T\}$$

where A(T) indicates the membrane activity at $T=T_{av}$, under isothermal ($\Delta T=0$) and non-isothermal ($\Delta T\neq 0$) conditions. The coefficient α represents the percentage of membrane activity increase when a temperature difference of 1 °C is applied across the catalytic membrane. The α values calculated through the results of the experiment in figure 6 B by means of equation (5) are reported in Table III, together with the α values found with the same enzyme, but other membrane systems.

Table III. Values of α and β coefficients, defined by equations (5) and (7) respectively, for various catalytic membrane systems using the β-galactosidase as catalyst.

Membrane system	T (°C)	α x 100 ($°C^{-1}$)	β x 100 ($°C^{-1}$)	Reference number
TF-450/MAA/HEMA/HMDA	20	2.1	16.6	This work
	25	2.0	15.5	
	30	1.8	14.4	
	35	1.6	12.2	
	40	1.5	11.5	
TF-200 interposed between IMMUNODYNE catalytic membranes	30	1.7	//	13
TF-200 interposed between IMMUNODYNE catalytic membranes	50	//	18	14
	55		14	
	60		11	
IMMUNODYNE catalytic membranes + TF-200	50	//	40	14
	55		32	
	60		26	
TF-450/MMA	20	//	21.7	24
	25		20.0	
	30		18.1	
	35		16.5	
IMMUNODYNE catalytic membranes + TF-450	25	3.3	25	10
	30	3.3	30	
	35	3.0	25	
GELATINE catalytic membrane + TF-200	20	1.6	20	10
	25	1.6	25	
	30	1.6	30	
IMMUNODYNE catalytic membranes + TF-450	20	3.3	5.2	9
	30	3.3	7.5	
	40	3.3	9.3	

No significant differences between this membrane and the other composite membrane systems used in the past were observed. For a correct evaluation of the magnitude of the observed effects, one must bear in mind that under non-isothermal conditions a flux of heat is present in the apparatus so that actual temperatures on the catalytic membrane surfaces are different from the ones registered by the thermocouples. In fact $\Delta T^* < \Delta T$. As specified in materials and methods the apex (*) relates to the corresponding values at the membrane surfaces. As the temperatures on the membrane surfaces were impossible to measure, we calculated them considering that under our experimental conditions the solution flow into

Table IV. Relation between the temperature values read by the thermocouples (indicated by the symbol T) and the ones calculated at the surfaces of the two catalytic membranes (indicated by T*). The subscripts w and c refer to the warm solution and to the cold solution, respectively.

Temperature (°C)	Δ T (°C)	T_C (°C)	T_W (°C)	T_C*(°C)	T_W*(°C)	T_{AV} (°C)	ΔT*(°C)
20	10	15	25	19.5	20.6	20	1.1
20	20	10	30	18.9	21.1	20	2.2
20	30	5	35	18.4	21.6	20	3.3
25	10	20	30	24.5	25.6	25	1.1
25	20	15	35	23.9	26.1	25	2.2
25	30	10	40	23.4	26.6	25	3.3
30	10	25	35	29.5	30.6	30	1.1
30	20	20	40	29.0	31.1	30	2.2
30	30	15	45	28.4	31.7	30	3.3
35	10	30	40	34.5	35.6	35	1.1
35	20	25	45	34.0	36.1	35	2.2
35	30	20	50	33.4	36.7	35	3.3
40	10	35	45	39.5	40.6	40	1.1
40	20	30	50	38.9	41.1	40	2.2
40	30	25	55	38.4	41.7	40	3.3

each half-cell is laminar ($R_e << R_e^{critical}$) and consequently the heat flow propagates through the solution and the catalytic membranes according to Fourier's law [13,14]. Under these conditions and the knowledge of thermal conductivity and thickness of both the filling solutions and membrane, we can calculated the effective temperatures on the membrane surface. The results of this calculation are reported in Table IV. On inspection of the table and observing that the system (solution + membrane) is symmetric, we can deduce the following simple set of equations:

$$6)\quad \begin{cases} T_W^* = T_W - a\ \Delta T \\ T_C^* = T_C - a\ \Delta T \\ \Delta T^* = \Delta T\ (1 - 2a) \end{cases}$$

In our case a = 0.445 ($°C^{-1}$). Using this, the effect of the actual temperature difference on the membrane activity was derived (Figure 7).

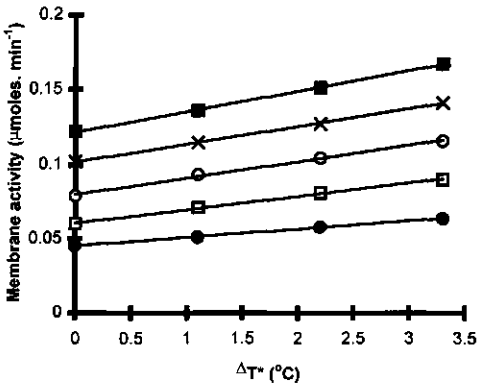

Figure 7. Membrane activity as a function of ΔT*. Curve parameter is T^*_W. Symbols: T_W=20 °C (•); T_W=25 °C (□); T_W=30 °C (o); T_W=35 °C (x); T_W=40 °C (■).

In this figure the membrane activity is reported as a function ΔT*, and T^*_W is the curve parameter. The figure confirms that the presence of the temperature gradient increases the enzyme reaction rate, but now the experimental parameters used are more significant because

of the reference to the actual temperatures on the catalytic membrane. The data of Figure 7 can now be expressed by means of a new equation similar to equation 5 thus:

$$7)\ \left[A(T)\right]_{\Delta T \neq 0}^{T = T_W^*} = \left[A(T)\right]_{\Delta T = 0}^{T = T_W^*} \{1 + \beta \Delta T\}$$

Here, the β coefficient is the percentage of catalytic membrane activity when the warm membrane surface is kept constant and the temperature of the cold membrane surface is lowered by 1°C. The β values calculated by applying equation 7 to the results of Figure 7 are listed in Table III, together with those obtained for other composite membrane systems. Also in this case no significant differences are observed between the β values calculated for the actual membrane and those calculated for the other composite membrane systems. This means that the substitution of a composite membrane system with a single membrane, both catalytic and hydrophobic, can be done successfully. Inspection of the actual β values indicates that catalytic membranes in non-isothermal bioreactors is an interesting tool for practical application in industrial processes.

All the experimental results reported above can be explained by analysis of the substrate (and product) traffic across the catalytic membrane under isothermal and non-isothermal conditions.

Under isothermal conditions only diffusion occurs. The diffusive substrate flux (moles m^{-2} s^{-1}) is expressed by the equation:

$$8)\quad J_S^D = D^* \frac{\Delta C}{\Delta x}$$

where D^* is the restricted diffusion coefficient (m^2 s^{-1}) and $\frac{\Delta C}{\Delta x}$ is the concentration gradient (moles m^{-4}).

Under non-isothermal conditions, i.e. when a temperature gradient is applied across a hydrophobic membrane, catalytic or not, the membrane is crossed by differential solvent and solutes fluxes, produced by the process of thermodialysis. Both matter fluxes are directly

proportional to the magnitude of the temperature gradient applied across the membrane. Under these conditions two separate matter fluxes are observed: a volume flux (water) from warm to cold, measured in (m^3 m^{-2} s^{-1}) and expressed by the equation:

9) $$J_{H_2O} = B \frac{\Delta T}{\Delta x}$$

and a solute flux from cold to warm, measured in (moles m^{-2} s^{-1}) and expressed by the equation:

10) $$J_S^{therm} = D' * C_c \frac{\Delta T}{\Delta x}$$

where $\frac{\Delta T}{\Delta x}$ is the temperature gradient across the membrane (K m^{-1}), C_c the actual concentration of solute in the cold half-cell (moles m^{-3}) and B the thermoosmotic coupling coefficient (m^2 s^{-1} K^{-1}).

Associated with the volume flux there is a solute flux, owing to the 'solvent drag', given by:

11) $$J_S^{drag} = J_{H_2O} C_W = V_{H_2O} C_W$$

where C_W is solute concentration in the warm half-cell, from which the water flux is coming and V_{H_2O} is the rate of water transport in (m s^{-1}). Of course, also under non-isothermal conditions the contribution of isothermal substrate diffusion still remains.

Summing up, under isothermal conditions the only substrate traffic is given by that represented by equation 8. Under non-isothermal conditions, the substrate traffic across the membrane is the result of three distinct contributions: the one expressed by equation 8 and those indicated by equations 10 and 11. A picture of the substrate traffic across the catalytic membrane operating under isothermal and non-isothermal conditions is given in Figure 8. Based on all these considerations it is easy to conclude that under non-isothermal conditions

the enzyme immobilized onto the hydrophobic-catalytic membrane 'feels' a higher substrate traffic and hence interacts with a substrate concentration higher than it would under isothermal conditions. The same reasoning is applicable to the product removal from the active site, because the thermodialysis process increases the rate of product removal from the catalytic site. Consequently, the presence of a temperature gradient increases the apparent turnover number of the enzyme reaction, thus increasing the rate of the enzyme reaction.

CONCLUSIONS

All the aims of this work have been achieved. A new grafted membrane, both catalytic and hydrophobic has been constructed. It is the first time that a correlation between phenomenological parameters, such as the thermodynamic coupling coefficients A and B, and membrane activity under non-isothermal conditions, has been made. From the study of the behaviour of the catalytic membrane under isothermal conditions the immobilization procedure has been shown to give more resistance to the enzyme at higher temperatures and in more acidic solutions.

The advantage of using non-isothermal bioreactors has been confirmed since these bioreactors have been more efficient with respect to their functioning under comparable isothermal conditions.

The negative aspects emerging from this research refer to the relatively low values of α and β coefficients. This one disadvantage was overcome by the use of a single membrane system instead of the double membrane system previously employed.

Studies are in progress in our laboratory aimed at increasing the values of the α and β coefficients.

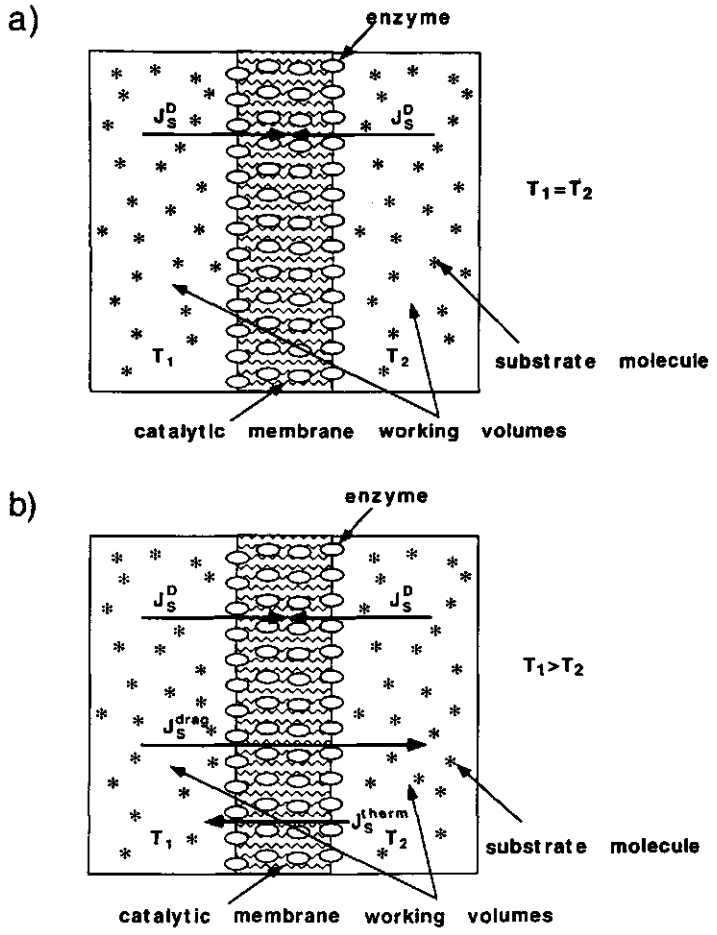

Figure 8. A not scale picture of substrate traffic across the catalytic membrane operating under isothermal (a) and non-isothermal (b) conditions.

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REFERENCES

1. Taylor, R.F. (1991). *Protein immobilization.* New York, Marcel Dekker.

2. van der Tweel, V. J. J., A. Arder & R.M. Buitelaar (1993). *Stability and stabilization of enzymes.* Amsterdam, Elsevier.

3. Shtilman, M.I. (1993).*Immobilization on polymer*. Utrecht, The Netherlands,VSP.

4. Tanaka, A., T. Toya & T. Kobayashi (1993). *Industrial application of immobilized biocatalysts.* New York, Marcel Dekker Inc.

5. Blum, L.J. & P.R. Coulet (1991). *Biosensors Principles and Applications.* New York, Marcel Dekker Inc.

6. Asenjo, J.A. & J.C. Merchuck (1995). *Biosensors system design.* New York, Marcel Dekker Inc.

7. Sheller, F.W. & F. Shubert (1992). *Biosensors.* Amsterdam, Elsevier.

8. Mita,D.G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69

9. Mita, D.G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F.S. Gaeta (1995). *Biotechnol. Appl. Biochem.* **22:** 281

10. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, F. Palumbo, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25

11. Russo, P., A. Garofalo, U. Bencivenga, S. Rossi, D. Castagnolo, A. D'Acunto, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141

12. Russo, P., A. De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F.S. Gaeta & D.G. Mita (1997). *Research in Microbiology*. **148:** 271

13. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D.G. Mita (1997). *J. Membrane Sci.* **129:** 175

14. Febbraio, F., M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta & D.G. Mita (1998). *Biotech. Bioeng.* **59:** 108

15. Gaeta, F. S. & D. G. Mita (1978). *J. Membrane Sci.* **3:** 191

16. Bellucci, F., E. Drioli, F. S. Gaeta, D. G. Mita, N. Pagliuca & F. G. Summa (1979). *Trans. Farad. Soc. II.* **75:** 247

17. Pagliuca, N., G. Perna, D. G. Mita, F. S. Gaeta, B. Karamanlis & F. Bellucci (1983). *J. Membrane Sci.* **16:** 91

18. Mita, D. G., U. Bencivenga, A. D'Acunto, N. Pagliuca, G. Perna, S. Rossi & F. S. Gaeta (1988). *Gazzetta Chimica Italiana.* **118:** 79

19. Gaeta, F. S., E. Ascolese, U. Bencivenga, J. M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi & D. G. Mita (1992). *J. Phys. Chem.* **96:** 6342

20. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 613

21. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 625

22. Mohy Eldin, M.S., M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. D'Uva, P. Canciglia, F. S. Gaeta & D.G. Mita (1999). Influence of the microenvironment on the activity of enzymes immobilized on Teflon membranes grafted by γ-radiation. In press in *J. Mol. Catal. B:Enzymatic.* 301

23. Mohy Eldin, M.S., A. De Maio, S. Di Martino, S. Rossi, U. Bencivenga, A. D'Uva, F. S. Gaeta & D.G. Mita (1999). *Adv. Polym. Technol.* **18:** 109

24. Mohy Eldin, M.S., A. De Maio, S. De Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Membrane Sci.***146:** 237

Chapter 7

CHARACTERIZATION OF THE ACTICITY OF PENICILLIN G ACYLASE IMMOBILIZED ONTO NYLON MEMBRANES GRAFTED BY MEANS OF γ-RADIATION

ABSTRACT

Penicillin G acylase (PGA) was immobilized onto nylon membranes grafted with methylmethacrylate (MMA) or diethyleneglycol dimethacrylate (DGDA) monomers by means of γ-radiation. Hexamethylenediamine (HMDA) was used as spacer between the grafted membranes and the enzyme and glutaraldehyde (GA) was used as crosslinking agent to couple both the HMDA to the grafted membrane and the enzyme to the HMDA. The catalytic membranes thus prepared were studied as a function of pH and temperature of the solution containing the substrate. The membranes showing the best characteristics were the ones grafted with DGDA. The dependence of the behavior of these membranes under different experimental conditions was studied, i.e. the temperature and duration of the aminoalkylation process, spacer concentration, the glutaraldehyde concentration and the enzyme concentration. The experimental conditions giving the best performance of the catalytic membranes have been deduced. The time required to obtain 50% of substrate conversion, i.e. hydrolysis of cephalexin, was investigated as a function of its initial concentration.

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INTRODUCTION

The production of valuable compounds in the chemical, pharmaceutical and food industry benefits from the use of enzymes as biocatalysts. The advantages of using immobilized enzymes promoted the exploitation of catalytic membranes in industrial processes. This was inspite of the fact that the immobilized biocatalyst [1] exhibited lower activity than that of an equivalent amount of the free form, essentially the result of the interactions between the macromolecule and the supporting matrix. The knowledge of these interactions became relevant together with the ways in which they affect the microenvironment in which the immobilized enzyme operates. This is why much effort has been put into obtaining new polymeric carriers and immobilization techniques capable of increasing the stability and yield of enzymatic processes [2-6].

Hydrophilic supports have been used because enzymes normally operate in aqueous media, and all industrial processes involving the use of immobilized enzymes are implemented under isothermal conditions.

A recent discovery reveals that the enzyme activity of a catalytic membrane coupled to a hydrophobic membrane increased when used in non-isothermal bioreactors and that the activity increase was proportional to the temperature difference imposed across the membrane [7-13]. A prerequisite for obtaining these results was the presence of a hydrophobic membrane with the ability to induce the process of thermodialysis [14-18], i. e. transmembrane matter transport under non-isothermal conditions. The process of thermodialysis is considered to be one of the physical causes responsible for the increased activity of the catalytic membranes in the presence of temperature gradients.

With the aim of obtaining new catalytic membranes for use in the process of thermodialysis, we have recently modified commercial teflon or nylon membranes, making them both catalytic and hydrophobic, by means of a double grafting technique and using γ-radiation [19,22]. One of these membranes, loaded with β-galactosidase [23], has given good reproducible results when employed in non-isothermal bioreactors. We used the β-galactosidase in view of its use in the process of lactose hydrolysis in milk.

In this work we characterize under isothermal conditions a new catalytic and hydrophobic membrane, obtained from a nylon sheet pre-activated by means of γ-radiation in the presence

of different solutions of suitable monomers and loaded with penicillin G acylase (PGA; EC.3.5.1.11). We have chosen PGA because this enzyme plays a relevant role in the pharmaceutical industry, catalyzing an important intermediate for the industrial production of semi-synthetic penicillins and cephalosphorins [24,25]. Besides this function, the enzyme can also be used in other biotechnological processes for resolution of racemic mixtures [26].

MATERIALS AND METHODS

MATERIALS

We used nylon Hydrolon membranes as solid support for grafting, a precious gift from the Italian Division of Pall (Pall Italia srl-Milano- Italy). These hydrophobic membranes have a nominal pore size of 0.2 mm. Pore size is related to the size of the minimum value of the diameter of the smallest particles that the membrane retains, since there is no 'classical' pores in the membrane, instead, it has irregular cavities crossing the membrane thickness. All the chemicals were purchased from SIGMA (Sigma Aldrich srl - Milano - Italy) and used without further purification. Diethyleneglycol dimethacrylate (DGDA) or methylmethacrylate (MMA) were used as grafting monomers. Hexamethylendiamine (HMDA) (70 % aqueous solution) was used as spacer between the grafted membrane and the enzyme. Glutaraldehyde (GA) aqueous solution (2.5 %) was employed as bi-functional coupling agent for covalently binding the HMDA to the solid support and the enzyme to the activated membrane.

The PGA and its substrate, i.e. cephalexin, were gifts from DSM, Geleen,The Netherlands. The enzyme's specific activity with cephalexin was 250 - 300 μMoles min^{-1} mL^{-1} of the original enzyme solution. The cephalexin had a purity of 92.5% w/w, 6% (w/w) being water and the remaining substances being impurities. The PGA hydrolyses the cephalexin to phenylglycine (PG) and 7-aminodeacetoxy cephalosporinic acid (7-ADCA).

METHODS

Catalytic membrane preparation

Membrane grafting was done by irradiation with γ-rays. The irradiation source was caesium 137 in a gamma cell 1000 Elite from Nordion International Inc., Canada. The average dose rate in the core of the radiation chamber (central dose rate) was 2.35 x 10^4 rad $hour^{-1}$.

The nylon membranes for grafting were immersed in a solution of ethanol in water (1:1 v/v) containing 10% (v/v) of DGDA or MMA and irradiated for eight hours. After treatment the membranes were washed with water to remove the homopolymers adhered to the membrane surface, and then soaked in acetone for about one hour. After a further washing with water the membrane was dried to estimate the amount of the grafting obtained. To evaluate the percentage of grafting we adopted the classical definition used 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 › according to the expression:

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

The result of these procedures is a nylon-polyDGDA or nylon-polyMMA grafted membrane ready to be activated for enzyme binding. The overall process of membrane activation and enzyme immobilization (procedure described below) is schematically presented in Figure 1.

Membrane activation

To activate the grafted membranes, they were soaked in a 2.5% glutaraldehyde aqueous solution at room temperature (25°C) for one hour and then rinsed with water. The membranes were then ready for the aminoalkylation process. This was done by immersing the membranes in a HMDA 10% (v/v) aqueous solution for one hour at room temperature. This temperature was used in all the aminoalkylation processes, except when stated differently. After a further washing with water the aminoalkylated membranes were again immersed, for one hour at room temperature, in a glutaraldehyde 2.5% (v/v) aqueous solution. At this point the membranes were activated and ready to bind the enzyme.

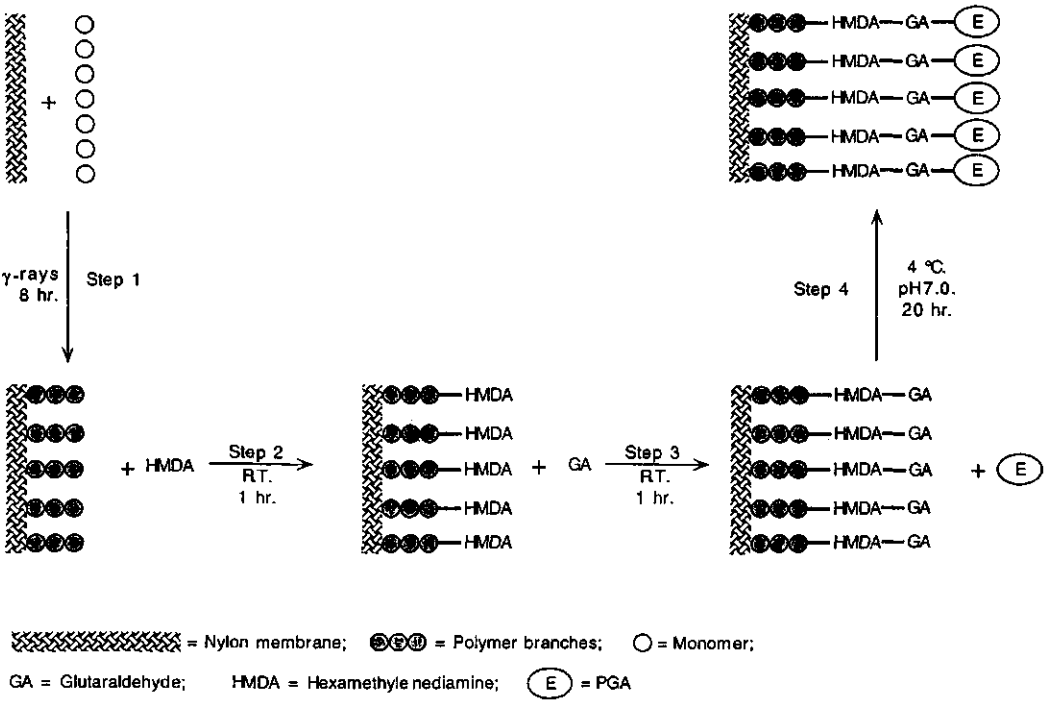

Figure 1. Schematic illustration of the processes of activation of nylon grafted membranes and enzyme immobilization.

PGA immobilization

To immobilize the enzyme, the activated membranes were immersed for 20 hours at 4°C in a 0.1 M phosphate-buffer solution of pH 7.0, 10% (v/v) of which consisting of the original enzyme solution. These experimental conditions were always applied, except when indicated otherwise. After rinsing (with water) the membranes were ready for use. When not used directly, the catalytic membranes were stored at 4°C in 0.1 M phosphate-buffer solution, pH 7.0.

Determination of catalytic membrane activity

Catalytic membrane activity was determined by putting the membrane in 25 ml 0.1 M phosphate-buffer solution, pH 7.0, containing a 20 mM cephalexin concentration. Temperature, pH and cephalexin concentration were changed according to the experimental needs. The enzyme activity was calculated using the amount of alkaline solution (0.5 N NaOH) needed to keep the cephalexin solution at the initial pH value. Membrane activity was expressed as μmoles min^{-1}. Each experiment usually lasted 30 minutes. Stability of the biocatalytic membranes was evaluated by analysis of their activity under the same experimental conditions each day. After two days, during which the membranes lost some activity, a stable condition was reached remaining unchanged for over two months. Only these stabilized membranes have been used in the comparative experiments reported herein. Under standard conditions, i.e. 25 mL of 0.1 M phosphate-buffer solution, pH 7.0 and T=30°C, 20 mM cephalexin concentration, the absolute membrane activity was 1 μmoles min^{-1}, corresponding to an activity of 285 μmoles min^{-1} per m^2 of membrane surface.

Treatment of the experimental data

Each experimental point reported in the figures represents the average of three experiments done under the same conditions. The experimental errors never exceeded 5 %.

RESULTS AND DISCUSSION

Biochemical characterization of the catalytic membranes

Effect of pH

Enzyme activity is markedly affected by environmental conditions, such as pH. The changes in optimum pH and the pH activity curve of immobilized enzymes with respect to free enzymes is dependent on the enzyme and/or the support charges. These changes are attributed to partition effects which, owing to electrostatic interactions with fixed charges on the support, cause different concentrations of charged species, such as substrate, products, hydrogen or hydroxyl ions etc., in the microenvironment of the immobilized enzyme than in the domain of the bulk solution. One of the main consequences of these partition effects is a shift in the optimum pH towards more alkalinity or acidity for negatively or positively

charged matrices, respectively [27]. Some authors also reported both a shift of optimum pH toward acidity and a change of the pH activity profile when the enzyme immobilization occurs by a Schiff's base formation [28]. In Figure 2 the relative activity of PGA as a function of pH is reported for the free enzyme and the immobilized form, either on the nylonPDGDA or on the nylonPMMA membrane. It is evident from Figure 2 then the free and immobilized enzyme have different pH values at which their optimum activity occurs. The optimum pH of the immobilized PGA shifts towards alkalinity, in spite of the fact that immobilization occurs by a Schiff's base formation. The optimum pH shifts about 3 pH units. Also the profiles of the activity pH curves are different. While the free enzyme exhibits the well-known bell shape, the immobilized enzyme presents a broader bell shape for the PGA immobilized on nylonPMMA. A sigmoidal curve emerges for PGA immobilized on nylonPDGDA. If 'optimum pH range' is the range at which the relative activity is comprised between 95 and 100%, this range occurs at pH=6.1-6.9 for the free enzyme; at pH=8.9-10.1 for the enzyme immobilized on nylonPMMA; and at pH=9.0-10.5 for the enzyme immobilized on nylonPDGDA.

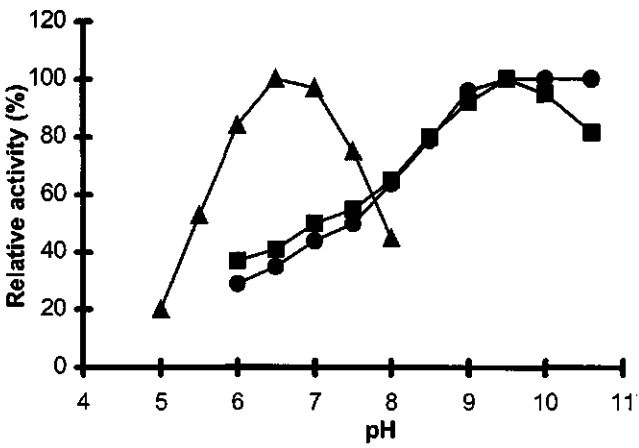

Figure 2. Relative activity of the catalytic membrane as a function of pH at temperature = 30 °C. Experimental condition for obtaining the catalytic membrane were: [DGDA] = [MMA] = 10% (v/v); [GA] = 2.5% (v/v); [HDMA] = 10% (v/v); $T_{aminoalkylation\ process}$ = 25 °C; duration of aminoalkylation process = 1 h; [Enzyme] =10% (v/v) of an enzyme solution of 250-300 μmoles min^{-1} mL^{-1} at pH 7; [S] = 20 mM at pH = 7. (▲) = free enzyme, (●) = DGDA membrane, (■) = MMA membrane

Effect of the temperature

Studies on the dependence of enzyme activity on the temperature usually produce a bell-shaped curve, showing thus an optimum of activity. The curve for the immobilized enzyme can be broader, narrower or equal to the one of the free enzyme, while the optimum activity generally shows a shift towards higher temperatures upon immobilization. This can mean a higher resistance to enzyme thermal deactivation, which is the case when the structure of the enzyme is strengthened by the immobilization procedure. Figure 3 presents the temperature dependence of the PGA activity for the two types of catalytic membranes employed. The temperature dependence of the activity of the free enzyme is also shown as reference curve. In all cases an approximately bell-shaped curve emerges. The free and immobilized enzymes exhibit a shared maximum activity at about 45 °C. Interestingly, a marked difference can be observed of the behaviour in enzyme immobilized on nylon-PDGDA membrane, which has a rather large temperature range in which the membrane has the optimum activity practically

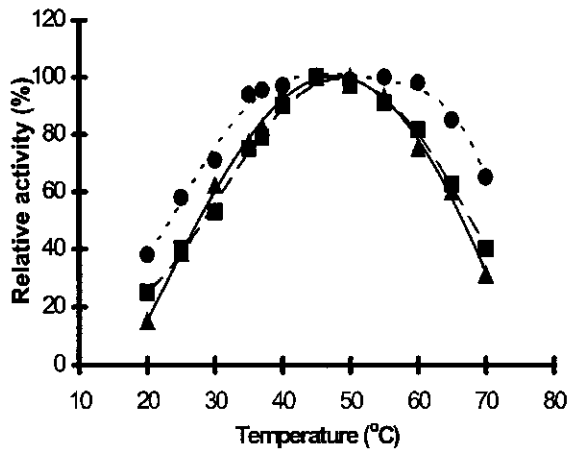

Figure 3. Relative activity of the catalytic membrane as a function of temperature at pH= 7. Experimental conditions required for obtain the catalytic membrane were the same of Figure 1. (▲) = free enzyme,(●) = DGDA membrane, (■) = MMA membrane

constant. Let us assume the 'optimum temperature range' to be the temperature range at which the relative activity is between 95 and 100%. For free enzyme the optimal temperature range would be 46 - 51 °C, for the enzyme immobilized on nylonPMMA 46 – 54 °C, and for the enzyme immobilized on nylonPDGDA 44 - 56 °C.

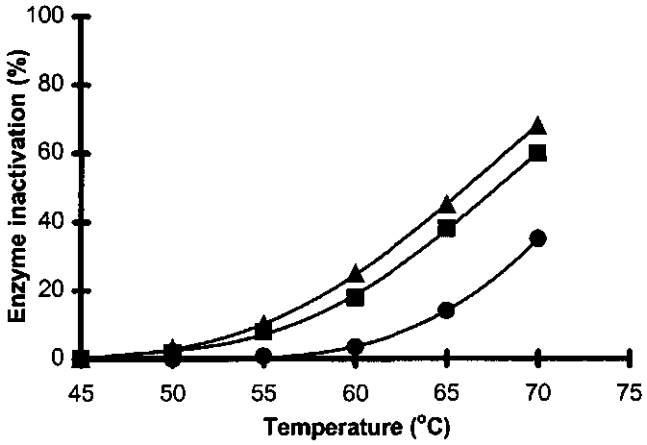

Figure 4. Percent of enzyme inactivation as a function of temperature at pH = 7. Experimental conditions for obtaining the catalytic membrane were the same of Figure 1. (▲) = free enzyme, (●) = DGDA membrane, (■) = MMA membran

The nylon-PDGDA membrane is more suitable than the others for industrial applications, especially those in which high operational temperatures are required. With this consideration in mind, the percentage of enzyme inactivation is given as a function of temperature (Figure 4). The reference temperature chosen is 45 °C because at this temperature the free and immobilized enzymes have a common maximum of relative activity. For instance, Figure 4 shows that at 70 °C the enzyme is reduced by about 70% for the free enzyme, 60% for the enzyme immobilized on nylon-PMMA membrane, and 35% for the PGA immobilized on nylon-PDGDA membrane. This interesting behaviour can be explained by the difference in branched structure of the PMMA and PDGDA graft chains. While the PMMA chains grow linearly, the DGDA monomers have two double bonds that allow the formation of branched and cross-linked polymeric structures. The latter results in a close-packed net structure, which

probably imits the thermal agitation of the bound enzyme molecules, thus reducing the amount of macromolecules inactivated by the temperature increase.

Optimization of the aminoalkylation process

Direct immobilization of PGA on grafted membranes produced no appreciable enzymatic activity. This was probably due to strong interactions of the enzyme with the electrostatic charges on the nylon membrane, altering the enzyme structure. That is why we used HMDA as spacer to bind the enzyme far enough away from the electric field of the membrane. The aminoalkylation process in this case gave positive results, since now the resulting catalytic membranes were active. For this reason we studied the yield of the catalytic membrane activity in relation to the temperature and duration of the aminoalkylation process and of the HMDA and glutaraldehyde concentration. All the experimental results reported below refer to nylon-PDGDA membranes, since these proved to be the most interesting for practical applications, as discussed above.

Dependence on the aminoalkylation temperature

Figure 5 depicts the relative activity of the catalytic membrane as a function of the temperature of the aminoalkylation process. The membranes prepared at 60°C exhibit optimum activity. The activity decrease at temperatures higher than 60°C can be explained by the fact that at these temperatures the amination process can also take place on the nylon matrix, so that the density of the immobilized PGA molecules on the surface of the membrane increases. In spite of this increase the membrane activity decreases since under these conditions, the occurrence of protein-protein interactions possibly reduces the number of active macromolecules.

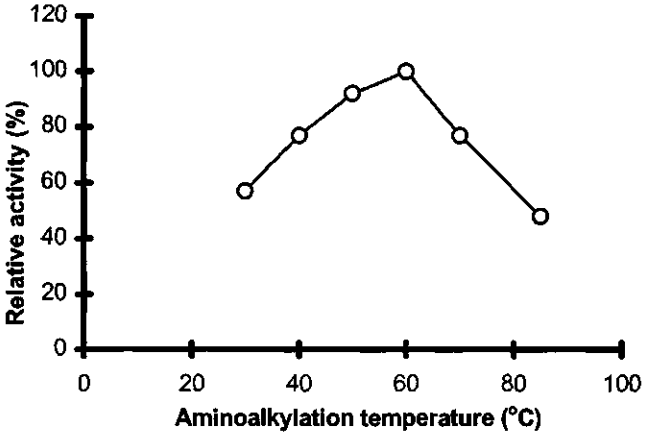

Figure 5. Relative activity of the catalytic membrane as a function of aminoalkylation temperature. Experimental condition for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5 % (v/v); [HDMA] = 10% (v/v); duration of aminoalkylation process = 1 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250-300 μmoles min^{-1} mL^{-1} at pH 7; [S] = 10 mM at pH. 9.5.

Dependence on the HMDA concentration

The relative activity of PGA immobilized on nylon-PDGDA membrane is presented in Figure 6 as a function of the HMDA concentration used in preparing the catalytic membranes. No significant effect is observed: the activity of the catalytic membrane remains practically constant or only shows a small decrease. This may be explained by the fact that the 10 % (v/v) HMDA concentration is sufficient to activate all the grafted polymeric branches on the membrane surface.

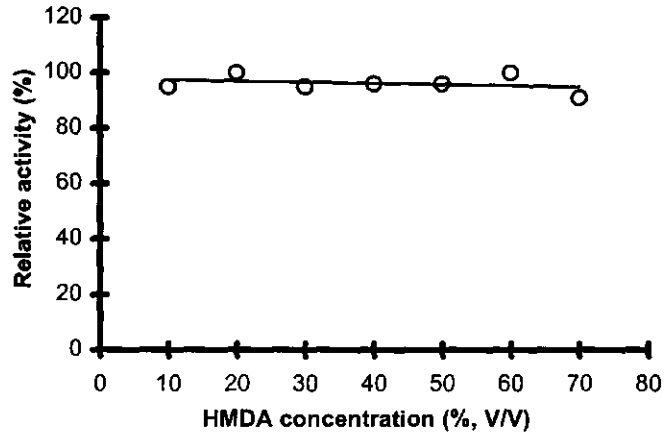

Figure 6. Relative activity of the catalytic membrane on the HMDA concentration. Experimental conditions for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5 % (v/v); $T_{aminoalkylation\ process}$ = 60 °C; duration of aminoalkylation process = 1 h; Enzyme concentration is 10% (v/v) of an enzyme solution of 250-300 μmoles min^{-1} mL^{-1} at pH 7 ; [S] = 10 mM at pH 9.5.

Dependence on the duration of aminoalkilation

Figure 7 shows the effect of the duration of the aminoalkylation process on the activity of the catalytic membranes. The results indicate that increasing duration slightly decreases the activity of the catalytic membrane. This behaviour can be explained by the long reaction times causing some aminoalkylation to occur directly on the nylon surface, giving rise to an increase in density of immobilized PGA. Should a protein-protein interaction take place, a slight decrease in activity could be the result.

Dependence on the glutaraldehyde concentration

The effect of glutaraldehyde concentration used in the activation of the grafted membranes on the activity of the catalytic membrane is shown in Figure 8. Glutaraldehyde concentration did not produced appreciable effects on the activity of the catalytic membranes. The same

behaviour was also observed by other authors when immobilizing PGA on acrylic copolymers [29].

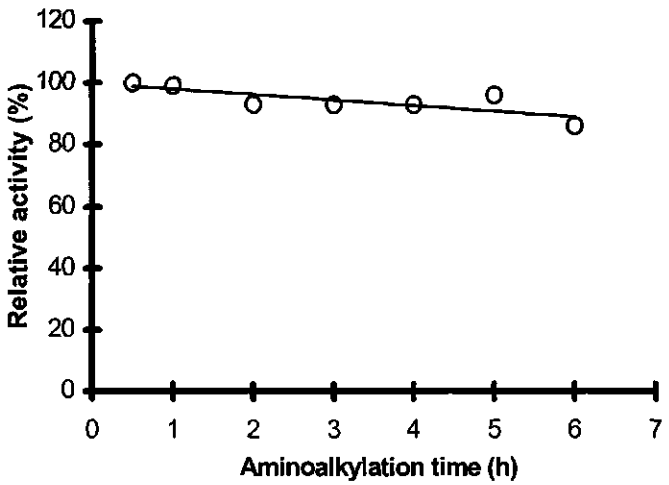

Figure 7. Relative activity of the catalytic membrane on the duration of aminoalkylation process. Experimental conditions for obtaining the catalytic membrane were the same as in Figure 5 with the exception of the duration of aminoalkylation.

Optimization of the immobilization process

Having found the best experimental conditions for the aminoalkylation process, what remained to be done for obtaining catalytic membranes giving high enzyme activities, was the optimization of the immobilization process. Therefore, a study was done of the most relevant factors affecting this process, namely the enzyme concentration and pH of the PGA solution used during the immobilization phase.

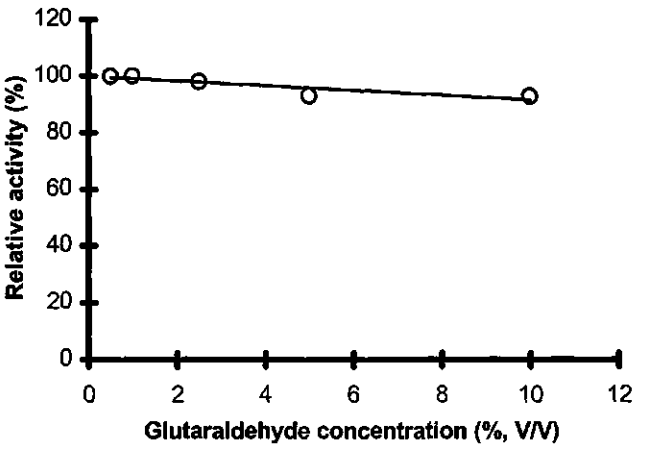

Figure 8. Relative activity of the catalytic membrane as a function of glutaraldehyde concentration. Experimental conditions for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); $T_{aminoalkylation\ process}$ = 60 °C; duration of aminoalkylation process = 0.5 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250-300 μmoles min^{-1} mL^{-1} at pH 7; [S] = 10 mM at pH 9.5.

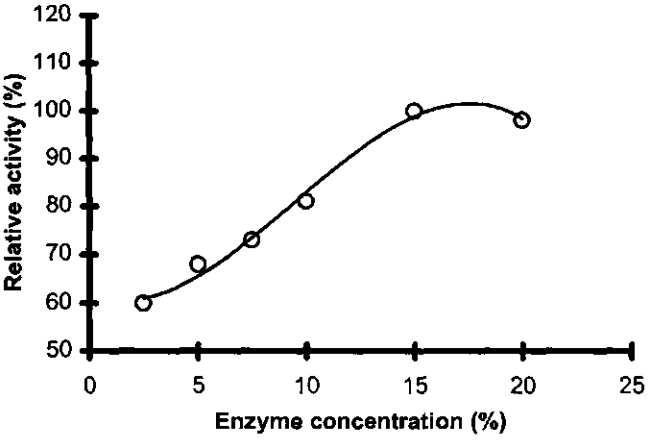

Figure 9. Relative activity of the catalytic membrane as a function of enzyme concentration. Experimental conditions for obtaining the catalytic membrane were [DGDA] = 10% (v/v); [GA] = 0.5 % (v/v); $T_{aminoalkylation\ process}$ = 60 °C; duration of aminoalkylation process = 0.5 h; [S] = 10 mM at pH. 9.5.

Dependence on the enzyme concentration

The effect of the PGA concentration used during the immobilization phase on the activity of the catalytic membrane is shown in Figure 9. Membrane activity increases with the increase of the enzyme concentration until a concentration of about 15 % is reached; a further increase in enzyme concentration has no effect. These results are in agreement with those found in the literature [30].

Dependence on the pH of the PGA solution

In Figure 10 the relative activity of different catalytic membranes is reported as a function of the pH of the PGA solution used in the immobilization phase. All the other experimental parameters were kept constant. The grafting percentage for each membrane was calculated. All the membranes had a grafting percentage value of 43 ± 3 %. Results in the figure show

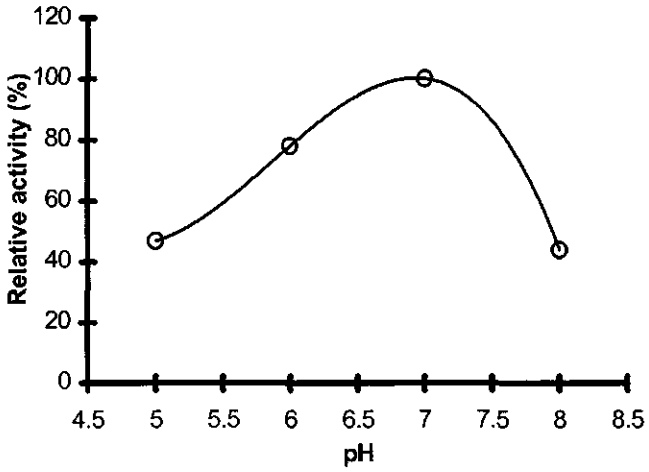

Figure 10. Catalytic membrane activity as a function of pH of the PGA solution used for the immobilization process. Experimental conditions for obtaining the catalytic membrane were [DGDA] = 10% (v/v); [GA] = 0.5 % (v/v); $T_{aminoalkylation\ process}$ = 60 °C; duration of aminoalkylation process = 0.5 h; [Enzyme] = 15 % (v/v) of an enzyme solution of 250-300 μmoles/(min. mL) [S] = 10 mM at pH. 9.5.

that the optimum membrane activity occurs using an enzyme solution at pH 7. The decrease of the membrane activity at pH values greater than this may be attributed to the instability of PGA at pH 8, as already reported by other authors [29].

Effect of the substrate concentration on the conversion

Since the enzyme should be used over longer times in industrial practice instead of measuring initial activities only, we also monitored the activity over a longer period, i.e. to 50% hydrolysis of cephalexin at different concentrations (Figure 11). These results indicate that increasing the cephalexin concentration exponentially increases the time required for obtaining 50 % of conversion, indicating for instance enzyme inactivation or inhibition. This lowers the potential for industrial application.

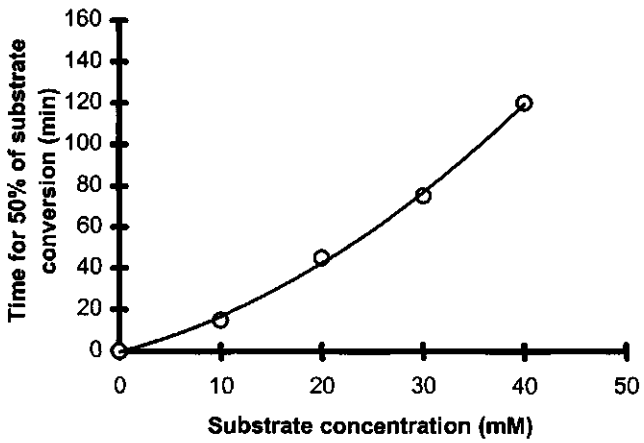

Figure 11. Time for 50% of substrate conversion as a function of the initial substrate concentration. Experimental condition for obtaining the catalytic membrane were: [DGDA] = 10% (v/v); [GA] = 2.5 % (v/v); [HDMA] = 10 % (v/v); $T_{aminoalkylation\ process}$ = 25 °C; duration of aminoalkylation process = 1 h; [Enzyme] = 10% (v/v) of an enzyme solution of 250-300 μmoles/(min. mL) at pH 7; [S] = 20 mM at pH 10.

CONCLUSIONS

Penicillin G acylase immobilized onto a grafted nylon membrane produces the highest yields if the membrane is first treated with a 10% (v/v) DGDA solution, and then a 2.5% (v/v) glutaraldehyde solution. After a 10% (v/v) HDMA solution treatment (at 60 °C for the aminoalkylation process with duration of 0.5 h), the last step is the application of a 15% (v/v) enzyme solution (the undiluted solution exhibiting an activity of 250-300 U/mL at pH 7).

Increasing the cephalexin concentration causes the times required for obtaining a 50% of substrate conversion to exponentially increase, which would then make our membranes useless for industrial processes. A method of reducing the bioconversion times comes from the technology of non-isothermal bioreactors [6-13], where the reaction rate is increased linearly with the temperature difference applied across the catalytic membrane. The efficiency of the bioreactor increases from 20 to 50% when a temperature difference of 1 °C is imposed across the membrane. The value of this efficiency increase depends on the enzyme and immobilization method. Experiments in this line of thinking using PGA immobilized on nylonPMMA membranes, are reported on in a different article [32].

ACKNOWLEDGEMENTS

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REFERENCES

1. Tanaka, A., T. Toya & T. Kobayashi (1993). *Industrial application of immobilized biocatalysts*. New York, Marcel Dekker Inc.

2. Taylor, R.F. (1991). *Protein immobilization*. New York, Marcell Dekker.

3. van der Twel, V. J. J., A. Arder & R.M. Buittelaar (1993). *Stability and stabilization of enzymes*. Amsterdam, Elsevier.

4. Bickerstaff, G.F. (1997). *Immobilization of enzymes and cells*. New Jersey, Totowa, Humana Press.

5. Shtilman, M.I. (1993). *Immobilization on polymer*. The Netherlands, Utrecht, VSP.

6. Fernandez-Lafuente, R., C. M. Rosell, G. Alvaro & J.M. Guisàn (1992). *Enzyme Microb. Technol.* **14:** 489.

7. Mita, D.G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69

8. Mita, D.G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F.S. Gaeta (1995). *Biotechnol. Appl. Biochem.* **22:** 281

9. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, F. Palumbo, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25

10. Russo, P., A. Garofalo, U. Bencivenga, S. Rossi, D. Castagnolo, A. D'Acunto, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141

11. Russo, P., A. De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F.S. Gaeta & D.G. Mita (1997). *Research in Microbiology*. **148:** 271

12. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D.G. Mita (1997). *J. Membrane Sci.* **129:** 175

13. Febbraio, F., M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta & D.G. Mita (1998). *Biotechnol. Bioeng.* **59:** 108

14. Gaeta, F. S. & D. G. Mita (1978). *J. Membrane Sci.* **3:** 191

15. Bellucci, F., E. Drioli, F. S. Gaeta, D. G. Mita, N. Pagliuca & F. G. Summa (1979). *Trans. Farad. Soc. II.* **75:** 247

16. Pagliuca, N., G. Perna, D. G. Mita, F. S. Gaeta, B. Karamanlis & F. Bellucci (1983). *J. Membrane Sci.* **16:** 91

17. Mita, D. G., U. Bencivenga, A. D'Acunto, N. Pagliuca, G. Perna, S. Rossi & F. S. Gaeta (1988). *Gazzetta Chimica Italiana*. **118:** 79

18. Gaeta, F. S., E. Ascolese, U. Bencivenga, J. M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi & D. G. Mita (1992). *J. Phys. Chem.* **96:** 6342

19. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 613

20. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Appl. Polym. Sci.* **68:** 625

21. Mohy Eldin, M.S., M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. D'Uva, P. Canciglia, F. S. Gaeta & D.G. Mita (1999). Influence of the microenvironment on the activity of enzymes immobilized on Teflon membranes grafted by γ-radiation. *J. Mol. Catal. B:Enzymatic.* In press.

22. Mohy Eldin, M.S., A. De Maio, S. Di Martino, S. Rossi, U. Bencivenga, A. D'Uva, F. S. Gaeta & D.G. Mita (1999). *Adv. Polym. Technol.* **18:** 109

23. Mohy Eldin, M.S., A. De Maio, S. Di Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). *J. Membrane Sci.* **146:** 237

24. Koteva, K.P. & K.D. Ganchev (1994). *Acta Biotechnol.***14:** 37

25. Bruggink, A., E. C. Roos & E. de Vroom (1998). *Organic Process Research and Development.* **2:** 128

26. Sovidge, T.A. (1984). In: *Biotechnology of Industrial Antibiotics,* (ed.) E.J. Vandamme, vol **22:** 171. New York. Marcel Decker.

27. Goldstein, L. (1976). *Methods in Enzymology.* **44:** 397

28. Cabral, J. M. S., J.F. Kennedy & J.M. Novais (1982). *Enzym. Microb. Technol.* **4:** 343

29. Bryjak, J. & A. Noworyta (1993). *J. Chem. Tech. Biotechnol.* **57:** 79

30. Bryjak, J., A. Trochimezuk & A. Noworyta (1993). *J. Chem. Tech. Biotechnol.* **57:** 73

31. Ospina, S. S. (1992). *J. Chem. Tech. Biotechnol.* **53:** 205

32. Mohy Eldin, M.S., M. Santucci, S. Rossi, U. Bencivenga, P. Canciglia, F. S. Gaeta, J. Tramper, A. E. M. Janssen, C. G. P. H. Schroen & D.G. Mita (1999). Non-isothermal caphalexin hydrolysis by penicillin G-acylase immobilized on grafted nylon membranes. *J. Mol. Catal. B: Enzymatic. In press.*

NON-ISOTHERMAL CEPHALEXIN HYDROLYSIS BY PENICILLIN G ACYLASE IMMOBILIZED ON GRAFTED NYLON MEMBRANES

ABSTRACT

A new catalytic membrane was prepared using a nylon membrane grafted by γ-radiation with methylmethacrylate (MMA) and 1,6-hexamethylenediamine (HMDA) as spacer. Penicillin G acylase (PGA) and cephalexin were employed as catalyst and substrate, respectively.

Cephalexin hydrolysis was studied in bioreactors operated under isothermal and non-isothermal conditions. An increase in the hydrolysis was found when the temperature of the warm membrane surface was kept constant and the temperature of the other membrane surface was kept low. The hydrolysis increase was linearly proportional to the applied temperature difference. Cephalexin hydrolysis increased by about 10 % when a temperature difference of 1 °C was applied across the catalytic membrane.

These results have been attributed to the non-isothermal cephalexin transport across the membrane, i.e. to the process of thermodialysis. In this way the enzyme immobilized on and into the membrane reacts with a substrate concentration higher than that occurring by simple diffusion under isothermal conditions

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INTRODUCTION

The design and construction of new catalytic membranes represents one of the growing areas in the study of immobilized enzyme application in biotechnological processes. Transferring the results from research laboratory to industry however sometimes creates unexpected problems [1]. The immobilization process is a critical fundamental step to solve these problems, since besides allowing easy separation of the enzyme from the reaction mixtures it often improves catalyst stability at elevated temperatures and extreme pH values.

One of the most interesting enzymes used in biotechnological processes is penicillin G acylase (PGA). This enzyme plays a dominant role in pharmaceutical industries as catalyst for the synthesis of important intermediates in the production of semi-synthetic penicillins and cephalosphorines [2,3], as well as in the resolution of racemic mixtures [4].

Many papers have been recently published [5-9] on the immobilization of penicillin acylase using different polyacrylic carriers and different immobilization methods. Our interest has also grow in the immobilization of this enzyme [10]. The biochemical and biophysical characterization of the immobilized enzyme derivatives, prepared until now, have so far only been done under isothermal conditions.

Recently, it has been found [11-17] that it is possible to increase the activity of a membrane-bound enzyme by operating under non-isothermal conditions. The activity of the catalytic membrane increases by 20 to 40% when a temperature difference of 1 °C is applied across it. The activity increase depends on the enzyme and immobilization methods used. The results referred to here were obtained with purified enzymes as well as with immobilized cells. In the latter case the activity of both internal and cell wall bound enzymes was studied.

A prerequisite for obtaining these results was the presence of a hydrophobic Teflon membrane coupled to a hydrophilic catalytic membrane. In the presence of a temperature gradient, the hydrophobic Teflon membrane induces transmembrane mass transport of substrate and products by the process of thermodialysis [18-22]. This process consists of selective mass transport across a hydrophobic membrane separating two identical or different aqueous solutions maintained at different temperatures. Thermodialysis is considered to be one of the

physical causes inducing the observed increase of the enzyme reaction rate when catalytic membranes are employed in non-isothermal bioreactors.

With the aim of obtaining membranes both catalytic and hydrophobic, we have recently succeeded in modifying commercial Teflon or nylon membranes, by means of a double grafting technique and using γ-radiation [23-26]. One of these membranes, loaded with β-galactosidase, has been successfully employed in non-isothermal bioreactors [27].

The present work aims to study under isothermal and non-isothermal conditions, the behavior of PGA immobilized onto a nylon membrane grafted with methylmethacrylate (MMA) and using 1,6-hexamethylene-diamine (HMDA) as spacer. We expect two advantages from this new membrane. The first concerns the replacement of the double membrane system, one hydrophobic and the other catalytic, employed until now in non-isothermal bioreactors, with a single membrane carrying out catalysis and thermodialysis simultaneously. The other concerns the realization of a process by which it is possible to increase the synthesis of cephalexin in industrial bioreactors, thus reducing the production time with all the consequent economical implications. Here the reverse reaction, the hydrolysis of cephalexin, is used as model reaction for practical reasons.

MATERIALS AND METHODS

The bioreactor

The apparatus employed consisted of two cylindrical half-cells (Figure 1), filled with the working solution and separated by a planar membrane. Solutions containing the substrate were recirculated in each half-cell by means of a peristaltic pump through hydraulic circuits starting and ending in the common cylinder C. The temperature in each half-cell was controlled at T_i (i=1, 2). When the apparatus worked under isothermal conditions T_1 was equal to T_2. Thermocouples, placed at 1.5 mm from each of the membrane surfaces, measured the temperatures inside each half-cell and allowed the calculation of the temperature profile

across the catalytic membrane when the apparatus was kept under non-isothermal conditions. The temperatures measured by the thermocouples are indicated by the symbol T, those calculated at the membrane surfaces are indicated by the symbol T*. The values related to the warm and cold side, respectively, will be indicated by the subscripts w and c. Thus we get

$\Delta T = T_w - T_c$ and $\Delta T^* = T_w^* - T_c^*$, as well as $T_{av} = \frac{(T_w + T_c)}{2}$ and $T_{av}^* = \frac{(T_w^* + T_c^*)}{2}$. In non-isothermal experiments $T_w^* < T_w$, $T_c^* > T_c$ and $\Delta T^* < \Delta T$.

The correlation between the temperatures read by the thermocouples and the actual temperatures on the catalytic membrane surfaces will be given in the experimental part.

Materials

Nylon Hydrolon membranes were used as solid support for grafting, a precious gift from the Italian Division of Pall (Pall Italia srl-Milano-Italy). These membranes are hydrophobic and have a nominal pore size of 0.2 mm. The pore size is related to the size of the minimum value of the diameter of the smallest particles that the membrane retains; the membrane has no 'classical' pores but irregular cavities crossing the membrane thickness. All the chemicals, excluding the enzyme and its substrate, were purchased from SIGMA (Sigma Aldrich srl-Milano-Italy) and used without further purification. Diethylene glycol dimethacrylate (DGDA) or methylmethacrylate (MMA) were used as grafting monomers. 1,6-Hexamethylenediamine (HMDA) (70 % aqueous solution) was used as spacer between the grafted membrane and the enzyme. A 2.5 % glutaraldehyde (GA) aqueous solution was employed as bi-functional agent for covalently coupling the enzyme to the activated membrane. The PGA and its substrate, cephalexin, were a gift from Gist-brocades, Delft and DSM, Geleen, The Netherlands. The enzyme's specific activity, with cephalexin, was 250 - 300 μmoles min^{-1} mL^{-1} for the original enzyme solution. The purity of cephalexin was 92.5% w/w, 6% (w/w) being water and the remaining substances being impurities. The PGA hydrolyses the cephalexin to phenyl glycine (PG) and 7-aminodesacetoxy cephalosporanic acid (7-ADCA).

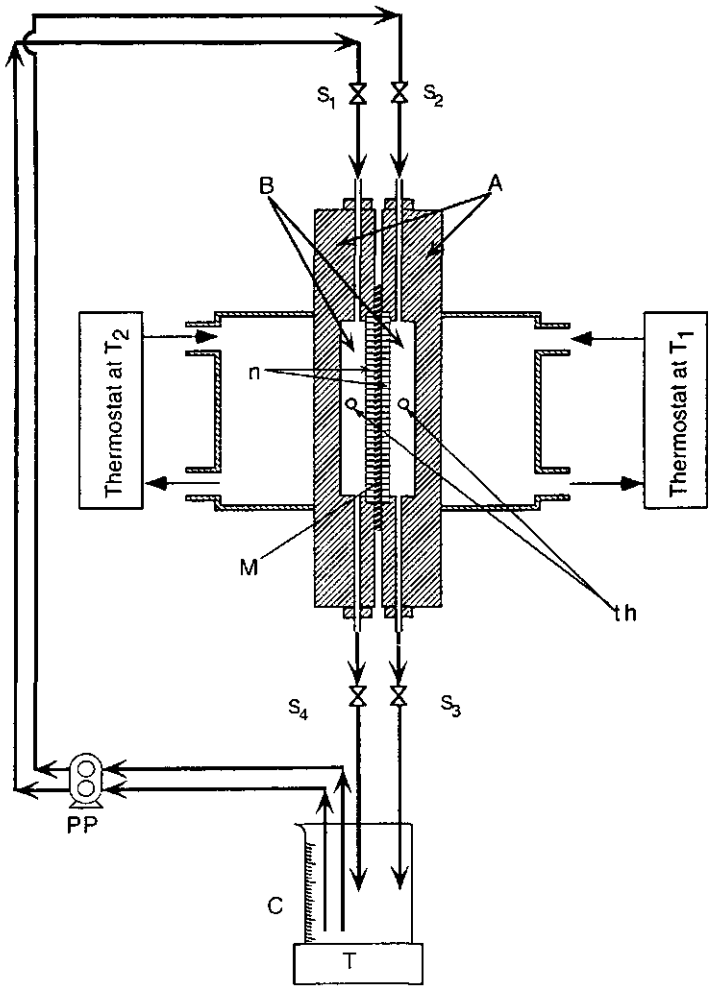

Figure 1. Schematic (not to scale) representation of the bioreactor. (A)=half-cells; (B)=internal working volumes; (C)=external working volume; (M)=membrane; (n)=supporting nets; (th)=thermocouples; (S_i)=stopcocks; (T)=thermostatic magnetic stirrer; (PP_i)=peristaltic pumps.

Methods

Membrane grafting

Membrane grafting was done by irradiation with γ-rays. The irradiation source was cesium 137 in a gammacell 1000 Elite from Nordion International Inc., Canada. The average dose rate in the core of the radiation chamber (central dose rate) was 2.35×10^4 rad $hour^{-1}$.
The nylon membranes to be grafted were immersed in a solution of ethanol:water (1:1 v/v) containing 10% (v/v) of MMA and irradiated for eight hours. After this treatment the membranes were washed with water to remove the homopolymers adhered to its surface and then soaked in acetone for about one hour to swell the membranes allowing the release of the included homopolymer. After a further washing with water the membrane was dried to estimate the value of the grafting percentage obtained. To evaluate the percentage of grafting we adopted the classical definition used for this parameter. The degree of grafting (X %) was determined by the difference between membrane mass before ($G_{B)}$ and after ($G_{A)}$ the grafting expressed:

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

The result of all these steps is a nylon-polyMMA grafted membrane ready to be activated for enzyme binding.

Membrane activation

To activate the grafted membranes, they were soaked in a 10% HMDA (v/v) aqueous solution for one hour at room temperature. After a further washing with water the aminoalkylated membranes were immersed for yet another hour at room temperature in a glutaraldehyde 2.5% (v/v) aqueous solution. The membranes thus obtained were activated and ready to bind the enzyme.

Enzyme immobilization

To immobilize the enzyme, the activated membranes were immersed for 20 hours at 4°C in a 0.1 M phosphate buffer solution, pH 7.0, containing 15% (v/v) of the original enzyme solution. After washing with water the membranes were ready for use. When not used, the catalytic membranes were stored at 4°C in 0.1 M phosphate buffer solution, pH 7.0.

Determination of the time stability of the catalytic membrane

Time stability of the biocatalytic membranes was assessed by daily analysis of their activity under the same experimental conditions. After two days, during which the membranes lost some activity, a stable condition was reached remaining unchanged for over two months. Only these stabilized membranes have been used in the comparative experiments reported herein. Under standard conditions, i.e. 25 mL of 0.1 M phosphate-buffer solution, pH 7.0 and T=30°C, containing 20 mM cephalexin, the absolute membrane activity was 1 μmoles min^{-1}, corresponding to an activity of 285 μmoles min^{-1} per m^2 of membrane surface.

Determination of the enzymatic activity of the membrane

All the experimental results reported below have been done by circulating 25 ml of a 20 mM cephalexin solution of pH 7.0 in the two half-cells of the bioreactor. The catalytic membrane activity was assessed from the amount of alkaline solution (0.5 N NaOH) needed to keep the treated cephalexin solution at the initial pH value. Membrane activity is expressed as μmoles min^{-1}. One experiment lasted 30 minutes.

Treatment of the experimental data

Each experimental point reported in the figures represents the average of three experiments done under the same conditions. The experimental errors never exceeded 5 %

RESULTS AND DISCUSSION

Isothermal characterization of the catalytic membrane

All the membranes used in the experimentation had, before the immobilization phase, a MAA grafting percentage value of 43 ± 3%. Figure 2 shows the catalytic membrane activity as a function of the temperature. The activity of the free enzyme is also given. The figure shows that the optimum temperature of the enzyme reaction of the free and the immobilized form of the biocatalyst is practically the same. Our results can be attributed to the presence of the HMDA, the spacer which binds the enzyme far enough away from the electric field generated by the amide groups, constituting the backbone of the nylon membrane, such that the enzyme structure apparently only was liable to small modifications.

Non-isothermal characterization of the catalytic membrane

Having characterized the catalytic membrane behaviour under isothermal conditions, the effect of temperature gradients on the activity of the immobilized PA was studied. Figure 3 shows the catalytic membrane activity as a function of the temperature difference read by the thermocouples. Each curve refers to a specific average temperature. The results indicate a

linear increase of the enzyme reaction rate with the applied temperature difference at each of the average temperatures used.

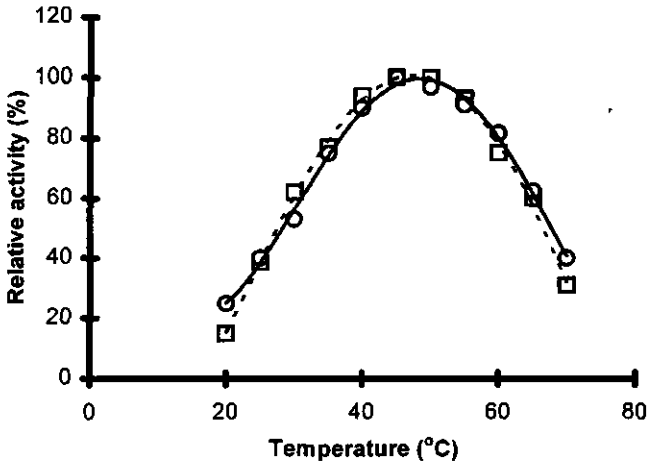

Figure 2. Relative activity for the free (O) and immobilized (□) PGA as a function of temperature.

A parameter giving information on the usefulness of non-isothermal bioreactors in industrial processes is the percentage activity increase (P.A.I.) defined at every average temperature as:

$$1) \quad P.A.I. = \frac{\left[\text{Activity}\right]_{T_{av}}^{\Delta T \neq 0} - \left[\text{Activity}\right]_{T=T_{av}}^{\Delta T = 0}}{\left[\text{Activity}\right]_{T_{av}}^{\Delta T = 0}} \times 100\,\%$$

where $\left[\text{Activity}\right]_{T_{av}}^{\Delta T \neq 0}$ and $\left[\text{Activity}\right]_{T=T_{av}}^{\Delta T = 0}$ are the catalytic membrane activities found at determined $T=T_{av}$ under non-isothermal and isothermal conditions, respectively. The P.A.I. values, calculated from the results of Figure 3, are given in Figure 4 as a function of ΔT. Inspection of figure 4 shows that the percentage activity increase is a linear function of the applied ΔT. At the same ΔT, P.A.I. decreases with increasing average temperature. This means that to obtain a significant increase of cephalexin hydrolysis it is sufficient to operate at low average temperatures and temperature gradients instead of higher average temperatures and gradients. From Figure 4 it is evident that a 50% activity increase is

obtained under the conditions T_{av}=25 °C and ΔT at 15 °C while at T_{av}=30 °C a ΔT of 22 °C is needed. It is unquestionable that the former conditions are more interesting for practical application of the technology of non-isothermal bioreactors in industrial processes.

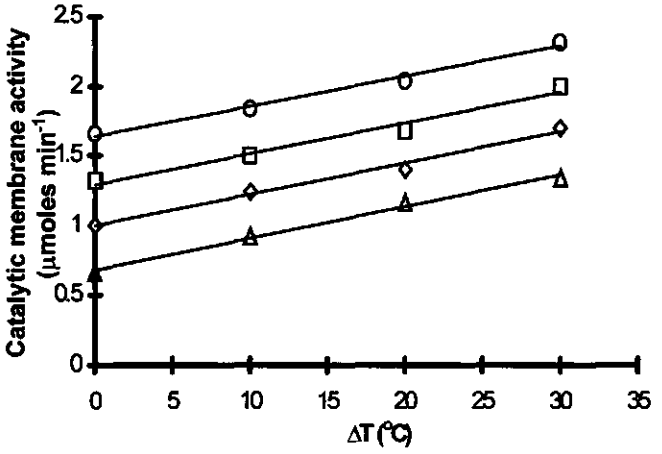

Figure 3. Catalytic membrane activity as a function of temperature difference read by the thermocouples. Curve parameter is the average temperature T_{av}= (T_W+T_C)/2; (O) = 40°C, (□) = 35 °C, (◊) = 30 °C, (Δ) = 25 °C.

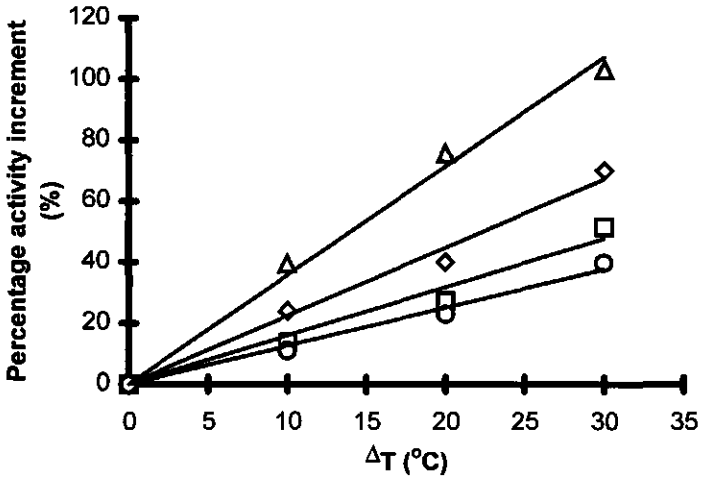

Figure 4. Percentage activity increment, calculated according to equation (1), as a function of temperature difference read by the thermocouples. Curve parameter is the average temperature; (O) = 40°C, (□) = 35 °C, (◊) = 30 °C, (Δ) = 25 °C.

In Figure 5 the catalytic membrane activity is given as a function of the system's average temperature. The temperature difference read by the thermocouples is the parameter of each of the curves. Here too it is interesting to observe how the catalytic membrane activity increases with the increase of the applied temperature difference at a constant average

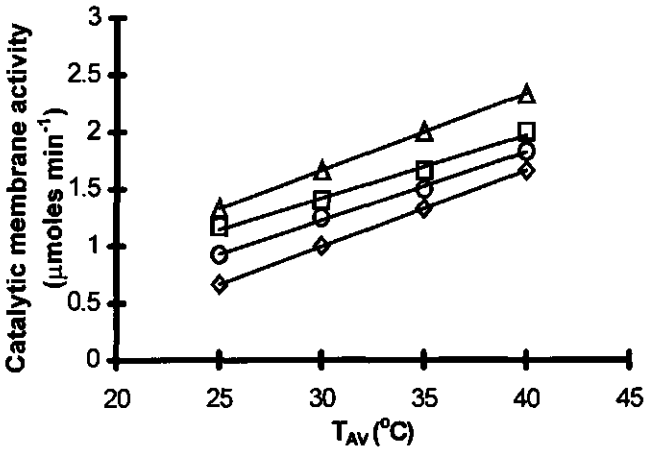

Figure 5. Catalytic membrane activity as a function of T_{av}. Curve parameter is ΔT, i.e. the temperature difference read by the thermocouples; (Δ) = 30 °C, ($\square$) = 20 °C, (O) = 10°C, ($\Diamond$) = 0 °C.

temperature. In Figure 6 the activity increase percentage of the catalytic membrane has been shown as a function of the average temperature at which the bioreactor is operated. Conclusions similar to the ones deduced from the results reported in Figure 4 can be drawn from the results of Figure 6. Figure 6 shows that at the same average temperature the percentage of increase is a function of the applied ΔT. The figure also shows how the same value of activity increase can be obtained by operating the bioreactor at a low average temperature and gradient or at a higher average temperature and gradient, the latter conditions being less convenient.

All the above results clearly show the effect of the temperature gradient on the cephalexin hydrolysis by immobilized PGA in non-isothermal bioreactors. Next it is important to measure the real magnitude of this effect by referring the catalytic membrane activities measured under non-isothermal conditions to the actual temperatures at the catalytic surfaces

rather than to the ones measured by the thermocouples. The heat flow across the bioreactor causes the actual temperature difference across the catalytic membrane ΔT^* to be less than

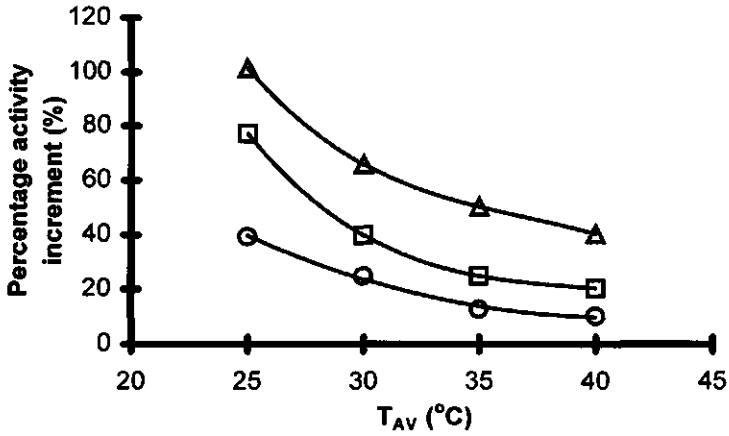

Figure 6. Percentage activity increase as a function of T_{av}. Curve parameter is the temperature difference read by the thermocouples; (Δ) = 30 °C, (□) = 20 °C, (O) = 10°C.

the temperature difference read by the thermocouples, ΔT. This means that all the observed effects attributed to a macroscopic ΔT must be related to the actual ΔT^*. Because it is impossible to measure the temperatures at the faces of the catalytic membrane, they must be calculated from the temperature measured at the thermocouples. It has been shown elsewhere [16] that the solution flow in each half-cell constituting the bioreactor is laminar, resulting in heat propagation in the two half-cell by conduction between isothermal liquid planes perpendicular to the direction of heat flow. This allows us to express the heat transport equation by Fourier's law: $J_q = K_i \left(\frac{\Delta T}{\Delta x}\right) = \text{const}$, where K_i is the thermal conductivity of the i-th medium crossed by the heat flow and $\left(\frac{\Delta T}{\Delta x}\right)_i$ is the temperature gradient in the same medium of a thickness x_i. Therefore, once the thermal conductivities and thicknesses of the cephalexin solutions and catalytic membrane are known, the temperature values at the membrane surfaces can be calculated. In Table I the results of these calculations are listed, with reference to one of the experimental conditions employed in this study. Table I shows

that a ΔT of 10 °C reduces to a ΔT^* of 1.1°C, similarly a ΔT of 20 °C to a ΔT^* of 2.2°C, and a ΔT of 30 °C to a ΔT^* of 3.2°C.

Table I. Correlation between temperature values read by the thermocouples (indicated by the symbol T) and those calculated at the surfaces of the two catalytic membranes (indicated by T*).

Temperature (°C)	ΔT (°C)	T_C (°C)	T_W (°C)	T_C^*(°C)	T_W^*(°C)	T_{AV} (°C)	ΔT^*(°C)
25	10	20	30	24.5	25.6	25	1.1
25	20	15	35	23.9	26.1	25	2.2
25	30	10	40	23.4	26.6	25	3.3
30	10	25	35	29.5	30.6	30	1.1
30	20	20	40	29.0	31.1	30	2.2
30	30	15	45	28.4	31.7	30	3.3
35	10	30	40	34.5	35.6	35	1.1
35	20	25	45	34.0	36.1	35	2.2
35	30	20	50	33.4	36.7	35	3.3
40	10	35	45	39.5	40.6	40	1.1
40	20	30	50	38.9	41.1	40	2.2
40	30	25	55	38.4	41.7	40	3.3

From the temperature values in the table enables us to write the following empirical equations:

$$2) \quad \begin{cases} T_W^* = T_W - a\ \Delta T \\ T_C^* = T_C + b\ \Delta T \\ \Delta T^* = \Delta T \left[1 - (a + b)\right] \quad b)\right] \end{cases}$$

where a and b are constants. Here, it concerns a symmetric system, a = b = 0.445 °C .
Knowing now the temperatures at the membrane faces listed in Table I, one can replot the results of Figure 5 to obtain the graph in Figure 7. The catalytic membrane activity in Figure 7

is shown as a function of the actual ΔT^* applied on the membrane. The curve parameter is T_W^*, which is the actual temperature of the catalytic membrane surface facing the warm half-cell. The results clearly indicate that it is possible to increase the reaction rate of cephalexin hydrolysis keeping constant the temperature of the warm side of the catalytic membrane and lowering the temperature of its opposite face. The amount of the reaction increase depends on the ΔT^*.

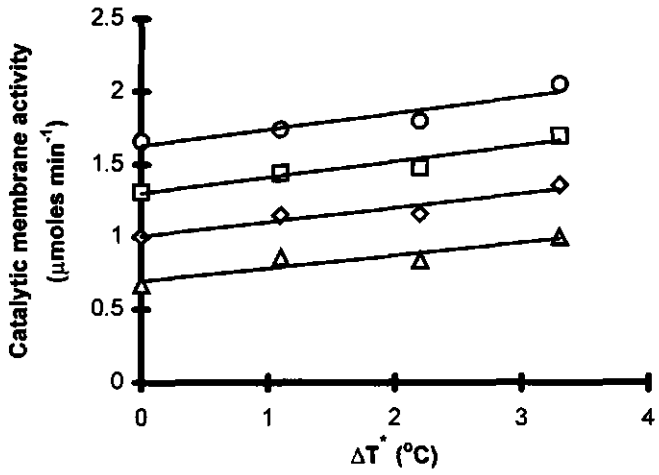

Figure 7. Catalytic membrane activity as a function of ΔT^*, the actual temperature difference across the membrane. Curve parameter is the temperature on membrane surface facing the warm half cell; (O) = 40°C, (□) = 35 °C, (◊) = 30 °C, (Δ) = 25 °C.

The linear behavior evidenced by Figure 7 allows us to write the following general equation:

$$3) \quad \left[\text{Activity}\right]_{T_w^*}^{\Delta T \neq 0} = \left[\text{Activity}\right]_{T_w^*}^{\Delta T = 0} \left(1 + \alpha^* \ \Delta T^*\right.$$

for each value of T_W^*. The α^* coefficients represent the relative increase of catalytic membrane activity when a temperature difference of 1 °C is applied across the membrane. The physical parameter α^* is an indication of the performance of the non-isothermal bioreactor. The α^* values calculated from equation (3) and applied to the results in Figure 7

are reported in Table II. These values clearly indicate the usefulness of executing cephalexin hydrolysis in non-isothermal bioreactors by means of immobilized PGA.

Table II. α^* values calculated from equation (3) using the results reported in Figure 7.

T_W^* (°C)	$\alpha^* \times 100$ (°C^{-1})
25	13
30	10
35	9
40	7

All the experimental results reported above can be explained from the way by which the substrate reaches the catalytic site of the enzyme immobilized on and into the membrane.
Under isothermal conditions only diffusion occurs. The diffusive substrate flux (moles cm^{-2} s^{-1}) is expressed by the equation:

4) $$J_S^D = D^* \frac{dc}{dx}$$

where the D^* is the restricted diffusion coefficient (cm^2 s^{-1}) and $\frac{dc}{dx}$ the concentration gradient (moles cm^{-4}).
Under non-isothermal conditions, i.e. when a temperature gradient is applied across a hydrophobic membrane, catalytic or not, the membrane is crossed by differential matter fluxes of solvent and solutes produced by the process of thermodialysis. Both matter fluxes are directly proportional to the magnitude of the temperature gradient applied across the membrane [18-22]. Under these conditions two separate matter fluxes can be observed: a volume flux (water) from warm to cold, measured in (cm^3 cm^{-2} s^{-1}) and expressed by the equation:

5) $$J_{H_2O} = L\frac{dT}{dx}$$

and a solute flux from cold to warm, measured in (moles cm^{-2} s^{-1}) and expressed by the equation:

6) $$J_S^{therm} = D' * C_c \frac{dT}{dx}$$

In these equations $\frac{dT}{dx}$ is the temperature gradient across the membrane (K cm^{-1}), C_c the actual concentration of solute in the cold half-cell (moles cm^{-3}), and L the thermo-osmotic coupling coefficient (cm^2 s^{-1} K^{-1}).

In association with the volume flux there is a solute flux, know as 'solvent drag', expressed by:

7) $$J_S^{drag} = J_{H_2O} C_c = V_{H_2O} C_c$$

where C_c is the solute concentration in the cold half-cell, from which the water flux originates and V_{H_2O} is the rate of water transport in cm s^{-1}. Of course also under non-isothermal conditions the contribution of isothermal substrate diffusion still remains.

Summing up, under isothermal conditions the only substrate traffic is given by that represented by equation 4; under non-isothermal condition the substrate traffic across the membrane is due to three distinct contribution: the one expressed by equation 4 and those indicated by equations 6 and 7. A picture (not to scale) of substrate traffic across the catalytic membrane operating under isothermal and non-isothermal conditions is given in Figure 8.

Based on these considerations it is easy to conclude, therefore, that under non-isothermal conditions the enzyme immobilized onto the hydrophobic-catalytic membrane 'faces' a higher substrate traffic and hence interacts with a substrate concentration higher than under isothermal conditions. The same reasoning is applicable to the product removal from the active site, since the thermodialysis processes increase the removal speed from the catalytic site. As a consequence of the presence of a temperature gradient the apparent turnover number of the enzyme reaction appears to increase, thus increasing the rate of the enzyme reaction.

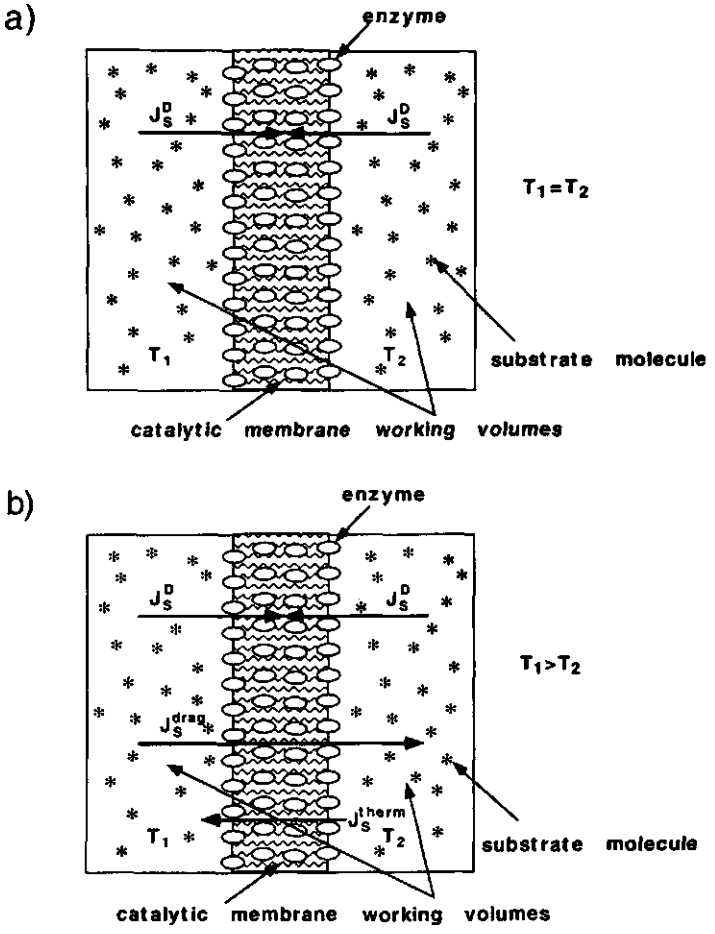

Figure 8. Picture (not to scale) of substrate traffic across the catalytic membrane operating under isothermal (a) and non-isothermal (b) conditions.

CONCLUSIONS

We can conclude that the aim of this work has been achieved since we have demonstrated that the rate of cephalexin hydrolysis depends on whether the bioreactor is operating under isothermal or non-isothermal conditions.

The positive effect of temperature gradients on the catalytic membrane activity, and hence on the amount of hydrolyzed cephalexin, is evident from all the experimental data presented. The magnitude of this effect quantified by the α^* coefficient appears to be attractive for practical applications. Incidentally, α^* values found with the present membrane are smaller but of the same order of magnitude as the ones obtained with the two-membrane system used in the past. This means that the idea of obtaining a single catalytic and hydrophobic membrane for use in non-isothermal bioreactors has potential.

Experiments are underway in our laboratory to construct other catalytic and hydrophobic membranes capable of increasing the yield of an enzymatic process executed in bioreactors operating under non-isothermal conditions.

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REFERENCES

1. Katchalsky Katzir, E. (1993). *Tibtech.* **11:** 471

2. Koteva, K.P. & K.D. Ganchev (1994). *Acta Biotechnol.* **14:** 37

3. Bruggink, A., E. C. Roos & E. de Vroom (1998). *Organic Process Research and Development*. **2:** 128

4. Sovidge, T.A. (1984). *Biotechnology of Industrial Antibiotics*, (ed.) E.J. Vandamme, vol **22:** 171. New York, Marcel Decker

5. Bryjack, J., A. Trochinezuk & A. Noworyta (1989). *Bioprocess Engineering*. **4:**159

6. Kolarz, B.N., A. Trochinezuk, J. Bryjack, M. Wojaczynska, K. Dziegielewski & A. Noworyta (1990). *Angew Makromol. Chem.* **179:** 173

7. Kolarz, B.N., A. Trochinezuk, M. Wojaczynska, J. Liesiene, J. Loborzewskii, A.A. Gorbunov & J. Bryjack (1992). *Reactive Polym.* **17:** 51

8. Bryjack, J., A. Trochinezuk & A. Noworyta (1993). *J. Chem. Technol. Biotechnol.* **57:** 73

9. Bryjack, J. & A. Noworyta (1993). *J. Chem. Technol. Biotechnol.* **57:** 79

10. Mohy Eldin, M.S., U. Bencivenga, S. Rossi, P. Canciglia, F. S. Gaeta, J. Tramper & D.G. Mita (1999). Characterization of the activity of Penicillin G Acylase immobilized onto nylon membranes grafted with different acrylic monomers by means of γ-radiation. *J. Mol. Catal. B: Enzymatic*. In press

11. Mita, D.G., M.A. Pecorella, P. Russo, S. Rossi, U. Bencivenga, P. Canciglia & F.S. Gaeta (1993). *J. Membrane Sci.* **78:** 69

12. Mita, D.G., M. Portaccio, P. Russo, S. Stellato, G. Toscano, U. Bencivenga, P. Canciglia, A. D'Acunto, N. Pagliuca, S. Rossi & F.S. Gaeta (1995). *Biotechnol. Appl. Biochem.* **22:** 281

13. Portaccio, M., S. Stellato, S. Rossi, U. Bencivenga, F. Palumbo, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **24:** 25

14. Russo, P., A. Garofalo, U. Bencivenga, S. Rossi, D. Castagnolo, A. D'Acunto, F.S. Gaeta & D.G. Mita (1996). *Biotechnol. Appl. Biochem.* **23:** 141

15. Russo, P., A. De Maio, A. D'Acunto, A. Garofalo, U. Bencivenga, S. Rossi, R. Annicchiarico, F.S. Gaeta & D.G. Mita (1997). *Research in Microbiology*. **148:** 271

16. Stellato, S., M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F. S. Gaeta & D.G. Mita (1997). *J. Membrane Sci.* **129:** 175

17. Febbraio, F., M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta & D.G. Mita (1998). *Biotechnol. Bioeng.* **59:** 108

18. Gaeta, F. S. & D. G. Mita (1978). *J. Membrane Sci.* **3:** 191

19. Bellucci, F., E. Drioli, F. S. Gaeta, D. G. Mita, N. Pagliuca & F. G. Summa (1979). Trans. Farad. Soc. II. 75: 247

20. Pagliuca, N., G. Perna, D. G. Mita, F. S. Gaeta, B. Karamanlis & F. Bellucci (1983). J. Membrane Sci. 16: 91

21. Mita, D. G., U. Bencivenga, A. D'Acunto, N. Pagliuca, G. Perna, S. Rossi & F. S. Gaeta (1988). Gazzetta Chimica Italiana. 118: 79

22. Gaeta, F. S., E. Ascolese, U. Bencivenga, J. M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi & D. G. Mita (1992). J. Phys. Chem. 96: 6342

23. Mohy Eldin, M.S., U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). J. Appl. Polym. Sci. 68: 613

24. Mohy Eldin, M.S., U., Bencivenga M. Portaccio, S. Stellato, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). J. Appl. Polym. Sci. 68: 625

25. Mohy Eldin, M.S., M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. D'Uva, P. Canciglia, F. S. Gaeta & D.G. Mita (1999). Influence of the microenvironment on the activity of enzymes immobilized on teflon membranes grafted by γ-radiation. J. Mol. Catal. B:Enzymatic. In press

26. Mohy Eldin, M.S., A. De Maio, S. Di Martino, S. Rossi, U. Bencivenga, A. D'Uva, F. S. Gaeta & D.G. Mita (1999). Adv. Polym. Technol. 18: 109

27. Mohy Eldin, M.S., A. De Maio, S. De Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M. Santucci, P. Canciglia, F. S. Gaeta & D.G. Mita (1998). J. Membrane Sci. 146: 237

Chapter 9

IMMOBILIZATION OF PENICILLIN G ACYLASE ONTO CHEMICALLY GRAFTED NYLON PARTICLES

ABSTRACT

Nylon particles, grafted with diethylene glycol dimethacrylate (DGDA) using potassium persulphate as initiator, were treated with 1,6-hexamethylene diamine (HMDA). The aminoalkylated particles were activated with glutaraldehyde and finally penicillin G acylase was immobilized to these activated particles.

Both the conditions of the aminoalkylation and the immobilization process were optimized. The hydrolysis of cephalexin was used as model conversion. The retention of activity of the immobilized enzyme was 12%. This value improved to 30% by adding phenyl acetic acid, as active-site protecting agent, to the enzyme solution. The results suggest formation of multipoint attachment between the enzyme and the matrix.

This chapter has been submitted by the authors:

M. S. Mohy Eldin, C.G.P.H.Schroen, A. E. M. Janssen, D. G. Mita and J. Tramper

INTRODUCTION

Immobilized penicillin G acylase (PGA) is one of the few immobilized enzymes applied on industrial scale for the production of semisynthetic antibiotics [1-4]. Many supports have been investigated for immobilization of the enzyme [5-8], including nylon [9]. Since nylon has few free end groups for covalent attachment of enzyme molecules, it must be pretreated to generate potentially reactive centers [10]. In spite of the great potential of the grafting technique to create reactive centers on copolymer matrices, very few articles were published about grafted nylon used for enzyme immobilization [11-15]. The value of graft copolymers is that a variety of matrices, possessing different physical, chemical and morphological characteristics, can be made. By careful selection of the matrix and the grafted monomer it is possible to vary the hydrophilic/hydrophobic nature of the immobilized support which could improve enzyme activity and stability and also give easier handling and storage. The structure of the immobilized enzyme system, using graft copolymers, suggests that the enzyme is more available for reaction than is the case with enzyme entrapped in a gel where problems of diffusion of reactants and products can arise especially if the products are competitive inhibitors. Additionally, the number of reactive groups used for coupling the enzyme can be more closely controlled. Recently the authors used nylon membranes grafted with different vinyl monomers to immobilize different enzymes [16-17].

In the present work nylon particles grafted with diethylene glycol dimethacrylate monomer was used as matrix for the immobilization of penicillin G acylase. The activation and the immobilization conditions were optimized and the efficiency of the immobilization process is reported on.

MATERIALS AND METHODS

MATERIALS

Nylon 6,6 pellets of 5 mm diameter were used as solid support to be grafted with diethylene glycol dimethacrylate (DGDA). Potassium persulphate (KPS) served as initiator for the grafting process. 1,6-Hexamethylene diamine (HMDA), 70 % aqueous solution, was used as

spacer between the grafted membrane and the enzyme. Glutaraldehyde (GA), 25 % aqueous solution, was used as coupling agent for covalently binding the enzyme to the activated HMDA-nylon beads. All the chemicals were purchased from Aldrich and used without any further purification. The enzyme, penicilline acylase (PA), and the substrate used here, i. e. cephalexin, were gifts from Gist-brocades, Delft and DSM, Geleen, The Netherlands, respectively. The enzyme specific activity, with cephalexin, was 2500 $IUmL^{-1}$ for the original enzyme solution. The cephalexin had a purity of 92.5% w/w and 6% (w/w) of water. Phenyl acetic acid (PAA), 98.5%, from Acros Organics, New Jersey, USA, was used as active- site protector.

METHODS

The overall process of particle grafting, activation and enzyme immobilization is schematically represented in Figure 1.

Nylon-particles grafting (Matrix Functionalization)

The nylon pellets were first grinded to 1mm in diameter and then immersed in 5% diethylene glycol dimethacrylate solution in ethanol:water (1:1) in the presence of 0.5% (w/v) potassium persulphate. The temperature was raised to 60°C and the mixture stirred for one hour. The obtained matrix was washed with ethanol to remove the unreacted monomer and then dried at 80°C. The percentage of grafting, X%, is defined as the difference between particle masses before, G_B, and after the grafting, G_A, according to the following equation:

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

Under these conditions a grafting percentage of about 8% is usually obtained.

Matrix activation

To activate the grafted particles, they were soaked in a 1,6-hexamethylene diamine aqueous solution of a defined concentration, reaction time and temperature as specified later in the text. After washing with water, the aminoalkylated nylon particles were immersed for one

hour at room temperature in a glutaraldehyde 2.5% (v/v) aqueous solution of specified pH. At this point the particles were activated and ready to bind the enzyme.

Enzyme immobilization

The activated particles (2 g) were immersed for 16 hours at 25°C in 20 ml of the enzyme solution, prepared by diluting ten times the original enzyme solution with 0.1 M phosphate-buffer solution pH 7.0; the suspension was gently stirred during immobilization. These experimental conditions were always applied, except where indicated otherwise. After washing with water and buffer the immobilized enzyme was ready for use. In case of studying the effect of adding phenyl acetic acid as active-site protector, it is added to the enzyme buffer solution with final concentration 15 mM.

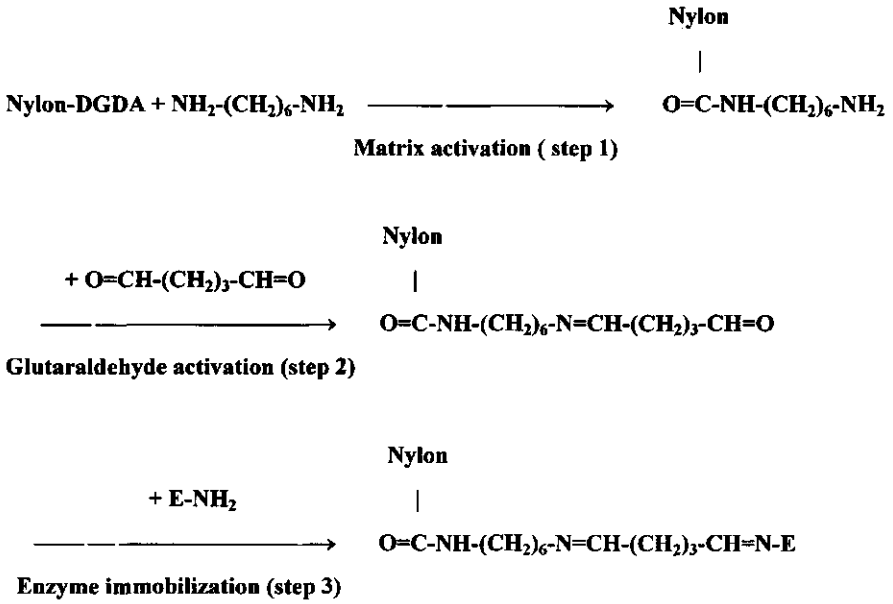

Figure 1: Scheme of the the particles activation and PA immobilization processes.

Determination of catalytic activity

Catalytic activity was determined by putting 2 g of the immobilized penicillin G acylase in 50 ml of 20 mM cephalexin pH 7.0 and 30 °C. The enzyme activity was measured through the amount of alkaline solution (0.05 N NaOH) needed to keep the treated cephalexin solution at

pH 7.0 using an automatic pH control unit. The activity is expressed per two grams of the immobilized enzyme as μmoles min^{-1}. The amount of enzyme immobilized on the matrix is expressed as percentage immobilization yield (IMY%), obtained from the difference between the enzyme activity in solution before, B, and after the immobilization, A, divided by activity in solution before immobilization, B, times 100%. The percentage of activity retention (RTA%) was calculated by dividing the measured immobilized enzyme activity by the expected activity (i.e. B-A), times 100%. When the catalytic matrices were not in use, they were stored at 4°C in 0.1 M phosphate-buffer solution, pH 7.0.

Reliability of the experimental data

The experimental errors fall in the range of 3-6 % for the grafting and in the range of 6-10% for the activity based on duplicate experiments.

RESULTS AND DISCUSSION

Since the activity of the immobilized enzyme depends on the amount of functional groups created on the matrix, optimization of the conditions of the activation process, including aminoalkylation, glutaraldehyde activation and enzyme binding, is required.

Optimization of the aminoalkylation process

The dependency of the catalytic activity of the immobilized enzyme on the concentration of HMDA, temperature and duration of the aminoalkylation process were therefore studied first.

Effect of the HMDA concentration

In Figure 2 the activity of immobilized pennicillin acylase is presented as a function of the HMDA concentration. The figure shows that the activity increases with the concentration of HMDA, reaching a maximum value at 4-6%. Further increase in the concentration to 10% leads to a decrease of the activity by 50%.

The thermal stability of immobilized enzyme was also investigated. The immobilized enzyme was incubated in buffer, pH 7.0, at 60 °C for one hour and then the residual activity % was

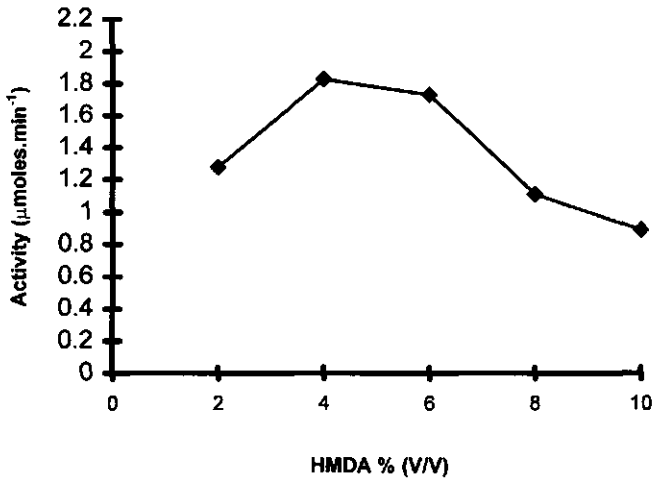

Figure 2: Activity of the catalytic matrix as a function of the HMDA concentration. Experimental conditions for obtaining the catalytic matrix were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); $T_{aminoalkylation\ process}$ = 25°C; duration of aminoalkylation process = 1 h; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

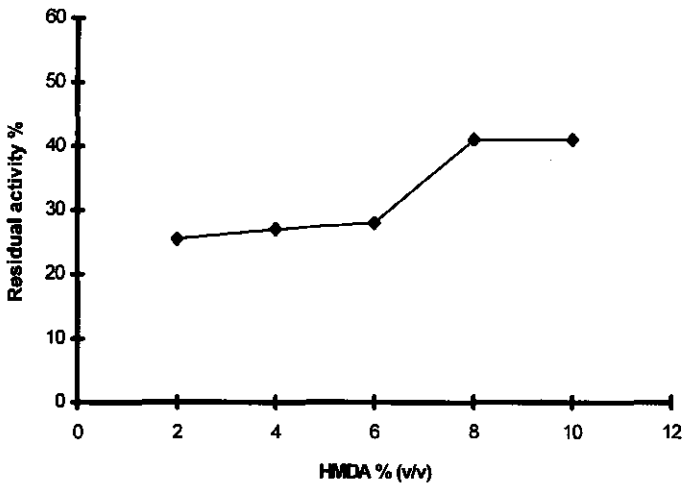

Figure 3. Effect of HMDA concentration on the thermal stability of the catalytic matrix. Experimental conditions for obtaining the catalytic matrix are the same as mentioned in Figure 2. Incubation temperature 60°C; duration of incubation one hour at pH 7.0.

measured (Figure 3). From the figure it is clear that the enzyme kept from 25 to 41% of its original activity, while the free enzyme lost all its activity under these conditions. The higher residual activity, i.e. higher stabilities, occurred at the higher HMDA concentrations. These results indicate the formation of multiple bonds between an enzyme molecule and the matrix; other authors [18] have found that carriers for acylase immobilization should not have a high amino-group concentration in order to prevent multi-point enzyme bonding, resulting in deactivation, but also stabilization of the retained activity. On the basis of the above results we concluded that the formation of multibonds between the enzyme molecule and the support is the likely cause for the decrease in activity of the immobilized enzyme when the HMDA concentration used is increased. Comparison of the thermal stability of our immobilized enzyme with that obtained by other authors [19], who immobilized penicillin G acylase on oxiran-acrylic beads, is favourable for our preparation, as the other one lost completely its activity after only 30 minutes at pH 8 and 60 °C.

Effect of the aminoalkylation temperature

The dependency of the catalytic activity on the temperature of the aminoalkylation process is shown in Figure 4. Optimum activity is observed at 55°C. Further increase of the temperature to 65°C, resulted in 40% less activity. The activity decrease at temperatures higher than 55°C may be explained considering that at these temperatures the amination process can take place also on the nylon matrix. As a result, the density of the amino groups and hence the immobilized penicillin G acylase molecules directly on the surface of the matrix may have increased. Enzyme molecules with their active sites oriented to the surface are likely to have a decreased activity.

Effect of the aminoalkylation reaction time

The obtained results indicate that increasing the reaction time from 7.5 to 75 minutes decreases the activity by 20%. The minimum handling time is 7.5 minutes and this cleary is enough to obtain the highest maximum activity which indicates that the process mainly occurred on the surface of the particles. This is in agreement with the non-porous nature of the nylon particles.

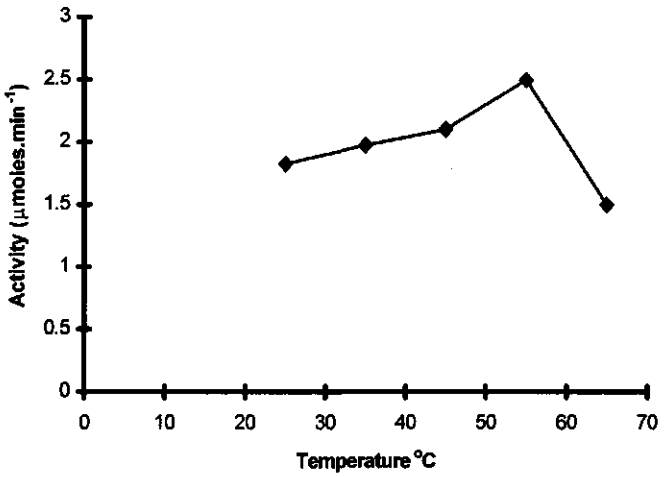

Figure 4: Activity of the catalytic beads as a function of aminoalkylation temperature. Experimental condition for obtaining the catalytic matrix were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); [HDMA] = 2% (v/v); duration of aminoalkylation process = 1 h; [Enzyme] = 2500 U at pH 7; ([S] = 20 mM at pH. 7.0.

Optimization of the glutaraldehyde-activation process

The effect of the activation conditions, i.e. glutaraldehyde concentration, reaction time, and reaction pH, were then optimized.

Effect of glutaraldehyde concentration

The effect of the glutaraldehyde concentration used for the activation of the aminoalkylated matrix on the activity is shown in Figure 5. Increasing the glutaraldehyde concentration increased the activity reaching its maximum value at 2.5%. Beyond this concentration the activity decreased with the glutaraldehyde concentration; at 5% glutaraldehyde the relative activity was 55%.

Effect of the activation time

No significant effect of the activation time was observed in the range of 1-3 hours. At 4h the activity started to decrease and dropped to 59% at 5h. Prolonging the reaction time to 24h

decreased the activity to 43%. This may be explained by one of two effects. First, the reaction of the aldehyde groups of both sides of the glutaraldehyde molecules with the amino groups on the matrix, reducing thereby the aldehyde groups available to react later with amino groups on the enzyme; this in turn leading to less immobilized enzyme. Second, multipoint attachment of enzyme to matrix can lead to a more rigid enzyme and hence reduction of the activity.

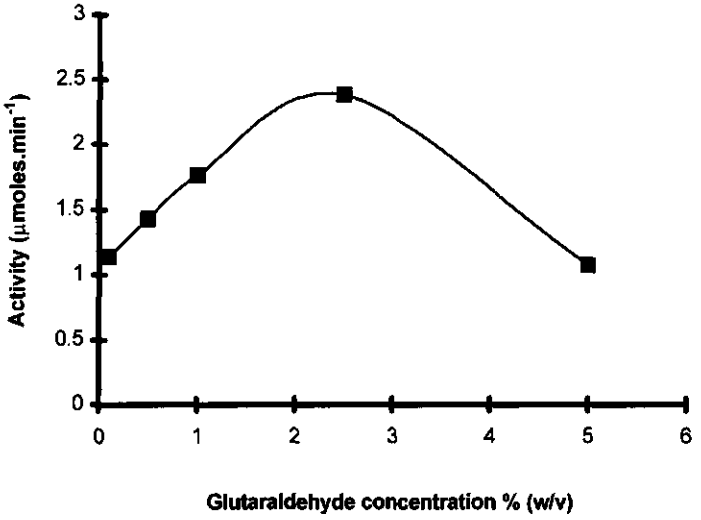

Figure 5. Activity of the catalytic matrix as a function of glutaraldehyde concentration. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); $T_{aminoalkylation\ process}$ = 55 °C; duration of aminoalkylation process = 7.5 minutes; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

Effect of pH of the glutaraldehyde solution

Figure 6 presents a clear effect of the pH of the glutaraldehyde solution found in the studied range of 6 to 10.The maximum activity was found at pH 9.0. Dramatic loss of activity occurred at both lower and higher pH, with only 50% of relative activity at pH 10 and 20% at pH 6. These results are in full agreement with those obtained by other authors who studied the glutaraldehyde activation step in detail with the immobilization of invertase on nylon 6 tubes. They used the O-alkylation technique to create active functional groups on the surface of the nylon tubes [20].

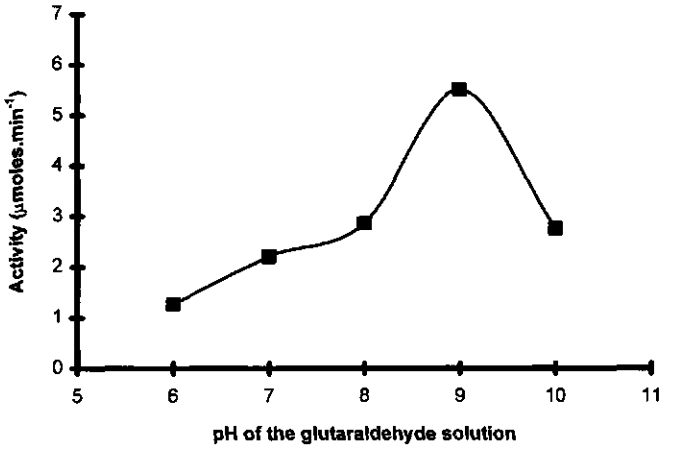

Figure 6. Activity of the catalytic matrix as a function of pH of the glutaraldehyde solution. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v); $T_{aminoalkylation\ process}$ = 55 °C; duration of aminoalkylation process = 7.5 minutes; [Enzyme] = 2500 U at pH 7; [S] = 20 mM at pH 7.0.

Optimization of the enzyme immobilization process

Finally, the immobilization conditions were optimized, i.e. enzyme amount, pH of the enzyme solution and the immobilization time. In addition, the effect of phenyl acetic acid as active-site protector was studied.

Effect of the enzyme concentration

The effect of the penicillin G acylase concentration used during the immobilization step on the catalytic activity is shown in figure 7. The activity initially sharply increases with enzyme concentration until 25 U per ml; thereafter further increase of enzyme concentration has no effect. These results agree with those found in the literature [21],where penicillin G acylase was immobilized onto a copolymer of butyl acrylate and ethylene glycol dimethacrylate.

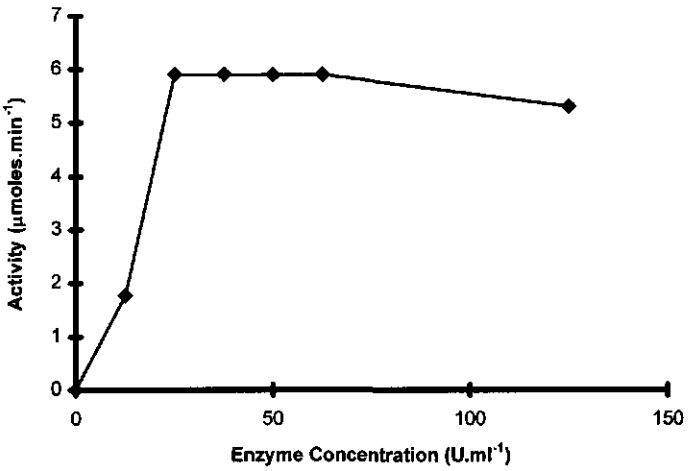

Figure 7: Activity of the catalytic matrix as a function of enzyme concentration. Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{aminoalkylation\ process}$ = 55 °C; duration of aminoalkylation process = 7.5 minutes; [S] = 20 mM at pH 7.0.

Effect of the pH of the enzyme solution

In Table 1 the activity of the immobilized enzyme, prepared using solutions with different pH, is presented. The results show maximum activity in the 7.0 to 8.0 range. Especially at the highest pH tested, i.e. 10, the activity drops to less than 25% of the maximum activity. This may be explained by more than one reason: a decrease of amount of enzyme immobilized at the pH as shown in Table 1; wrong orientation of the immobilized enzyme at the alkaline pH; possibility of formation of multipoint attachment to the matrix with loss of activity; and instability of penicillin G acylase at this high pH, as already reported by other researchers [22], which is the most likely reason. The results in the table also show that the trend of the retained activity (RTA%) is the same as the activity, reaching a maximum at neutral pH. This could be because of the effect of the pH on the immobilization yield, which increased at a higher rate than that of the activity and has its maximum at alkaline pH. The retained activity is the result of dividing the measured activity by the theoritical activity of the immobilized enzyme as indicated in the methods.

Table 1 : Effect of the pH of the immobilization solution on the activity, IMY% and RTA% Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{aminoalkylation\ process}$ = 55 °C; duration of aminoalkylation process = 7.5 minutes; [Enzyme] = 250 U;[S] = 20 mM at pH 7.0.

pH	Activity / 2 g ($\mu moles.min^{-1}$)	IMY(%)	RTA(%)
6.0	4.87	10.6	8.2
7.0	5.60	20.0	11.3
8.0	5.60	24.0	9.0
9.0	4.55	81.3	2.3
10.0	1.38	42.5	1.3

Effect of immobilization time

In Table 2 the effect of the immobilization time on the activity is shown. One hour of immobilization gives an activity of 2 $\mu moles.min^{-1}$ (30% of maximum); doubling this time increases this value to 4.85 $\mu moles.min^{-1}$, reaching 5.78 and 6.3 $\mu moles.min^{-1}$ after four and six hours, respectively. Further increase of the immobilization time to sixteen hours had no effect. This result is easy to explain by the fact that the amount of immobilized enzyme (IMY%) shows the same tendency (Table 2). As mentioned in the methods, the retained activity depends on both the activity and the amount of immobilized enzyme (IMY%), and they exhibit the same behaviour. This caused the retained activity of the system to be affected slightly by changing the immobilization time.

Effect of the phenyl acetic acid

From the results in Tables 1 and 2 it is clear that most of the enzyme activity is lost in the immobilization process. Therefore, phenyl acetic acid (PAA), an inhibitor of the enzyme, was added to protect the active site during the immobilization process. The effect of PAA on the activity and RTA% at different immobilization times is also shown in Table 2. The results show that both the activty and RTA% increased by adding PAA to the immobilization solution as protecting agent for the enzyme active site. The increase by a factor 2 indeed indicates that bonds are formed with loss of activity between amino groups in the active site

Table 2 : Effect of the immobilization time on the activity, IMY% and RTA%.
Experimental conditions for obtaining the catalytic beads were: [DGDA] = 5% (v/v); [GA] = 2.5 % (v/v) at pH 9.0; $T_{aminoalkylation\ process}$ = 55 °C; duration of aminoalkylation process = 7.5 minutes; [Enzyme] = 250 U;[S] = 20 mM at pH 7.0; [PAA] = 15 mM.
ND = Not determined

Immobilization time (h)	IMY(%)	Activity(- PAA) ($\mu moles.min^{-1}$)	Activity(+ PAA) ($\mu moles.min^{-1}$)	RTA(%) (- PAA)	RTA(%) (+PAA)
1h	ND	2.0	ND	ND	ND
2h	16	4.85	12	12	30.77
4h	18	5.78	11	15	27.5
6h	21	6.3	11.5	12	21.7
8h	20	6.1	ND	12	ND
16h	21	6.0	ND	11.3	ND
24h	ND	ND	11.73	ND	22.3

as well as the aldehyde groups on the matrix in the absence of PAA. This result is in agreement with the results obtained by another group [22], which immobilized penicillin G acylase into an agarose matrix. They found that the retention of activity of the prepared derivative in the absence of PAA was 51% but improved to 98% by the addition of PAA to the immobilization matrix. This marked loss of activity by not adding PAA to the matrix, could be attributed to the severe distortion induced in the enzyme as a result of a greater number of covalent enzyme-support linkages. In the case of the derivatives prepared in the presence of PAA, it is deduced that when such material is bound to the active site it produces a shielding effect that enables the enzyme to retain a higher proportion of its activity.

CONCLUSIONS

Penicillin G acylase was immobilized onto poly diethyleneglycol dimethacrylate (pDGDA) grafted nylon of 8% percent grafting. After optimization of the factors affecting the activity of the immobilized enzyme, 12% of the activity is retained. Binding in the wrong orientation of

the immobilized enzyme, multipoint attachment to the matrix, and binding with groups in the active site, are the probable reasons for the loss of activity. Increasing the amino group concentration on the matrix surface was found to have a negative influence on the activity, but a possitive influence on the thermal stability. By protecting the active site of the penicilin acylase through the immobilization process using PAA, both the activity and the retention of activity were improved by a factor of two, the latter reaching 30%.

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REFERENCES

1. Vandamme, J. (1993). *J. Chem. Tech. Biotechnol.* **31:** 637

2. Vandamme, J. (1983). *Enzyme Microb. Technol.* **5:** 403

3. poulsen, B. (1984). *Biotechnol. Genetic Engin. Reviews.* **1:** 121

4. Ospina, S. S. (1992). *J. Chem. Tech. Biotechnol.* **53:** 205

5. Warburton, D., K. Balasingham, P. Dunill & D. Lilly (1972). *Biochimica Et Biophysica Acta.* **284:** 278

6. Kalal, J. (1978). *J. Polymer Sci., Polymer Sym.* **62:** 251

7. Braun, J., P. Le Chanu & F. Le Goffic (1989). *Biotechnol. Bioeng.* **33:** 242

8. Bryjak, J., A. Trochimezuk & A. Noworyta (1989). *Bioprocess Eng.* **4:** 159

9. Boccu, E., T. Gianferrara & L. Gardossi (1990). *IL Farmaco.* **45:** 203

10. Hornby, W. E. & L. Goldstien (1976). *Methods Enzymol.* **44:** 118

11. Abde-Hey, F. I., J. T. Guthrie, C. E. J. Morish & C.G. Beddows (1979). *Polym. Bull.* **1:** 755

12. Abde-Hey, F. I., C.G. Beddows & J. T. Guthrie (1980). *Polym. Bull.* **2:** 607

13. Beddows, C.G., R A. Mirauer, J. T.Guthrie, F. I. Abde-Hey & C. E. J. Morish (1979). *Polym. Bull.* **1:** 749

14. Abde-Hey, F. I., C.G. Beddows & J. T. Guthrie (1981). *Makromol. Chem.* **182:** 717

15. Beddows, C.G., J. T.Guthrie & F. I. Abde-Hey (1981). *Biotechnol. Bioeng.* **23:** 2885

16. Mohy Eldin, M. S., A. De Maio, S. Di Martino, S. Rossi, U. Bencivenga, A. D'Uva, F. S. Gaeta & D.G. Mita (1999). *Adv. Polym. Technol.* **18:** 109

17. Mohy Eldin, M.S., U. Bencivenga, S. Rossi, P. Canciglia, F. S. Gaeta, J. Tramper & D.G. Mita (1999). Characterization of the activity of Penicillin G Acylase immobilized onto nylon membranes grafted with different acrylic monomers by means of g-radiation. *J. mol. catal. B: Enzymatic*. In press

18. Kolarz, B. N., M. Wojaczynska, J. Bryjak & J. Lobarzewski (1993). *Macromolecular Rep.* **A30:** 201

19. Erslan, A. & A. Guray (1991). *J. Chem. Tech. Biotechnol.* **51:** 181

20. Onyezili, F. N. (1987). *Biotechnol. Bioeng.* **29:** 399

21. Bryjak, J., A. Trochimezuk & A. Noworyta (1993). *J. Chem. Tech. Biotechnol.* **57:** 73

22. Rosell, C. M., R. Fernandez-Lafuente & J. M. Juisan (1995). *Biocatalysis and Biotransform.* **12:** 67

Chapter 10

GENERAL DISCUSSION

INTRODUCTION

This thesis presents graft copolymers as promising matrices for enzyme immobilization and for enzymatic reaction under non-isothermal conditions as a solution to reduce the effect of the diffusion-limitation problems.

Two different enzymes, β-galactosidase and penicillin G acylase, were immobilized onto two different grafted membranes i.e. grafted Teflon and grafted nylon respectively. γ-Radiation was used as initiation system for grafting the membranes. β-Galactosidase has been immobilized by two techniques: entrapment through the grafting of the enzyme with 2-hydroxyethyl methacrylate (HEMA) solution to grafted Teflon membranes and covalent binding to the carboxylic or hydroxylic groups after activation. On testing the activity of these catalytic membranes under non-isothermal conditions, only the enzyme immobilized by covalent means worked under non-isothermal conditions. The enzyme entrapped by grafting leaked from the membranes during the first experiment and no residual activity was detected on the membranes. This could be improved by using a crosslinker in the second grafting step and by prolonging the irradiation time for better crosslinking. In addition to the benefits of preventing enzyme leakage from the matrix, the reduction of matrix hydrophilicity enhances the functioning of the membrane under non-isothermal conditions.

Penicillin G acylase was covalently immobilized to grafted nylon membranes and particles. The enzyme immobilized on membranes was tested under non-isothermal conditions.

The activity of both immobilized enzymes, β-galactosidase and penicillin G acylase, increased when non-isothermal conditions were used.

β-Galactosidase immobilization

Entrapment method

The very low activity obtained by the direct grafting of enzyme-HEMA solution to un-grafted Teflon membranes doubled by grafting the membranes first with acrylic acid (AA). The factors affecting the grafting process were optimized. Increase of the ferrous ammonium sulfate (FAS) concentration during the first grafting step with AA did not affect the percentage of grafting. However, the activity of the immobilized enzyme decreased sharply. At the time we wrote Chapter 3 we believed that protein-protein interactions caused this effect. In fact, this effect could also be explained otherwise. The carboxylic groups of the AA branches, can easily exchange their protons with Fe^{2+} cations. As a result, the microenvironment of the enzyme entrapped in the matrix will be rich with Fe^{2+} cations. A very simple experiment designed to study the effect of the presence of Fe^{2+} cations on the activity of the enzyme in the free form is shown in Figure 1.

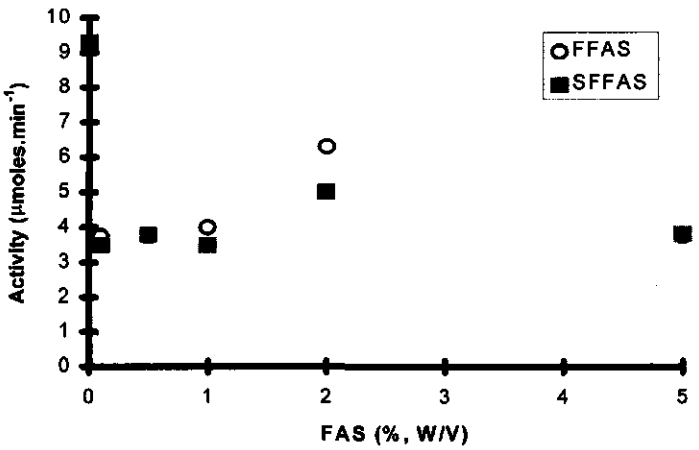


Figure 1. Effect of FAS concentration on the activity of free β-galactosidase in buffer solution. Activity before (FFAS) (o) and after (SFFAS) (■) centerfugation.

The figure shows that 50% of the enzyme activity was lost as a result of the presence of Fe^{2+} cations in the medium. The activity of the enzyme solution remained the same before and after centrifugation indicating that no enzyme precipitated as a result of the presence of Fe^{2+}

cations. Not protein-protein interactions, but FAS is thus the likely cause for the observed loss in activity. Other interesting results emerged when the enzyme was dissolved in bidistilled water instead of phosphate buffer. In that case very little loss of activity was observed upon addition of FAS (Figure 2).

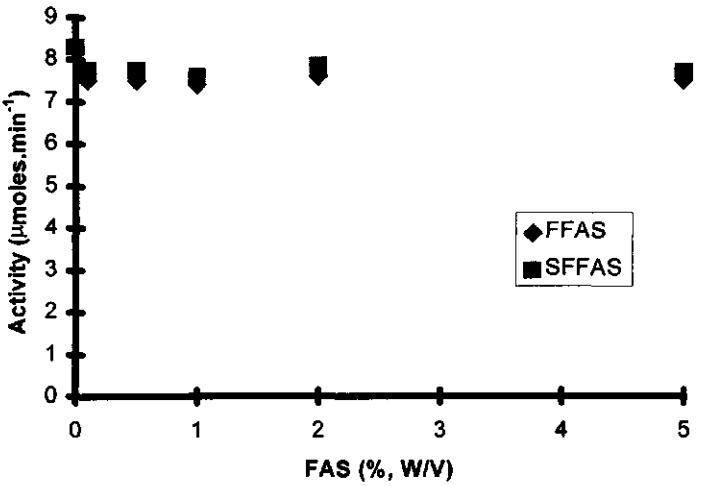


Figure 2. Effect of FAS concentration on activity of the free β-galactosidase in water solution. Activity before (FFAS) (◆) and after (SFFAS) (■) centerfugation.

This means that not the presence of the Fe^{2+} cations, but the interaction between the Fe^{2+} cations and the buffered medium gave the negative effect on the activity of the enzyme.

Substituting the AA in the first grafting step with methacrylic acid (MAA) or acrylamide (Am) affected the activity and the properties of the immobilized enzyme by inducing changes to the microenvironment of the enzyme. As result of diffusion limitation a lower V_m and higher K_m compared to the free enzyme for all the immobilized forms were found. No changes in the pH optimum of the enzymatic reaction were found, except for the enzymes immobilized on membranes without the first grafting step. For the temperature the case was different, since all immobilized forms had a higher optimum reaction temperature with the exception of the Teflon-AA. The latter is explained by the high degree of swelling (300%) of the Teflon-AA derivative, which makes the microenvironment match that of the free enzyme. The effects of the temperature on the activity at different pH values were modeled and yielded

a simple equation. This equation allows the planning of the best experimental conditions for obtaining the highest yield of the enzyme reaction when one of the two parameters, i. e. temperature or pH, is fixed.

Covalent method

One of the membranes prepared for enzyme entrapment by grafting (Chapter 4) was prepared free of enzyme with the aim to covalently attach the enzyme to this membrane later. Because of the membrane's two functional groups, i.e. -COOH groups of the MAA branches and -OH groups of the HEMA branches, two possible sites for enzyme attachment were available.

Attachment of the enzyme to -COOH groups shifted the optimum reaction temperature to higher values. No such shift was noticed in case of enzyme immobilized to -OH groups. Shift of the pH optimum to the more acidic region, as compared to the free enzyme, was found in both cases. The formation of Schiff's bases explains the case for the enzyme attached to -COOH groups. The enzyme attached to -COOH groups has a higher V_m and lower K_m in comparison with the enzyme entrapped by grafting. This illustrates the possible effects of varying the immobilization method on the kinetic parameters of the resulting immobilized enzyme.

Penicillin G acylase immobilization

Penicillin G acylase was covalently attached to grafted-nylon supports. Two physical forms of nylon were used: membranes and particles. Two monomers were used for the membrane grafting, i.e. methyl methacrylate (MMA) and diethylene glycol dimethacrylate (DGDA) using γ-radiation as initiation system. The ester groups of both membranes were activated using 1,6-hexamethylene diamine, introducing terminal primary amino groups. The temperature of the activation process was found to be the most influential factor. No shift of the optimum enzymatic reaction temperature was observed in the case of the MMA-grafted membranes, while a noticeable shift to higher temperatures was found with the DGDA-grafted membranes. Another interesting result was the pronounced shift of the pH optimum of the

enzymatic reaction to the alkaline side. This was contrary to the expected shift to the acidic side due to the formation of Schiff's bases. This could be explained by the presence of high electrostatic charges on the membranes. These negative charges are not compensating the positive charge of Schiff's bases.

The nylon particles were grafted only with one monomer (DGDA) using a chemical initiation system: potassium persulphate. The grafted particles were activated by the same method as the nylon membranes. The temperature of the activation process and the pH of the glutaraldehyde solution were found to be the most decisive factors affecting the activity. The difference between nylon membranes and nylon particles can be shown by comparing the obtained activities of one gram of immobilized enzyme preparation, which was 13 $\mu moles.min^{-1}$ for the membranes and 0.45 μ $moles.min^{-1}$ for the particles. This might be due to the difference in the grafting percentage or in other words the number of reactive groups created on the support. The grafting percentage achieved was 40 and 8% for the nylon membranes and particles, respectively. Improving the grafting percentage of the nylon particles to a higher limit could thus a significant positive effect on the activity. Moreover, the surface area, which in the case of nylon membranes is very high owing to the porosity, might also have played a significant role in the widely varying activities.

The operational stability of enzyme immobilized on two different membranes was investigated (Figure 3 and 4).

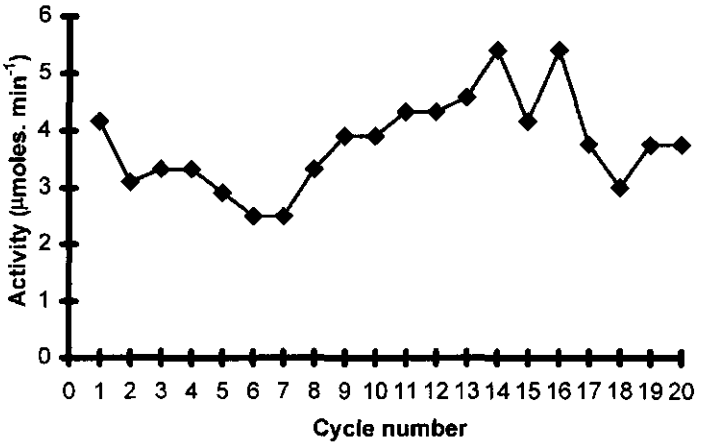


Figure 3. Operational stability of penicillin G acylase immobilized onto pMMA-grafted nylon membranes

The membranes were repeatedly used and washed after each of the first ten cycles with bidistilled water and after each of the last ten cycles with the same buffer as was used for the reaction; 10mM of cephalexin in glycin buffer pH 9.0 at 30°C for 30 minutes were the experimental conditions for these experiments. Both membranes, pMMA-grafted nylon and pDGDA-grafted nylon did not loose their activity after 20 repeated cycles (10 hours). The poor reproducibility may be explained by the reversible pore blockage which occasionally occurs with porous carriers. Although the reproducibility of the activity is not good, the positive effect of using buffer instead of water for washing the membranes after each cycle is clear. Using nylon particles that are non-porous could have the advantage of a better reproducible activity for measuring the operational stability. More important, the diffusion limitation could possibly be reduced because the enzyme is immobilized only on the surface of the particles.

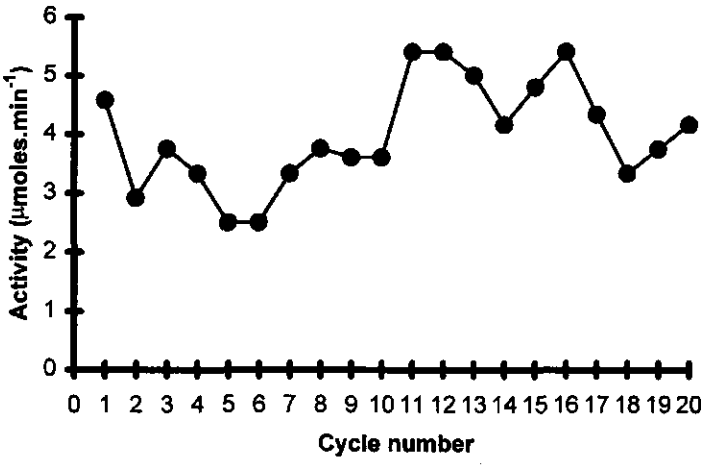


Figure 4. Operational stability of penicillin G acylase immobilized onto pDGDA-grafted nylon membranes

Addition of the inhibitor phenyl acetic acid (PAA) to the enzyme solution during the immobilization process as protecting agent for the enzyme active sites was found to have a significant effect on both the activity and the retention of the activity. In addition to the pDGDA-grafted nylon particles, the enzyme was also immobilized onto other matrices i.e.,

polyethylene-co-maleic anhydride and Eupergit, with different nature and having different functional reactive groups. Table 1 shows the activity and the retention of the activity for the enzyme immobilized onto these carriers. From the table we can see that both the activity and the retention of the activity for all the immobilized forms were significally improved by addition of PAA.

The effect of the presence of high concentrations of amino groups on the surface of the grafted nylon particles on the activity of the immobilized enzyme was investigated. For that, nylon particles were grafted with a different monomer, acrylic acid, which was activated by using 1,6-hexamethylene diamine, as well as by using thionyl chloride. The activity of the immobilized form prepared by using the thionyl chloride was found to be 100% higher than the immobilized form prepared by using 1,6-hexamethylene diamine as activator of the matrix. These results confirm the negative effect of the presence of primary amino groups on the immobilized enzyme activity. High concentrations of amino groups on carriers should thus be avoided for the immobilization of PGA.

Table 1. Effect of the phenyl acetic acid on the activity and the retention of activity of the immobilized enzyme.

Carrier name	Activity (μmoles.min^{-1})			Retention of activity (%)		
	PAA-	PAA+	Increment (%)	PAA-	PAA+	Increment (%)
pDGDA-g-nylon	5.80	9.90	70	15	29	93
PE-co-MaA	3.90	14.90	278	5.2	19.6	280
Eupergit	7.40	14.33	94	15.4	28.7	86

Non-isothermal bioreactors

Testing the activity of enzymes immobilized on membranes in non-isothermal bioreactors gave promising results. The activity under non-isothermal conditions was found to be higher than under isothermal conditions. The activity increment reached 100% with immobilized

penicillin G acylase operating at T_{AV} = 25 °C and ΔT = 30 °C and 70% with immobilized β-galactosidase under the same conditions. A direct relation has been found between the hydrophobicity of the membranes and the activity increment. In case of the more hydrophobic membranes, the effect of the non-isothermal conditions is higher (Chapter 6). Even higher values of the activity increment can be expected if the membrane is produced without the second grafting step. In general, the activity increment under non-isothermal conditions is mainly due to the reduction of diffusion limitation. The non-isothermal conditions thus present a new solution for the diffusion-limitation problem. Despite the benefits from using non-isothermal bioreactors, this type of reactor still faces some difficulties to be applied on industrial scale: 1) the control of the temperature difference when using large volume bioreactors, 2) surface area limitation. Overcoming these difficulties could be achieved by circulating the reaction volume with higher velocity and using tubular membrane bioreactors instead of flat membrane bioreactors.

SUMMARY

Immobilized enzymes can be reused, easily separated from the reaction medium, and are more stable in most of the cases. Despite of these advantages, there are still some problems facing the usage of the immobilized enzymes in industry. One of those problems is diffusion-limitation of both the reactants and the products. This problem becomes even more serious when the products are inhibitors of the enzymes. Different strategies for overcoming this problem have been discussed in Chapter 2

To overcome the problem of diffusion limitation, the enzymatic reaction can for instance be executed in non-isothermal bioreactors. For that the enzymes have to be immobilized on a hydrophobic membrane. Both β-galactosidase and penicillin G acylase have been immobilized onto different hydrophobic membranes, i.e. Teflon and nylon. The membranes were functionalized first using the grafting technique to create reactive groups on it. In Chapter 3 and Chapter 4 the Teflon membranes were grafted using a two-steps technique by which β-galactosidase was entrapped. The effect of the factors controlling the grafting process on the enzyme activity was studied (Chapter 3). The effect of variation of the microenvironment through changing the type of monomer used in the first grafting step on the properties and the activity of the immobilized enzyme is described in Chapter 4. The immobilized enzyme shows good activity under isothermal conditions, but it completely failed to work under non-isothermal conditions, since all the immobilized enzyme leaked out the membrane matrix during the first experiment in non-isothermal bioreactors.

The enzyme was then immobilized by a covalent method to one of those membranes (Chapter 5) using different functional groups on the membrane. The kinetic parameters and the properties of the immobilized enzyme were determined in dependence of the microenvironment of the immobilized enzyme.

One of those membranes was tested in a non-isothermal bioreactors (Chapter 6). The hydrolysis of the lactose under these conditions was found to be higher than the corresponding

isothermal ones. The activity increase reached 70%. The magnitude of the effect of the non-isothermal conditions was found to be dependent on the hydrophobicity of the membranes.

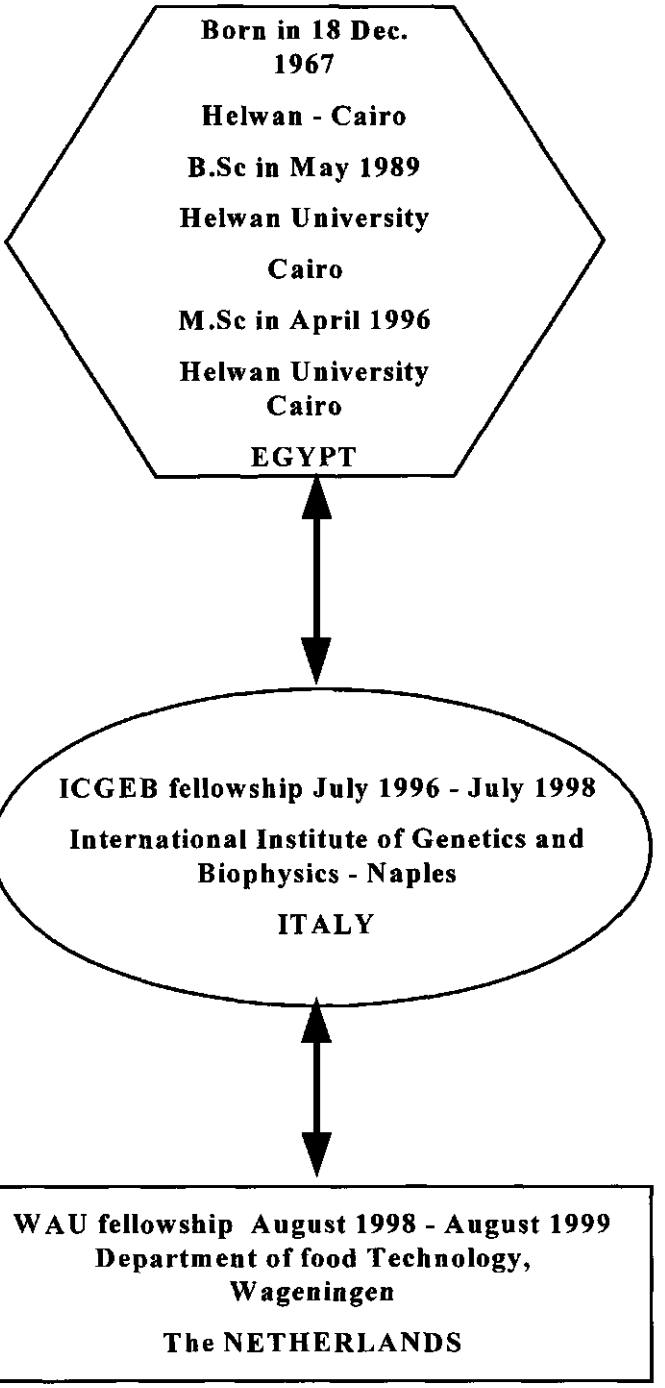
The second enzyme, penicillin G acylase, was immobilized on nylon. The membranes were first grafted with different acrylate monomers, followed by activation to bind the enzyme covalently (Chapter 7). The properties of the immobilized enzyme were studied and compared with the free enzyme.

A selected membrane was tested in non-isothermal bioreactors for the hydrolysis of cephalexin. The activity under these conditions was found to increase by a factor 2 as compared to the corresponding isothermal conditions (Chapter 8). In general, the application of the non-isothermal conditions seems to be promising to solve the diffusion-limitation problems.

In addition to that, the effect of the physical form of the matrix on the activity of the immobilized penicillin G acylase has been investigated (Chapter 9). Nylon particles have been grafted using a chemical initiator instead of γ-rays. The processes of the matrix activation and enzyme immobilization were partially optimized. The activity of the particle-immobilized enzyme was found to be very low in comparison with the activity of the enzyme immobilized on membranes.

In the general discussion (Chapter 10) some topics are present that were not discussed before.

CURRICULUM VITAE



List of publications

[1] **M.S. Mohy Eldin**, U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M., Santucci, P. Canciglia, F. S. Gaeta, and D.G. Mita, Characterization of the activity of b-galactosidase immobilized on teflon membranes preactivated with different monomers by g-radiation. J. Appl. Polym. Sci. 68, (1998) 613-623.

[2] **M.S. Mohy Eldin**, U. Bencivenga, M. Portaccio, S. Stellato, S. Rossi, M.Santucci, P. Canciglia, F. S. Gaeta and D.G. Mita, b-galactosidase immobilization on premodified teflon membranes using g-radiation grafting. J. Appl. Polym. Sci. 68, (1998) 625-636.

[3] **M.S. Mohy Eldin**, A. De Maio, S. De Martino, M. Portaccio, S. Stellato, U. Bencivenga, S. Rossi, M.Santucci, P. Canciglia, F. S. Gaeta and D.G. Mita, Non isothermal bioreactors utilizing catalytic teflon membranes grafted by γ-radiation. J. Membrane Sci.146, 237-248 (1998)

[4] M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, **M.S. Mohy Eldin**, P. Canciglia, F. S. Gaeta and D.G. Mita, Galactose competitive inhibition of β-galactosidase (*Aspergillus oryzae)* immobilized on chitosan and nylon supports. Enzy. Microb. Technol. 23,(1998) 101-106.

[5] **M. S. Mohy Eldin**, A. De Maio, S. Di Martino, S. Rossi, U. Bencivenga, A. D'Uva, F. S. Gaeta, and D.G. Mita, Immobilization of β-galactosidase on Nylon membranes grafted with diethylene glycol dimethacrylate (DGDA) by γ-radiation: Effect of the membrane pore size. Adv. Polym. Technol. 18(2), 109-123 (1999).

[6] **M. S. Mohy Eldin**, , M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. D'Uva, P. Canciglia, F. S. Gaeta, and D.G. Mita, Influence of the microenvironment on the activity of enzymes immobilized on Teflon membranes grafted by γ-radiation. In press in J. Molecular Catalysis (1999).

[7] **M.S. Mohy Eldin**, U. Bencivenga, S. Rossi, P. Canciglia, F. S. Gaeta, J. Tramper and D.G. Mita, Characterization of the activity of Penicillin G Acylase immobilized onto nylon membranes grafted with different acrylic monomers by means of γ-radiation. In press in J. Molecular Catalysis (1999).

[8] **M.S. Mohy Eldin**, C.G.P.H.Schroen, A.E.M.Janssen, S. Rossi, U. Bencivenga, P. Canciglia, F. S. Gaeta, J. Tramper and D.G. Mita, Non-isothermal caphalexin

hydrolysis by penicillin G-acylase immobilized on grafted nylon membranes. In press in J. Molecular Catalysis (1999).

[9] **M.S. Mohy Eldin**, C.G.P.H.Schroen, A.E.M.Janssen, D.G. Mita, and J. Tramper, Immobilization of penicillin acylase onto chemically grafted nylon matrix. Submitted to J. Molecular Catalysis (1999).

[10] **M. S. Mohy Eldin**, A. De Maio, S. Di Martino, N. Diano, V. Grano, N. Pagliuca, S. Rossi, U. Bencivenga, F. S. Gaeta, D. G. Mita, Isothermal and non-isothermal lactose hydrolysis by means of β-galactosidase immobilized on a single double-grafted teflon membrane. Sumitted to J. Membrane Sci., (1999).