Designing Acrylamide- and Methacrylate-Based Novel Supports for Lipase Immobilization

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ABSTRACT: To design efficient polymeric supports for lipase immobilization, two series of hydrogels based on acrylamide and three methacrylates were prepared via crosslinking with ethylene glycol methacrylate and *N*,*N*-methylenebisacrylamide. The three methacrylates used to prepare these hydrogels had different alkyl chain lengths: C_1 (methyl methacrylate), C_{12} (dodecyl methacrylate), and C_{18} (octadecyl methacrylate). In the reaction scheme, only the feed concentration of the hydrophobic component (methacrylate) was varied. The characterization of the hydrogels was carried out with Fourier transform infrared, scanning electron microscopy, and nitrogen analysis to establish their structural aspects and to obtain evidence for network formation; the swelling and water uptake of the hydrogels were studied as functions of the time, temperature, and pH. Lipase immobilization on selected hydrogels was studied as a function of the concentration of the methacrylate used in the feed and the nature of the crosslinker. The activity of the hydrogel series that showed the highest activity of the immobilized lipase was investigated further as a function of the methacrylate feed concentration, pH, and temperature. Some organic solvents were studied to investigate the effect of the nature of the solvent on the activity of the immobilized lipase. The activity of the immobilized lipase was more than that of the free lipase and was affected by the structural attributes of the polymeric supports and by the nature of the solvent. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 105: 3006–3016, 2007

Key words: crosslinking; enzymes; hydrogels; morphology; networks

INTRODUCTION

In view of the increased interaction of polymer science and biotechnology, lipases immobilized on polymeric supports are experiencing increased use in many industrial applications. The extent of the immobilization and activity of immobilized lipases is affected by the nature of the support and by environmental factors such as the pH, temperature, and nature of the reaction medium. Thus, to design a suitable support for lipase immobilization, all these aspects have to be considered. Immobilized lipase performs better in hydrophobic environments.[1] A combination of hydrophilic and hydrophobic monomers in a hydrogel is desirable for higher mechanical and chemical stability.[2] Tailoring hydrogels by the proper selection of the hydrogel components broadens their applicability for use in adverse pH and strongly ionic solutions.[3,4] The nature of the reaction medium is another important factor that affects the activity of immobilized lipases. A support of moderate hydrophilicity often offers higher conformational stability to lipases and also increases the surface area of hydrogels.[5] A lipase acts as an effective hydrolase in an aqueous medium[6] and as an esterase in hydrophobic organic solvents with limited water.[7] Hence, the nature of the reaction medium influences the lipase activity from kinetic and thermodynamic points of view.[8,9] The activity of immobilized lipases is usually low in comparison with the activity reported for other enzymes.[10,11] Therefore, it is necessary to design suitable supports for lipase immobilization for higher activity of immobilized lipases, especially under harsher conditions such as adverse pHs and higher temperatures.

The use of polyacrylamide [poly(AAm)]-based hydrogels and other natural and synthetic polymers as supports for enzyme immobilization has been reported by many workers.[12–16] Poly(AAm) hydrogels absorb large amounts of water because of the presence of water-solubilizing amide groups, but at the same time they suffer from poor hydrolytic stability and low tensile strength.[17] Lipases have been immobilized on partially hydrolyzed poly(AAm) beads.[18] Many methacrylate (MA)-based supports have also been reported as supports in lipase immobilization.[4,19] The properties of poly(AAm) hydrogels can be improved by copolymerization with a hydrophobic monomer as the second component. Copolymer

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networks with combination of hydrophobic and hydrophilic characteristics are desirable for the high activity of immobilized lipases.

Because of this and as a continuation of our earlier work,[15,16] in this communication we report the design of a novel support with most of the desirable attributes for the immobilization of a well-characterized lipase. Two series of networks have been prepared by the copolymerization of acrylamide (AAm) with three MAs and have been crosslinked by two crosslinkers with different hydrophobic/hydrophilic natures, that is, ethylene glycol dimethacrylate (EGDMA) and *N,N*-methylenebisacrylamide (*N,N*-MBAAm). Methyl methacrylate (MMA), dodecyl methacrylate (DMA) and octadecyl methacrylate (ODMA) have been used, the latter two having long alkyl side chains (C_{12} and C_{18}, respectively). There is not much information on the use of these two monomers in specialty applications such as enzyme immobilization. The use of these two long-chain MAs (being highly hydrophobic) as components of these hydrogels is expected to increase the gel–lipase interactions.

EXPERIMENTAL

Materials

AAm, *N,N,N,N*-tetramethylethylenediamine (S.D. Fine, Mumbai, India), *N,N*-MBAAm (Qualigens Fine Chemicals, Mumbai, India), ammonium persulfate (Sarabhai Chemicals, Vadodra, India), DMA, ODMA, and EGDMA (Merck, Schuchardt, Germany) were used as received. Precharacterized lipase from *Bacillus coagulans* (MTCC-6375; the accession number was accorded by IMTECH, Chandigarh, India) was obtained from the Department of Biotechnology of Himachal Pradesh University (Shimla, India).

Synthesis of the networks

According to a procedure reported elsewhere, the hydrogels were prepared by the separate copolymerization and crosslinking of a fixed amount of AAm and five different concentrations of different MAs, a crosslinker, and fixed amounts of an initiator–accelerator system that comprised ammonium persulfate (1 m*M*) and *N,N,N,N*-tetramethylethylenediamine in a 1:1 water/acetone solvent system.[20] A model set of reactions can be described as follows. AAm (28 m*M*) and MMA (3.5 m*M*) were placed in 10.0 mL of acetone/water (1:1) along with ammonium persulfate (1 m*M*) and EGDMA (2.8 m*M*); this was followed by the addition of *N,N,N,N*-tetramethylethylenediamine (0.5 m*M*). The reaction system was allowed to stand at 25°C for 30 min. In the four other reactions, only the concentration of MMA was varied. The synthesis of networks with DMA or ODMA as the comonomer was carried out with the aforementioned scheme, and so was the preparation of *N,N*-MBAAm-crosslinked hydrogels.

Separation of the hydrogels

Insolubilized polymers were separated from the reaction system by filtration. The sol fractions trapped inside the networks, if any, were separated from the networks by separate treatments: they were refluxed with water and acetone, shifting from the solvent of higher polarity to the one of lower polarity and spending 1.0 h in each solvent. Such a treatment was required because the reaction system had both water- and acetone-soluble components. The product was dried in a vacuum oven for 24 h to obtain a xerogel. The xerogels were cut into small pieces of equal size with a calibrated chopper. The network formation efficiency (%*E*) was calculated and defined as follows:[20]

$$\%E = (\text{Weight of the xerogel})/[\text{Weight of the monomers (MA + AAm + Crosslinker)}] \times 100$$

The networks are designated poly(AAm-*co*-MA)-*cl*-*N,N*-MBAAm and poly(AAm-*co*-MA)-*cl*-EGDMA, where -*cl*- stands for crosslinked.

Scanning electron microscopy (SEM), nitrogen analysis, and Fourier transform infrared (FTIR) studies

SEM images of the networks were recorded on a JEOL JSM 6100 (Scotia, NY), and FTIR spectra were recorded in KBr pellets on a PerkinElmer (Waltham, MA) instrument. Elemental analysis (nitrogen only) was recorded on a Carlo Erba EA-1108 (Midland, Canada).

Swelling studies of the hydrogels

To optimize the time and temperature for swelling, networks of a known weight (0.1 g) were immersed in deionized water, and the water uptake was measured gravimetrically, as reported earlier.[20] The hydrogels showed maximum swelling at 120 min at 45°C. The effect of the pH (4.0 and 9.2) was studied with buffer tablets at 45°C for 120 min. The swelling ratio (S_r) of the hydrogels can be expressed with the following relationship:

$$S_r = (\text{Weight of the swollen hydrogel})/(\text{Weight of the xerogel})$$

Enzyme immobilization study

The lipase was immobilized by the equilibration of 100 mg of the hydrogels in a Tris–HCl buffer at

8.5 pH. The purified lipase (50 mg, equivalent to 1 mg/mL) was coupled with the respective hydrogels under continuous shaking at 8°C, and the hydrogels were placed on Whatman filter paper (Aldrich, Steinheim, Germany). The lipase activity was determined by the measurement of *p*-nitrophenol released from *p*-nitrophenyl palmitate at a wavelength of 410 nm on a Shimadzu (Japan) ultraviolet–visible spectrophotometer with the modified Winkler and Stuckmann method.[21] Along with the polymeric matrix, a mixture of *p*-nitrophenyl palmitate (20.0 m*M*, containing 75.0 μL), Tris–HCl buffer (0.05*M*, pH 8.5), and 100.0 mg of immobilized enzyme was added, and the total volume was increased to 3.0 mL with a 0.1 *M* Tris–HCl buffer (pH 8.5). Each test tube was incubated at 45°C for 20 min. The corresponding concentration was determined from a *p*-nitrophenol standard graph. The control contained 75.0 μL of the substrate, 2.925 mL (0.1*M*) of the Tris–HCl buffer, and 100.0 mg of the matrix. One unit of lipase activity is defined as the micromoles of *p*-nitrophenol released by 1.0 mL of the free enzyme per minute (or 1.0 g of the immobilized matrix) at 45°C under the assay conditions; this is equivalent to the IU/g value of the hydrogel. All the experiments were run twice, and high repeatability was observed throughout; the results were compared with those for the free enzyme.

RESULTS AND DISCUSSION

The composition of the hydrogel and its interaction with the medium significantly affect the extent of lipase immobilization and its resultant activity. Hence, the structure of these hydrogels is investigated as a function of the variation of the molar ratio of the monomers and the nature of the crosslinkers. Because monomers and crosslinkers have very different water interaction profiles, the structural aspects of the networks are expected to be of interest. The reported monomer reactivity ratio of AAm with MMA supports the idea that AAm is less reactive with itself than with a comonomer, and this is in agreement with results obtained for copolymers of AAm with MMA.[20,22] More or less the same reactivity behavior is expected for the copolymerization of DMA and ODMA although the effects of these two as comonomers are expected to be less than that of MMA because of their very long alkyl side chains and the consequent increase in the hydrophobic nature of the reaction system. The contribution of the hydrophobic component in the network is expected to affect the degree of lipase immobilization.

Effects of the MA concentration and nature of the crosslinker on the network yield

The effects of different reaction parameters on the network yield have been evaluated. In this study, the AAm concentration was kept constant (28 m*M*) in all experiments. Only the MA concentration was varied (five times over the concentration range). %*E* decreases sharply with an increase in the MA concentration. An increase in the hydrophobic contents of the system affects the polymerization of AAm as this trend is more pronounced in the presence of EGDMA, which along with MA is hydrophobic in nature [Fig. 1(a,b)]. %*E* decreases with progressive increases in the MA concentration in the feed. This conclusion is in agreement with earlier work reported on the copolymerization of MMA and DMA by Stahl et al.[22]

Characterization of the hydrogels

The hydrogels were characterized with SEM, nitrogen analysis, and FTIR studies to obtain evidence for the copolymerization of the different components present in the reaction system and network formation.

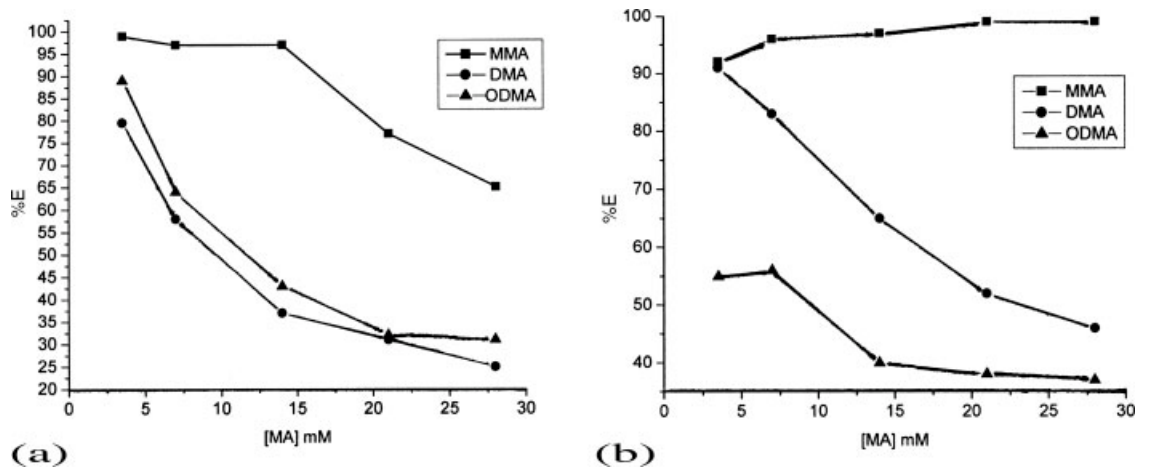


Figure 1 %*E* for (a) EGDMA-crosslinked series and (b) *N*,*N*-MBAAm-crosslinked series as a function of [MA].

TABLE I
Elemental (Nitrogen) Analysis of Different Networks

Network	Weight (g)	N (%)	N (g)	AAm (g)[c]	MA (g)	MA/AAm
Poly(AAm-*co*-MMA)-*cl*-EGDMA[a]	2.214	11.22	0.248	1.246	0.968	1.093
Poly(AAm-*co*-MMA)-*cl*-EGDMA[b]	2.324	4.81	0.111	0.562	1.762	4.41
Poly(AAm-*co*-MMA)-*cl*-*N,N*-MBAAm[b]	2.392	9.37	0.224	1.136	1.256	1.55
Poly(AAm-*co*-DMA)-*cl*-EGDMA[a]	2.206	14.58	0.321	1.631	0.575	1.26
Poly(AAm-*co*-DMA)-*cl*-EGDMA[b]	2.246	14.25	0.320	1.622	0.624	1.37
Poly(AAm-*co*-DMA)-*cl*-*N,N*-MBAAm[b]	2.017	12.52	0.252	1.280	0.737	2.06
Poly(AAm-*co*-ODMA)-*cl*-EGDMA[a]	3.052	10.42	0.318	1.61	1.442	2.88
Poly(AAm-*co*-ODMA)-*cl*-EGDMA[b]	2.467	5.48	0.135	0.685	1.782	4.47
Poly(AAm-*co*-ODMA)-*cl*-*N,N*-MBAAm[b]	2.090	2.89	0.060	0.306	1.784	4.80

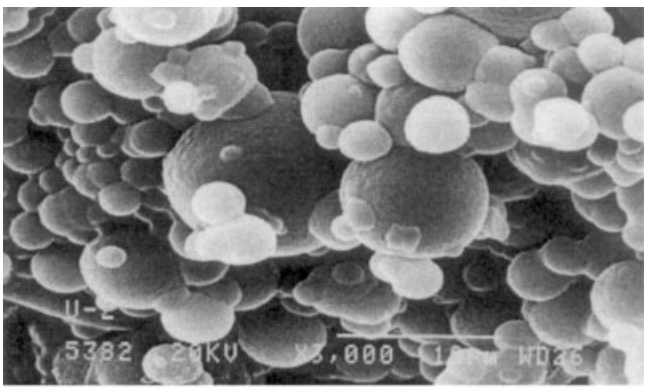
[a] Lowest MA concentration.
[b] Highest MA concentration.
[c] Includes the weight of *N,N*-MBAAm when it appears.

Elemental (nitrogen) analysis study

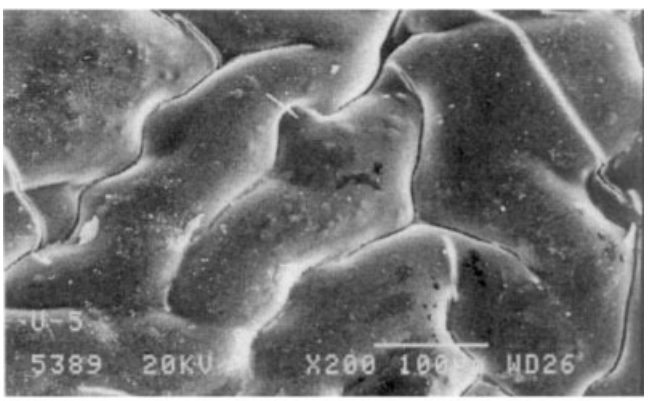
The nitrogen analysis of the hydrogels and functionalized hydrogels provides evidence of the incorporation of both monomers in the hydrogels. The ratio of MMA to AAm in the network is fairly high and increases with an increase in the MMA concentration in the feed. This is further supported by the fact that the percentage of nitrogen decreases in these networks with an increase in the concentration of MMA in the feed. The incorporation of MA increases with its availability in the feed. However, the ratio is almost constant for DMA and ODMA, and this means that the reactivity of MA decreases with an increase in the alkyl substituents of the ester moiety and a consequent increase in the hydrophobicity of the reaction medium (Table I).

SEM study

The effect of the crosslinker nature on the network structure is visible in SEM images (Fig. 2). The effects of the crosslinking and interaction of the hydrophobic and hydrophilic regions are clearly visible in the SEM images of the hydrogels. For poly(AAm-*co*-MMA)-*cl*-EGDMA (1 : 1 molar ratio of the monomers in the feed), most of the particles are globular, and the particle size distribution lies between 2 and 5 μm. There are only a few globules whose diameter is greater than 10 μm [Fig. 2(1a)]. The effect of the crosslinker nature on the hydrogel structure is apparent from the SEM image of poly(AAm-*co*-MMA)-*cl*-*N,N*-MBAAm, for which more intense crosslinking results in film formation [Fig. 2(1b)]. In the case of poly(acrylamide-*co*-dodecyl methacrylate) networks, the long, hydrophobic side chains of DMA are uniformly placed as coils as a result of their interactions with water in the synthetic stage. This also results from the repulsive environment provided by the polar hydrogel component (AAm) and the crosslinker (*N,N*-MBAAm). Thus, the surface morphology is distinct with uniformly aligned, long, and flexible MA chains [Fig. 2(2)]. Such an alignment of the hydrophilic and hydrophobic regions in the hydrogel, as reflected in their surface morphology, should provide suitable anchorage to the lipase molecules. The pore formation is also distinctly visible at the higher magnification [Fig. 2(2b), (2c)]. In the case of poly(AAm-*co*-ODMA)-*cl*-*N,N*-MBAAm (prepared with a 1 : 1 molar ratio of the monomers in the feed), bigger pores are visible along with the orientation

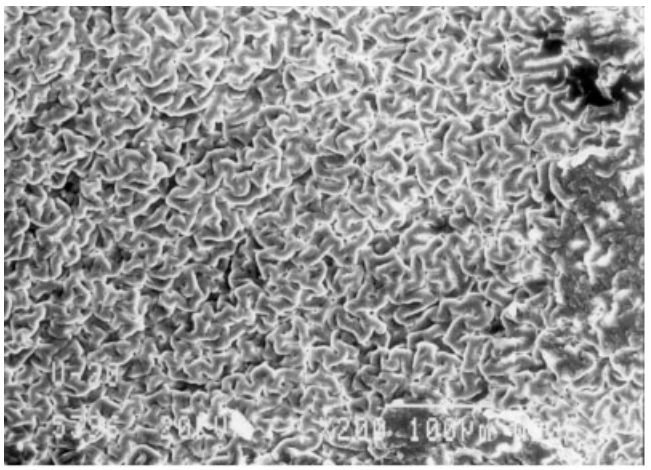


(1a)

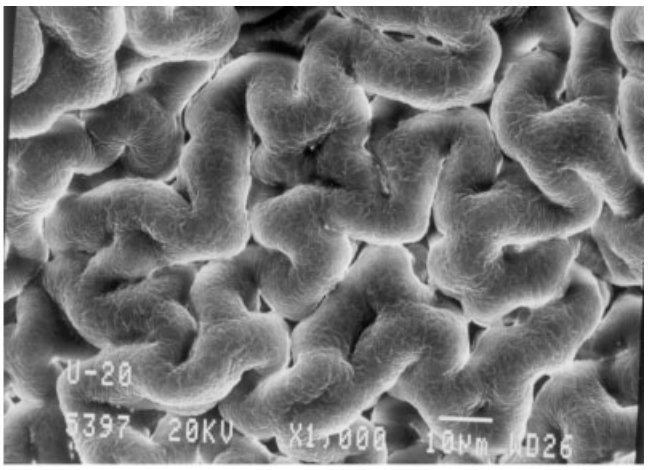


(1b)

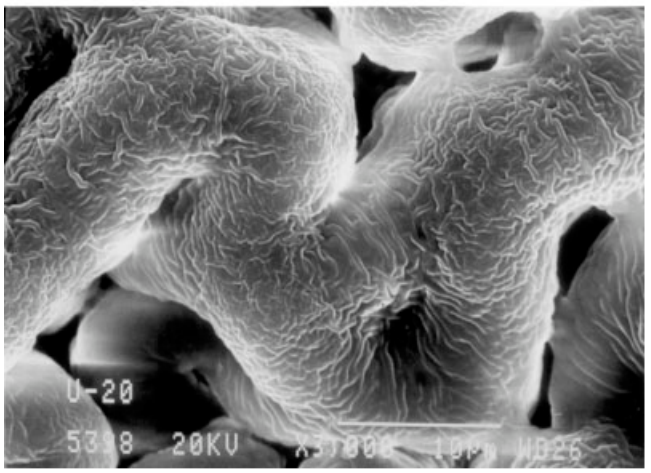
Figure 2 SEM images of (1a) poly(AAm-*co*-MMA)-*cl*-EGDMA, (1b) poly(AAm-*co*-MMA)-*cl*-*N,N*-MBAAm.



(2a)

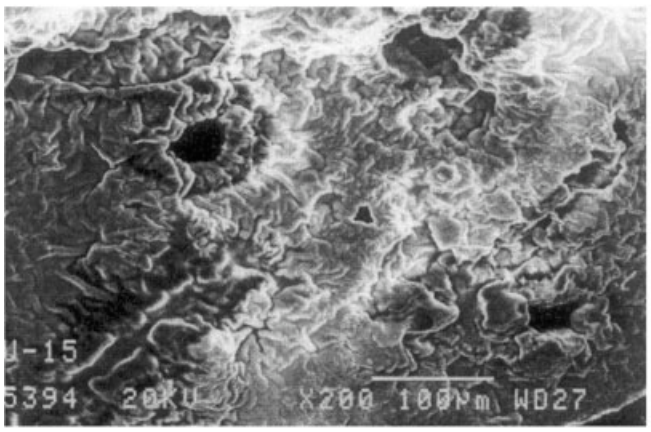


(2b)

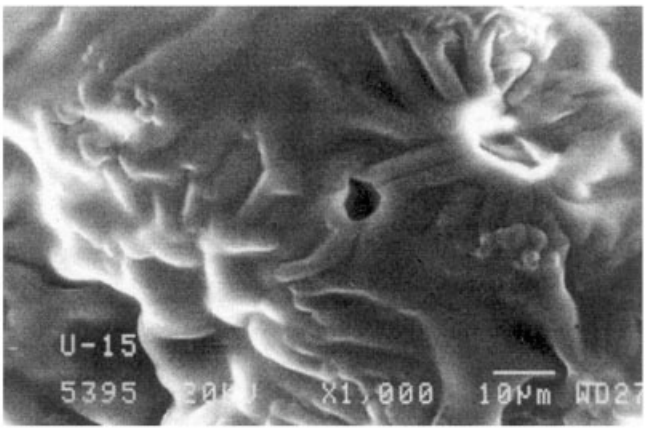


(2c)

Figure 2 (*Continued from the previous page*). (2a), (2b), (2c): Poly (AAm-*co*-DMA)-*cl*-EGDMA-N,N-MBAAm at the three diffent magnifications as given in the micrograph, (3a), (3b): Poly(AAm-*co*-ODMA)-*cl*-EDGMA-N,N-MBAAm at two different magnification as given in the micrograph.



(3a)



(3b)

of the long MA side chains as a result of hydrophobic interactions on the surface of the network [Fig. 2(3a), (3b)]. The differences in the surface structures of these hydrogels, as reflected by SEM, are expected to affect lipase adsorption to different extents.

FTIR spectroscopy

The FTIR spectrum of poly(AAm-*co*-MMA)-*cl*-*N,N*-MBAAm prepared with the highest MMA concentration shows characteristic absorption peaks at 3429.2

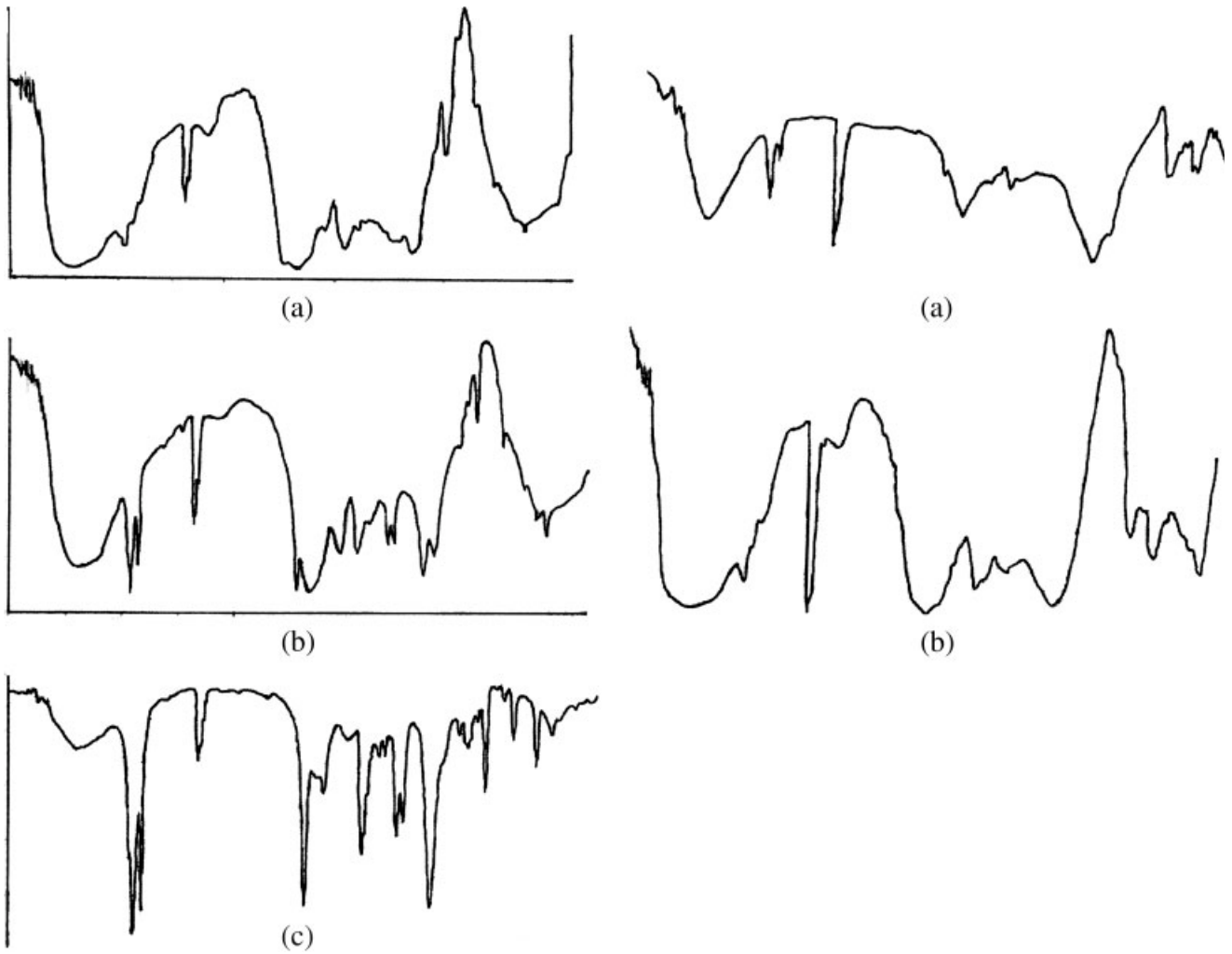


Figure 3 (1) FTIR spectra of *N,N*-MBAAm-crosslinked networks prepared with (a) MMA, (b) DMA, and (c) ODMA at the highest MA concentration and (2) FTIR spectra of poly(AAm-*co*-DMA)-*cl*-EGDMA at DMA concentrations in the feed of (a) 28 and 3.5 m*M*.

(absorption = 96.21%), 2951.5 (absorbance = 88.26%), 1700.0 (absorption = 92.0%), and 1671.7 cm^{-1} (absorption = 96.48%); they can be ascribed to the stretching vibrations of the aforementioned groups [Fig. 3(1a)]. The FTIR spectrum of the corresponding DMA network shows these absorption peaks at 3391.9 (absorption = 83.41%), 2925.4 (absorption = 93.41%), 1722.1 (absorption = 92.11%), and 1662.0 cm^{-1} (absorption = 96.84%), respectively; they are ascribed to the stretching vibrations of the aforementioned groups [Fig. 3(1b)]. The poly(AAm-*co*-ODMA)-*cl*-*N,N*-MBAAm shows the same characteristic peaks, and the trends for the quantitative incorporation of AAm are similar to those discussed for the other networks [Fig. 3(1c)]. The FTIR spectra of the network synthesized with the lowest EGDMA concentration and the highest DMA concentration and crosslinked with EGDMA are presented for the sake of comparison and for the incorporation of the crosslinker [Fig. 3(2a,b)].

Water uptake behavior of the hydrogels at different pHs

The water uptake level of polymeric supports for use in lipase immobilization is an important aspect. In this study, a combination of good gelling and nongelling monomers was designed to obtain a hydrogel of desirable swellability, especially in media of different pHs. After evaluating the optimum time and temperature (120 min and 45°C) for all the hydrogels, we studied the water uptake at different pHs. Most of the hydrogels take up an appreciable amount of water within 10 min. However, they do not respond much to pH changes, so S_r does not change dramatically. The order for S_r in solutions of different pHs is as follows for both series of hydrogels based on poly(acrylamide-*co*-methyl methacrylate): pH 7.0 < pH 4.0 < pH 9.2. The water uptake decreases with an increase in the MMA concentration in the feed [Fig. 4(1a,b)]. In the case of the poly(dodecyl methacrylate)-based hydrogels, S_r increases with the pH from pH 4 to pH 9.2 [Fig. 4(2a,b)].

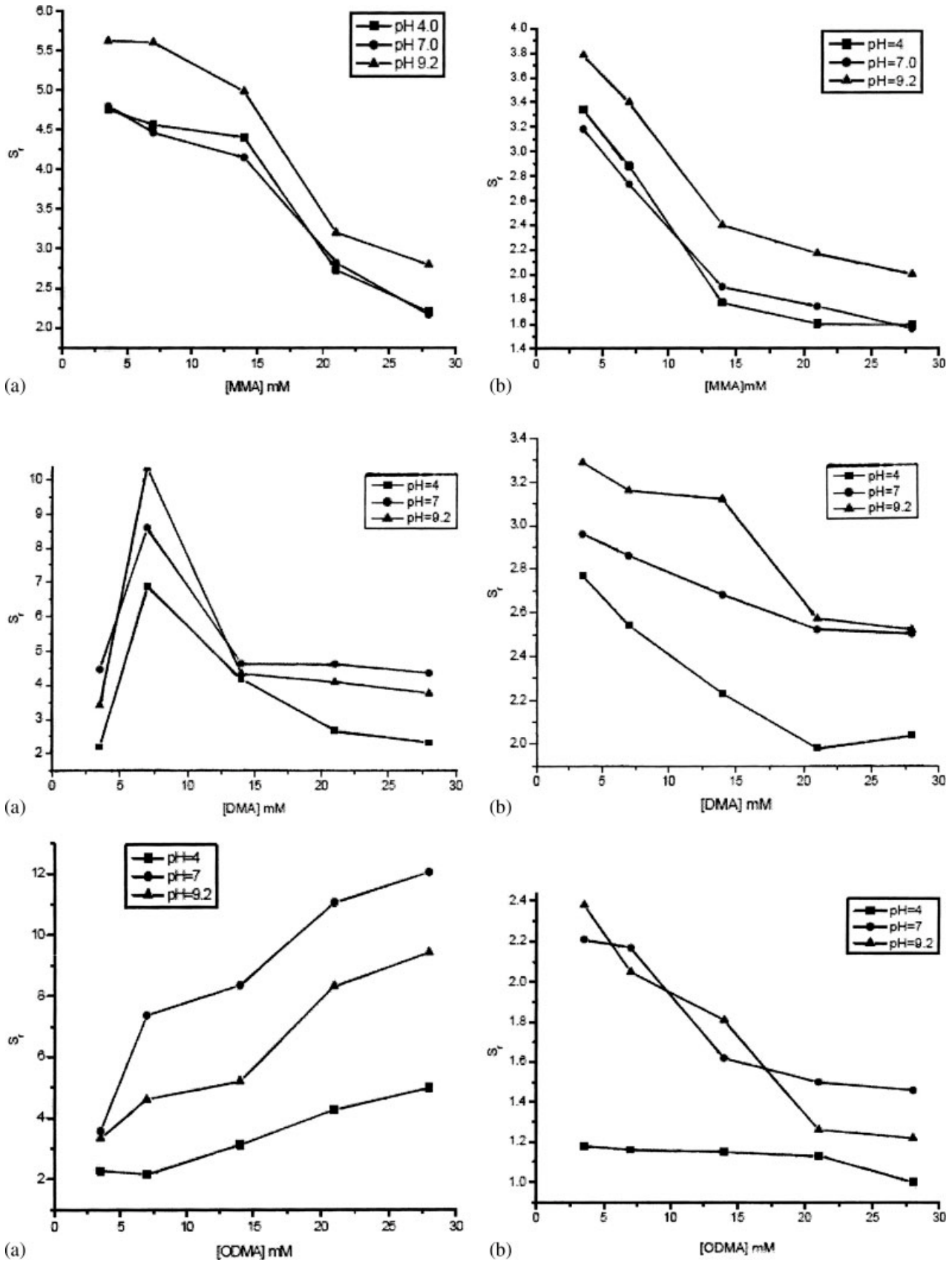


Figure 4 (1) Effect of the pH on S_r of (a) poly(AAm-*co*-MMA)-*cl*-EGDMA and (b) poly(AAm-*co*-MMA)-*cl*-*N*,*N*-MBAAm as a function of [MMA] in the feed, (2) effect of the pH on S_r of (a) poly(AAm-*co*-DMA)-*cl*-EGDMA and (b) poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm as a function of [MMA] in the feed, and (3) effect of the pH on S_r of (a) poly(AAm-*co*-ODMA)-*cl*-EGDMA and (b) poly(AAm-*co*-ODMA)-*cl*-*N*,*N*-MBAAm as a function of [MMA] in the feed.

TABLE II
Activity of Immobilized Lipase

Network	[MA] (m*M*)	Activity (IU/g)
Poly(AAm-*co*-MMA)-*cl*-EGDMA	3.5	60.2
Poly(AAm-*co*-MMA)-*cl*-EGDMA	28.0	160.0
Poly(AAm-*co*-MMA)-*cl*-*N*,*N*-MBAAm	3.5	150.0
Poly(AAm-*co*-MMA)-*cl*-*N*,*N*-MBAAm	28.0	145.0
Poly(AAm-*co*-DMA)-*cl*-EGDMA	3.5	81.0
Poly(AAm-*co*-DMA)-*cl*-EGDMA	28.0	182.0
Poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm	3.5	212.0
Poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm	28.0	120.0
Poly(AAm-*co*-ODMA)-*cl*-EGDMA	3.5	65.5
Poly(AAm-*co*-ODMA)-*cl*-EGDMA	28.0	192.0
Poly(AAm-*co*-ODMA)-*cl*-*N*,*N*-MBAAm	3.5	82.4
Poly(AAm-*co*-ODMA)-*cl*-*N*,*N*-MBAAm	28.0	42.0

The order for S_r at different pHs is as follows for both hydrogels series: pH 4.0 < pH 7.0 < pH 9.2. At the lower DMA concentration, swelling is appreciable, but it decreases with an increase in the DMA concentration. The effect of the nature of the crosslinker is very well marked for poly(AAm-*co*-ODMA)-*cl*-EGDMA, as it has been observed that S_r increases in a linear fashion with an increase in the ODMA concentration in the case of EGDMA-based series, whereas it decreases progressively for the *N*,*N*-MBAAm series [Fig. 4(3a,b)]. Such results emanate from the fact that the ratio of ODMA to AAm is almost the same in this case; hence, a proper balance of hydrophobic and hydrophilic forces favors more water uptake than for the other series of hydrogels. The SEM images of the ODMA-based series of hydrogels also reveal that these networks have large pores that can retain large amounts of water [Fig. 2(3)]. However, the same is not true for the more intensely crosslinked hydrogels based on the *N*,*N*-MBAAm-crosslinked series. The effect of the pH also follows a different order as the highest values for both series are obtained at pH 7.0.

From the results for water uptake in solutions of different pHs, it follows that the water uptake level of the hydrogels is moderate to low and is dependent on both the nature of the monomer and the nature of the crosslinker. These results suggests that at the low swelling time of 2 h, the interaction of the ionic species is not sufficient to cause hydrolysis of the ester or amide groups, so the water uptake does not change appreciably with a change in the pH. Furthermore, less intense crosslinking with EGDMA results in larger pores, so higher water uptake can be observed. In contrast, the more intensely crosslinked and film-like surfaces of *N*,*N*-MBAAm-crosslinked hydrogels do not allow sufficient water to penetrate the bulk of the network.

Enzyme immobilization

The immobilization of lipase was studied as a function of the structural aspects of hydrogels and environmental factors such as the pH, temperature, and time of incubation used for immobilization. The hydrogel that afforded better results was investigated further. The maximum lipase immobilization capacity of the hydrogels was found to be 65.4% in hydrogels showing 212 IU/g activity.

Effect of the time of incubation

The percentage of lipase immobilization was studied at different time intervals from 1 to 10 h. The immobilization of lipase increased with the incubation period from 1 to 10 h at pH 8.5 and 8°C. The highest lipase immobilization percentage was observed at 5 h (64.7%) and was selected for further work. A further increase in time (up to 10 h) did not change the lipase uptake much. Thus, these processes are initially time-dependent, as the lipase requires time to come in contact with the supports. The lipase immobilization of an enzyme is by way of adsorption on the hydrogel surface as it cannot easily diffuse in the interior of the hydrogel because of the large size of its molecule (98 kD). Thus, the lipase activity is affected by the nature of the monomer and by the crosslinker, as is evident from Table II. At the lower crosslinker concentration, the activity is high for poly(AAm-*co*-MMA)-*cl*-EGDMA. However, for poly(AAm-*co*-MMA)-*cl*-*N*,*N*-MBAAm prepared at a low or high MMA concentration, no selectivity or differentiation can be observed in the activity. For poly(acrylamide-*co*-dodecyl methacrylate)-based hydrogels, the effect of the nature of the crosslinker can be observed in a marked manner as poly (AAm-*co*-MMA)-*cl*-EGDMA shows higher activity at the highest DMA concentration or, in other words, in a more hydrophobic regime, whereas for poly (AAm-*co*-MMA)-*cl*-*N*,*N*-MBAAm, the results are just the reverse, with the hydrogel prepared at the lowest DMA concentration showing the highest activity (IU/g = 212) of all the hydrogels studied. For poly(AAm-*co*-ODMA)-*cl*-EGDMA and poly(AAm-*co*-ODMA)-*cl*-*N*,*N*-MBAAm, although the trends for the activity are the same as those observed for the DMA-based hydrogels,

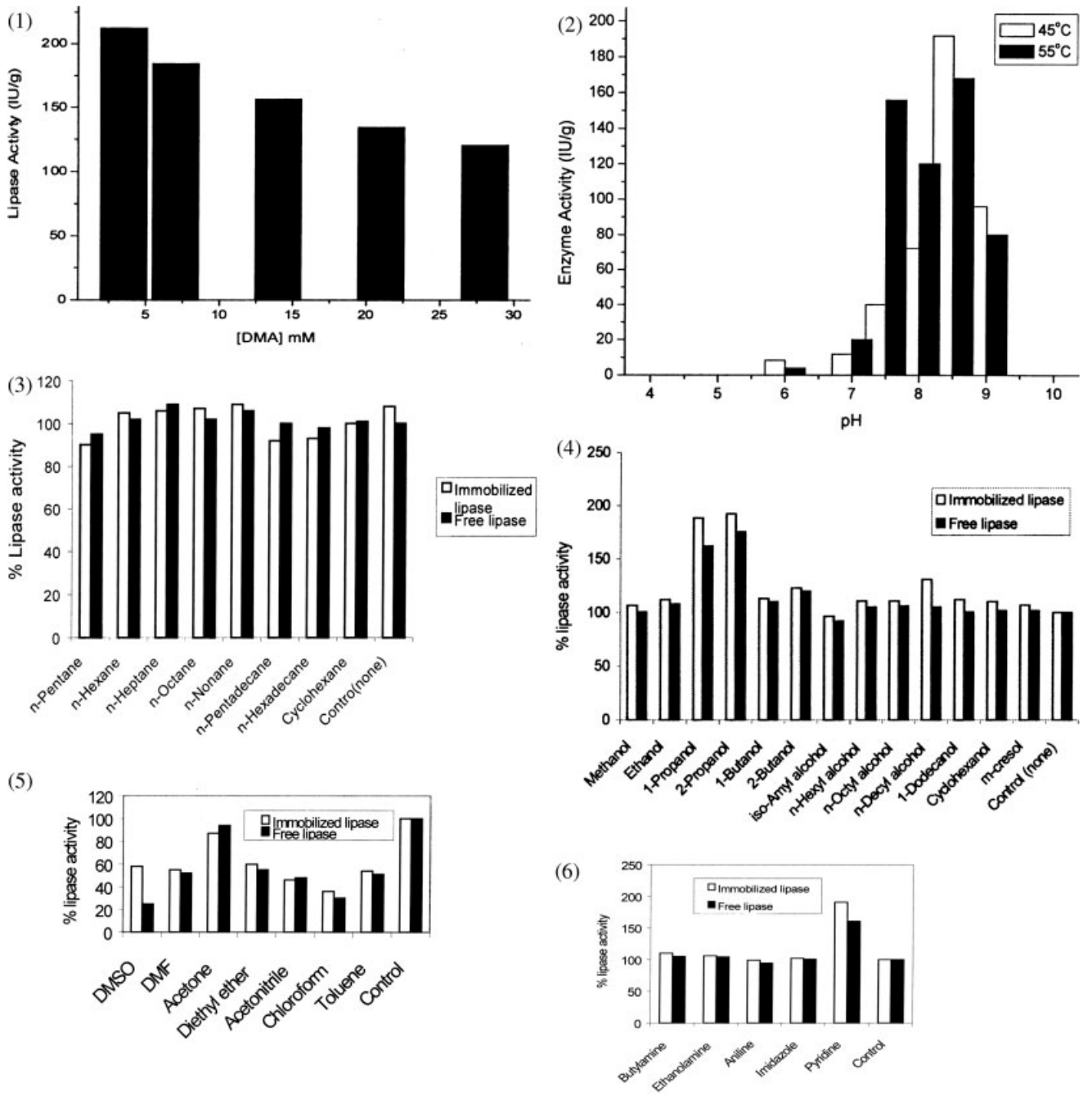


Figure 5 (1) Effect of the feed DMA concentration on the lipase activity at pH 8.5 and 45°C, (2) effect of the pH and temperature on the lipase activity of poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm ([DMA] in the feed = 3.5 m*M*), (3) effect of the alkane chain length on the activity of free and immobilized lipase, (4) effect of the alcohol chain length on the activity of free and immobilized lipase, (5) effect of some solvating and polar solvents on the activity of free and immobilized lipase, and (6) effect of some basic solvents on the activity of free and immobilized lipase.

higher activity is shown by the former, and much less activity is observed for the latter.

From the results obtained from the nitrogen analysis and investigation of the surface morphologies of these hydrogels, we assume that the higher activity and higher stability of the lipases are best manifested as follows: (1) the presence of hydrophobic and hydrophilic components in the hydrogel; (2) an appropriate carbon chain length for the hydrophobic component (ester group) that results in the maximum lipase–hydrogel interaction and hence maximum activity; (3) contrasting trends in the protein interactions because *N*,*N*-MBAAm and EGDMA show contrasting crosslinking properties; and (4) the highest activity of all 12 hydrogels studied because poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm combines all these aspects, as it has well-aligned hydrophobic regions and hydrophilic regions with a smooth surface. The surface morphol-

ogy of this network is most suitable for the adsorption processes of the immobilized lipase as it provides the enzyme optimum conformational stability and properly orients the active sites for better substrate interaction. After the evaluation of the highest activity for poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm prepared with 3.5 m*M* DMA in the feed, the whole series of poly(-AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm was studied for the effect of the feed DMA concentration. The trends shown by this investigation reveal a decrease in the lipase activity with the DMA concentration increasing in the feed [Fig. 5(1)].

Effect of the pH and temperature on the activity of lipase immobilized on poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm

To study the effects of external environmental factors such as the pH and temperature on the activity of immobilized lipase, the immobilization was carried out on poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm prepared with 3.5 m*M* DMA by the variation of the pH from 4.0 to 10.0 at 45–55°C in 10 h. At a lower (acidic) pH, no activity was observed at all at either temperature. Because the lipase is alkaline, a strong interaction and consequent denaturation of the protein are possible. The lipase activity increases with the pH, showing a maximum at pH 8.5 and 45°C. However, at 55°C, it decreases at all pHs studied, and two maxima can be observed at pHs 7.0 and 8.5 [Fig. 5(2)].[23] A further increase in the pH reduces the lipase activity.

Effect of the nature of the solvent on the lipase activity

The nature of the organic medium is an important factor in design a biocatalyst system based on an immobilized lipase. A small amount of water is absolutely essential to obtain sufficient enzyme conformational flexibility for enzyme activity. At higher water contents, the competition between the enzyme and support causes diffusion limitations for the substrates and a reduction in the reaction rate. Lipases show higher reactivity in small amounts of water and nonpolar solvents. In this study, some groups of the solvents were studied for their effect on the activity of both immobilized and free lipase. In this study, it has been observed that alkanes enhance the activity of the immobilized lipase but not in a marked manner. Even the most investigated solvent for lipase (*n*-hexane) does not increase lipase activity in a marked manner, despite its high log *P* value (3.5). The activity of the immobilized lipase in other solvents having log *P* values less than 2.5 is understandable because these distort the water layer around the enzyme, which is required to maintain the tertiary structure. With an increase in the carbon chain length or nonpolar character, the activity of both free and immobilized lipases increases with the carbon chain length up to C_9 (*n*-nonane) but decreases as higher alkanes with C_{15} and C_{16} are used. In any case, the enhancement of the activity does not exceed the control value [Fig. 5(3)]. On the other hand, most of the alcohols increase the activity of the free and immobilized lipases [Fig. 5(4)]. The activity of the lipases increases with an increase in the carbon chain length up to C_3 as both 1-propanol and 2-propanol show extraordinary increases, the latter showing higher activity. However, the activity decreases thereafter in the presence of higher chain and cyclic alcohols. These results can be explained by the fact that in the presence of moderately polar solvents, an immobilized lipase orients to a more favorable conformation. Chamorro et al.[24] drew similar conclusions concerning the effect of short-chain organic solvents, including 2-propanol, on *Candida rugosa* lipase. Furthermore, in such a system, the amount of water is minimal, that is, just enough to maintain the tertiary structure of the enzyme. Almost all other solvents studied of a solvating type in nature, such as dimethylformamide (DMF) and dimethyl sulfoxide (DMSO), reduce the activity in a drastic manner for both categories of lipases, and the denaturation of the lipases is not discounted [Fig. 5(5)]. In the presence of some basic or nitrogen-containing solvents, pyridine has shown a remarkably high value in comparison with other basic solvents such as imidazole. The trend in the results is a manifestation of their basic strength [Fig. 5(6)].

CONCLUSIONS

The characteristics of the prepared polymeric supports are of interest for biocatalysis in esterification reactions. These supports do not show superabsorbency in water, but they absorb sufficient water to maintain the hydrophilicity of the immobilized lipase and to act as self-removers of water from the reaction mixture to shift the equilibrium to favor ester formation. Although the immobilized lipase shows good activity on many networks, poly(AAm-*co*-DMA)-*cl*-*N*,*N*-MBAAm prepared with the lowest DMA concentration has shown the highest activity. This network has a well-defined surface morphology with distinctly aligned hydrophilic and hydrophobic regimes, which are suitable for the adsorption of lipase for better substrate interaction. The immobilization improves the lipase activity compared with that of the free lipase. The lipase activity is affected by the pH and temperature and shows maximum activity at the alkaline pH, as expected for an alkaline lipase. The results obtained for the effect of the nature of the solvent on the activity of the immobilized lipase are interesting. The lipase activity increases initially with an increase in the chain length of the solvent but decreases thereafter in a drastic manner in the presence of different

organic solvents. Alcohols have been observed to enhance the lipase activity more than alkanes. Solvating solvents such as DMSO and DMF reduce the lipase activity, and so do many amines. 2-Propanol, *n*-nonane, and pyridine have been observed to enhance the lipase activity in a significant manner. The high lipase activity in alcohols is an encouraging aspect of this study for the potential use of these supports in esterification reactions in the absence of auxiliary solvents.

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