Characterization and Application of Immobilized Lipase Enzyme on Different Radiation Grafted Polymeric Films: Assessment of the Immobilization Process Using Spectroscopic Analysis

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ABSTRACT: Lipase has been immobilized onto different films, polypropylene and poly(tetrafluoroethylene-perfluoro-propyl vinyl ether) using glutalaradehyde as a crosslinker. Differential scanning calorimetery, Fourier transform infrared spectroscopic, x-ray diffraction, and scanning electron microscopy measurements were carried out to confirm the structure of the polymer films as well as the immobilization process of the enzyme onto the polymeric carrier. The activity and stability of the resulting biopolymers produced by lipase have been compared to those for the native lipase. The experimental results showed that the optimum temperature and pH were 40°C and 8.0, respectively. The activity of the immobilized lipases varied with lipase concentration and with the yield of grafting. Subjecting the immobilized enzymes to a dose of γ-radiation of (0.5–10 Mrad) showed complete loss in the activity of the free enzyme at a dose of 5 Mrad. A leakage of the enzyme from the irradiated membranes was not observed in the repeated batch enzyme reactions. The operational stability of the free and immobilized lipase in *n*-hexane showed that the immobilized enzyme was much more stable than the free one. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 90: 155–167, 2003

Key words: immobilization; spectroscopic measurements; lipase; esterfication

INTRODUCTION

Enzyme immobilization methods are continuously being developed for uses in variety of areas such as the food, chemical, pharmaceutical, and agricultural industries. Most immobilization strategies are designed for enzyme function in mainly aqueous environments. However, the need for use of enzymes in organic media has dramatically increased in recent years, and its is now necessary to develop new methodologies to enable the synthesis of enzyme preparations that are stable in nonconventional environments, including nonaqueous solvents. One such approach is to form a protein-containing polymer.[1–3]

Lipases have been used for ester synthesis as an alternative to classical chemical methods.[4–8] A range of fatty acid esters is now being produced commercially using immobilized lipase in nonaqueous solvents and the interest in industrial processes employing lipase biocatalysts to synthesize more such esters is still growing due their important and multiple applications.[10,11] The chemical industrial process for synthesis of esters used in organic catalyst at high temperature, giving rise to the formation of undesirable by-products, colors, and odors. This requires cumbersome and costly purification procedures. The enzymatic process has some advantages. The use of lipases as catalysts allows for the synthesis of high purity esters, with much easier and cheaper downstream protocols.[12,13] The use of immobilized enzymes, such as immobilized lipases, as biocatalysts may offer many significant advantages. These include the use of any hydrophobic substrate, higher selectivity, milder processing conditions, and the ease of product isolation and enzyme reuse.[14,15]

The selection of the appropriate support for enzyme immobilization is of the utmost importance, since the efficiency of the enzyme depends largely on the nature of the support and its bonding with the enzyme. In addition to its high affinity or capacity for enzymes, a suitable chemical structure of support maximizes the degree of interaction between the enzyme and the substrate. The support should also be thermally stable, chemically durable, resistant to contamination, and available at a reasonable cost.[16]

In the present study, we report on the preparation and characterization of lipase membranes from

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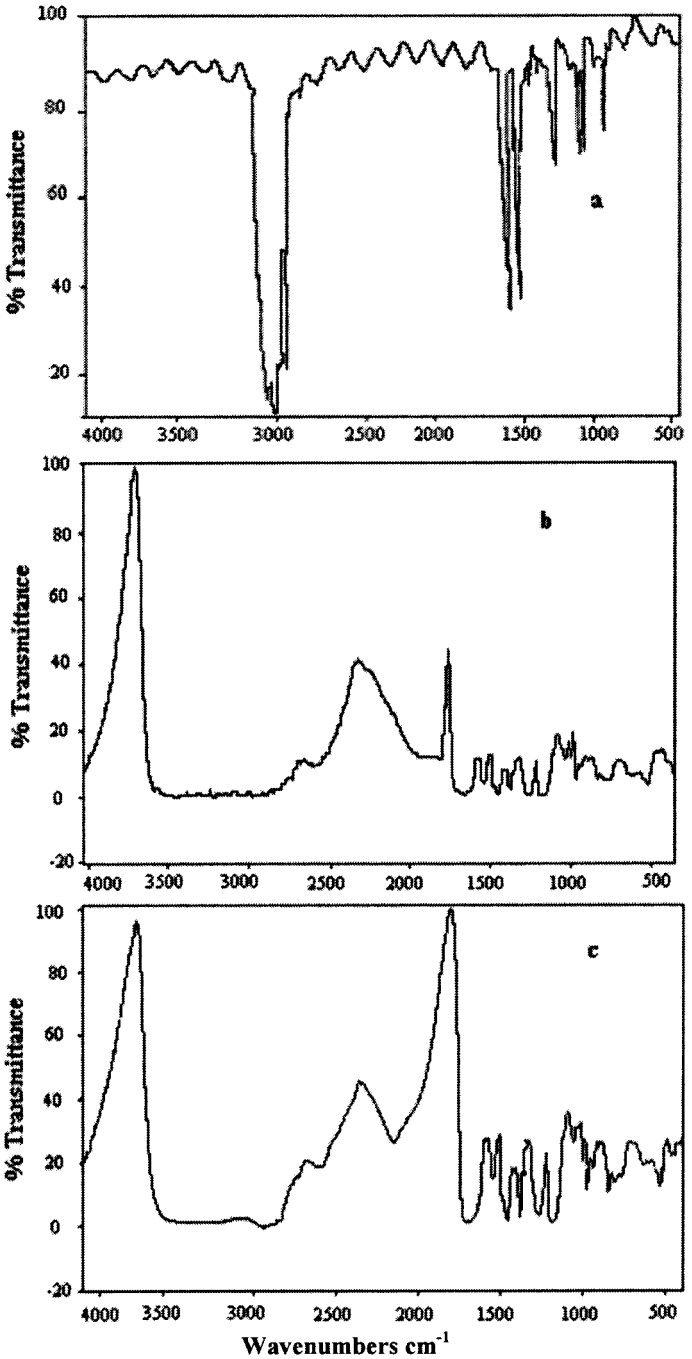

Figure 1 IR spectra of (a) PP film (blank), (b) PP-g-P(MAAc) having *G* % = 178, and (c) PP-g-P(MAAc)\(enzyme).

polypropylene (PP) and poly(tetrafluoroethylene-perfluoro-propyl vinyl ether) (PFA) films with different degrees of grafting using enzyme crosslinking with glutaraldehyde followed by γ-irradiation to increase the degree of crosslinking. The direct irradiation induced grafting of methacrylic acid (MAAc) onto polyolefine, e.g., PP films, and fluorinated polymer, e.g., PFAc films, was also investigated in order to prepare films with different degrees of grafting.

EXPERIMENTAL

Materials

Lipase from hog pancreas was supplied by Fluka (Buchs, Switzerland). PFA, 100 μm thick, supplied by Hoechst, Germany, and PP films, 20 μm, supplied by National Co., Cairo, Egypt, were washed with acetone and dried in vacuum oven at 40°C. Methacrylic acid of purity 99% (Merck, Darmstadt, Germany), stabilized

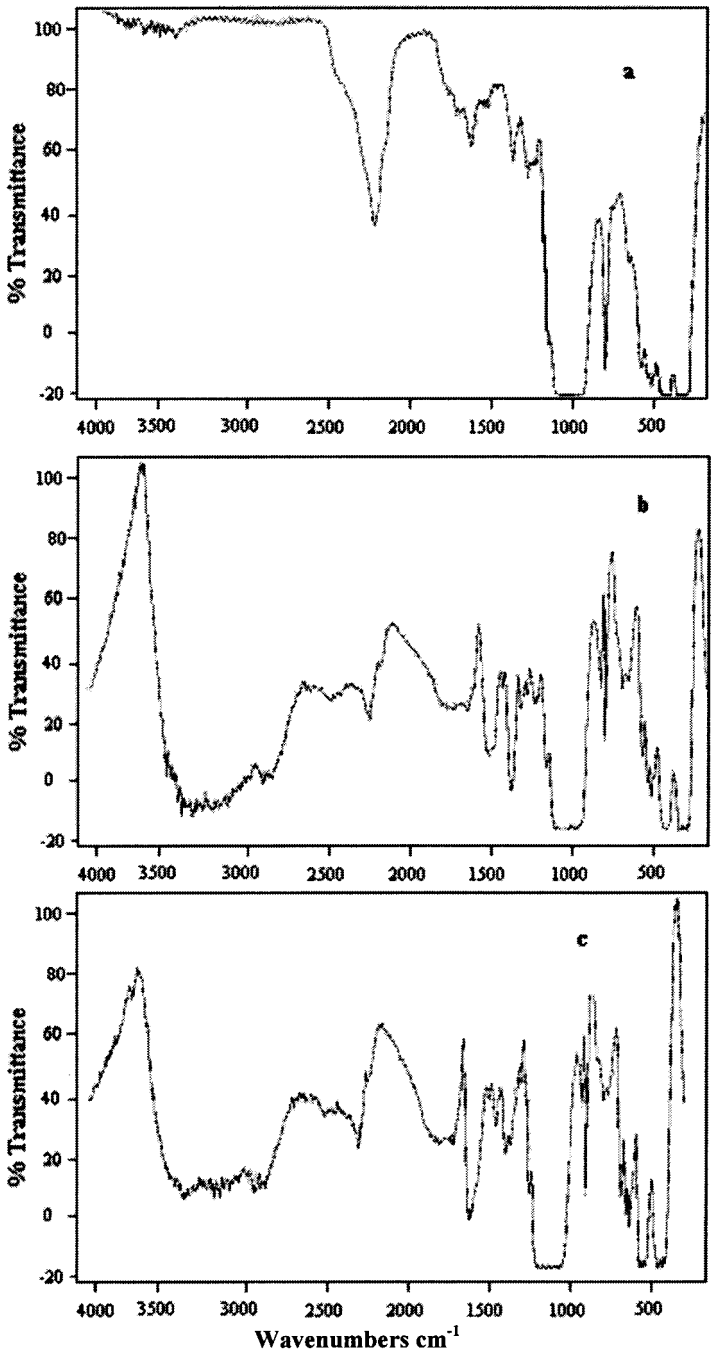

Figure 2 IR spectra of (a) PFA film (blank), (b) PFA-g-P(MAAc) having *G* % = 178, and (c) PFA-g-P(MAAc) \(enzyme).

with 200 ppm hydroquinone, was used without further purification. All other chemicals were of reagent grade and were used as received.

Methods

Graft polymerization

Strips of (PFA, PP) films were washed with acetone, dried in a vacuum oven at 40°C, weighted, and then immersed in an aqueous solution of monomer: methanol/water (70:30) in glass ampoules.[18,19] In the case of the grafting in nitrogen atmosphere, the glass ampoules that contain the films and monomer solution were deareated by bubbling the rough nitrogen gas for 5 min and sealed. The direct radiation grafting method was used, the glass ampoules were then subjected to Co^{60} γ-rays at a dose rate of 1.85 Gy/s. Different doses (0.5, 1, 2, 3) Mrad and different monomer concentra-

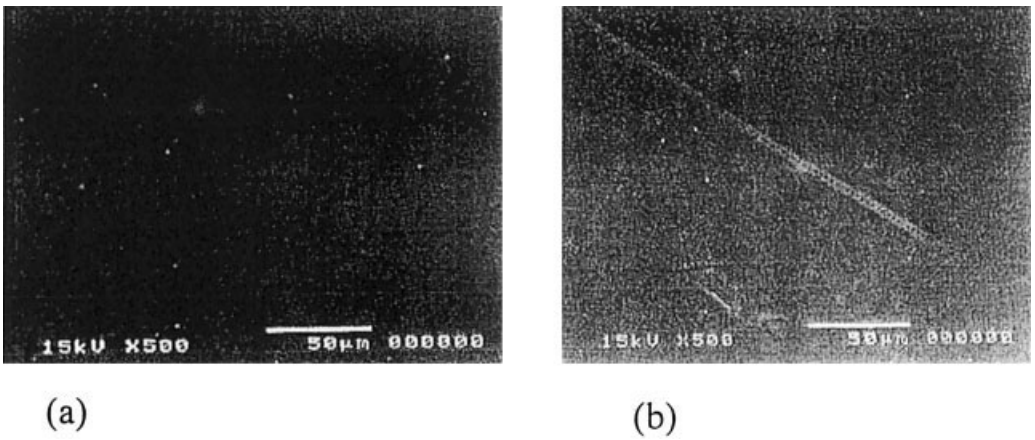

Figure 3 SEM for (a) PP film (blank) and (b) PFA film (blank).

tions (20, 40, and 60%) were used. The grafted films were washed thoroughly with hot distilled water and soaked overnight in water to extract the residual monomer and the homopolymer occluded in the films. The films were then dried in a vacuum oven at 50°C for 24 h and weighted. The degree of grafting was determined by the percentage increase in weight as follows:

$$\text{Degree of grafting } (\%) = (W_g - W_0/W_0) \times 100$$

where W_g and W_0 represent the weights of grafted and initial films, respectively.

Crosslinking with glutaraledehyde

Membranes with diameters of 50 mm were immersed in a fresh solution of glutaraldehyde [1:1 (v/v)] mixture of 25% glutaraldehyde solution and phosphate buffer pH 7, and incubated on a shaker (100 rpm) at 22–25°C for 6 h, thereafter, the samples were removed and washed with distilled water.

Lipase immobilization

The glutaraldehyde-activated membrane were further reacted in a sodium phosphate buffer pH 7, containing lipase for 24 h at 22–25°C. The membranes were washed with sodium phosphate buffer pH 7 thoroughly to remove the loosely bound lipase until no lipase could be detected in the wash solution. The membranes were dried in vacuum at 20°C to produce immobilized lipase membranes, to give preparation I and II, respectively.

γ-Irradiation of lipase-immobilized membranes

Lipase-immobilized membranes were placed in test tube with stoppers and exposed to 2.5 Mrad from Co^{60} at a dose rate of 1.85 Gy/s, to give preparation III and IV, respectively.

Enzyme assay

The assay procedure, described by Vorderwülbecke et al.,[17] was modified as follows: Olive oil–gum arabic emulsion was used as a substrate. Substrate emulsion was prepared by dissolving 16.5 g of gum arabic in 130 mL of reagent grade water. Once the materials in solution was diluted to a final volume of 165 mL with reagent grade water, 20 mL of reagent grade olive oil and 15 g crushed ice was added. The mixture was blended in a Waring blender at low speed and the emulsion was filtered through glass wool. The following was pipetted into a suitable titration vessel: gum arabic–olive oil emulsion (5 mL); 3.0*M* sodium chloride (2 mL); 0.*075M* $CaCl_2$ (1 mL); and 0.027*M* sodium taurocholate).

The pH of the reaction mixture was adjusted to 8 and the volume of titrant required to maintain the pH at 8 for 5–6 min was recorded. The "blank rate" of the reaction was determined as the volume of titrant added per minute from the linear portion of the curve. For the sample's activity determination, at zero time, diluted enzyme (or the equivalent amount of immobilized enzyme) was added appropriately and the pH was adjusted to 8 if necessary. The volume of titrant required to maintain the pH at 8 for 5–6 min was recorded. Then we determined the "sample rate" as the volume of titrant added per minute from the linear portion of the curve.

Calculation:

$$\text{Units/mg} = \frac{(\text{sample} - \text{blank}) \times \text{normality of base} \times 1000}{\text{mg enzyme in reaction mixture}}$$

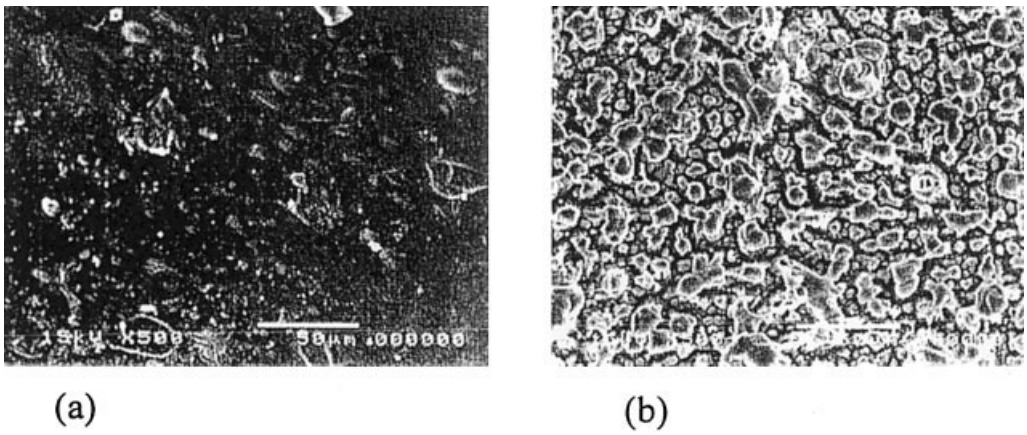

Figure 4 SEM for (a) PP-g-P(MAAc) having $G\ \% = 178$ and (b) PFA-g-P(MAAc)having $G\ \% = 6$.

Differential scanning calorimeter (DSC) measurements

The thermal measurements were determined for a graft sample using a DSC Shimadzu, DSC-50 apparatus (Japan).

Infrared (IR) spectroscopic measurements

IR spectra were determined for the grafted films using a Fourier transform infrared (FTIR), a Pye Unicam SP 2000 type (England).

X-ray diffraction measurements

The studies of x-ray diffraction were performed with a Philips diffractometer (type PW 1050/25), England.

Scanning electron microscopy (SEM) measurements

The surface topography of the grafted and immobilized enzyme films was studied using a JEOL ISM-5400 Scanning Microscope (JEOL, Japan).

Esterification method

Esterification was carried out by using 1 cm^3 membrane-containing lipase, 0.15*M* methanol and 0.15*M* olive oil in 5 mL *n*-hexane. The reaction mixture was incubated at 40°C with magnetic stirring. Samples were drawn at various times to determine the concentration of substrate. Control experiments were conducted without the addition of the biocatalyst. The methyl ester was also analyzed by thin layer chromatography (TLC). The developing solvent was composed of hexane : ethyl acetate : acetic acid = 90 : 10 : 1 (v/v). A solvent consisting of sulfuric acid:methanol = 1:1 (by weight) was sprayed onto the TLC plate and spots were detected by baking it.

Protein measurements

Protein was determined using a modification of the Lowry method.[20] Bovine serum albumin was used as a standard for calibration.

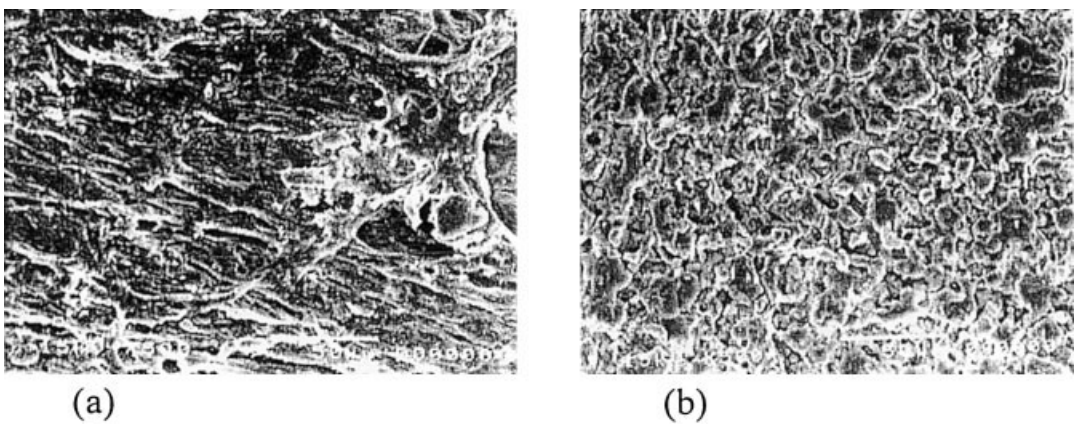

Figure 5 SEM for (a) PP-g-P(MAAc) \(enzyme) and (b) PFA-g-P(MAAc)\(enzyme).

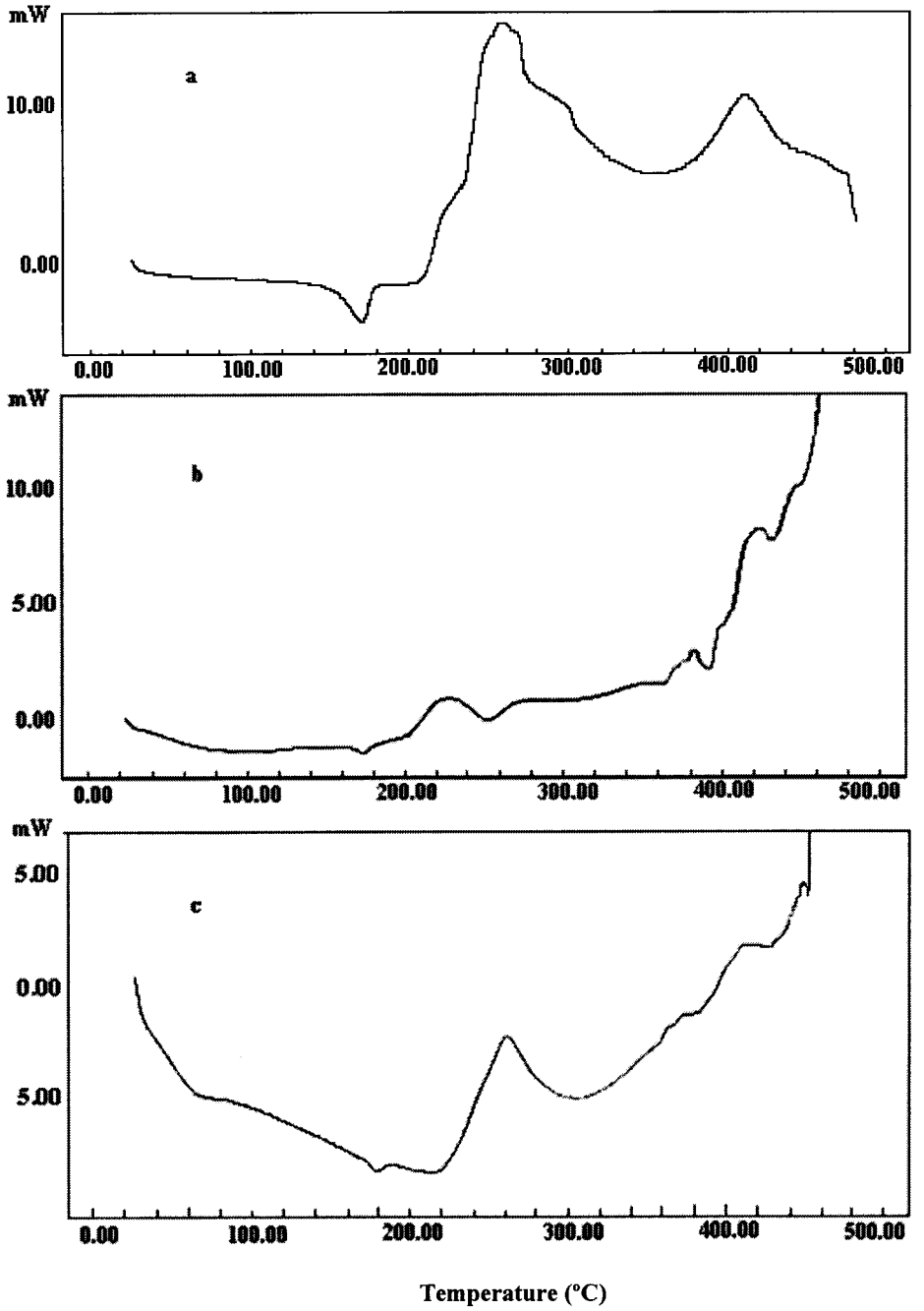

Figure 6 DSC thermal diagram of (a) PP film (blank), (b) PP-g-p(MAAc) having $G\% = 178$, and (c) PP-g-p(MAAc)\(enzyme).

RESULTS AND DISCUSSION

IR measurements

IR spectra of the original and grafted polypropylene (PP) and poly(tetrafluoroethylene-perfluoro-propyl vinyl ether (PFA) films with and without immobilized enzyme were made to investigate the physical and chemical changes that could occur due to the grafting and immobilization of enzyme and to characterize the structure of the films. Figure 1(a–c) shows the spectra of the original PP film and the PP-g-P(MAAc) with immobilized enzyme and PP-g-P(MAAc) having degree of grafting 178%. Figure 1(a) shows the spectra of the original (PP) film of thickness 20 μm. The characteristic bands of PP appeared clearly at 2850 and 2900 cm^{-1} for the CH_2 and CH_3 groups. Peaks at 1370 and 1460 cm^{-1} were characteristic for the symmetrical bending of the CH_2 group, the band at 1490 cm^{-1} also corresponding to crystalline structure. Figure 1(b) shows the spectra of the PP-g-P(MAAc) films, a strong broad band at 3000–3500 cm^{-1}

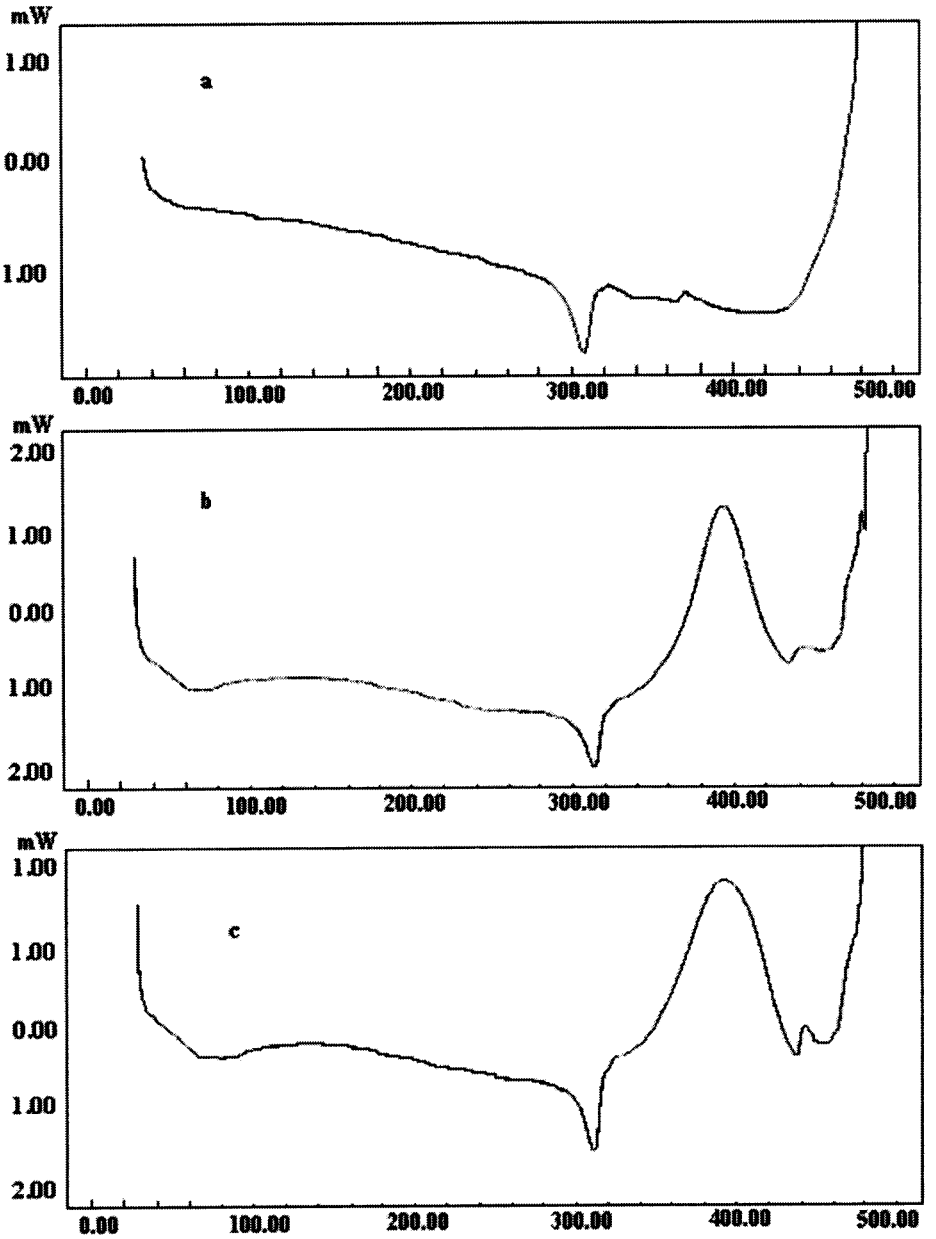

Figure 7 DSC thermal diagram of (a) PFA film (blank), (b) PFA-g-p(MAAc) having *G*% = 6, and (c) PFA-g-P(MAAc)\(enzyme).

characteristic of PP-g-P(MAAc) structure due to the hydrogen bonding and hydrogel groups.[17]

The band at (1725) cm^{-1} confirmed the existence of carbonyl groups and increased as the degree of grafting increased, the absorption around 2100 cm^{-1} is corresponding to free (OH) of acid, and 2900 cm^{-1} was assigned to the —CH_2-assymmetric stretching groups. Figure (1. c) shows the spectra of PP-g-P(MAAc) with the immobilized enzyme. The immobilized enzyme increased the broad band of hydroxyl group and shift the band of the carbonyl group. 1600 cm^{-1} broad band for NH group.[17,18]

Figure 2(a–c) shows the spectra of the original (tetrafluoroethylene perfluoro vinyl ether) PFA film, PFA-g-P(MAAc) having degree of grafting 6% and the grafted membrane immobilized enzyme. Figure 2 (a) shows the characteristic bands of PFA film appeared at 2400 cm^{-1} for the CO group ether, a strong broad band at 1200–1300 cm^{-1} for the CF_2 group.

Figure 2(b) shows the spectra of the grafted membrane as described previously. Figure 2(c) shows the spectra of the grafted immobilized membrane appeared at 1550 cm^{-1} for the enzyme indicating the presence of CH_2 group.

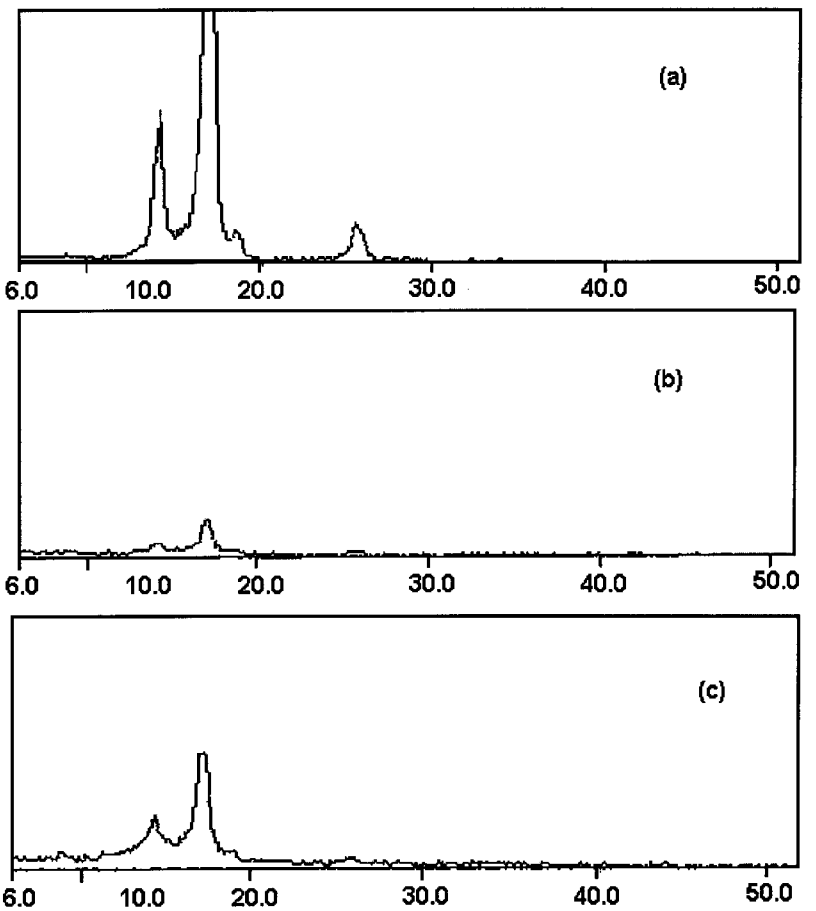

Figure 8 XRD pattern of (a) PP film (blank), (b) PP-g-P(MAAc) having *G* % = 178, and (c) PP-g-P(MAAc)/(enzyme).

Scanning electron microscopy

The immobilization of the enzyme molecules as a result of crosslinking formed between the PP-g-P(MAAc) chains and glutaraldehyde. Microscopic observation was adopted for the blank polymer, grafted film, and immobilized enzyme in the grafted film to estimate the porous structure. SEM shows the surface morphology of the original polymer PP and PFA shown in Figure 3(a,b), the grafted films with MAAc monomer of PP at graft 178% and for PEA at graft 6% Figure (4. a, b). It can be seen that the visible particles are in the pore structure of the grafted membrane and the largest particles were seen to be those mean distance to the outer surface, as shown in Figure 4(a,c), respectively.

Figure 5(a,b) shows that the polymer formed a microsphere from which was expected to be advantageous for the surface enzyme reaction with the substrate. Due to the immobilized enzyme particle is a complex entity, the enzyme exists in a microenvironment on the surface of the membrane.

Differential scanning calorimetry

The thermal stability of the membranes is an important factor for their durability during practical use.

The DSC spectra of the blank and grafted membrane (copolymer with MAAc) and immobilized enzyme membrane as in Figures 6 and 7 show the characteristic transition temperatures. The decomposition temperatures are observed from the endothermic peaks. The melting point determined (T_m) for the ungrafted PP film was 170°C. However, due to the introduction of PP-g-P(MAAc), an increase in the crystalline melting point on the grafted membrane at about (5°C) and the immobilized enzyme on the membrane increased the melting point by about 10°C. The observed increase in melting point indicated that grafting and immobilization affects the crystalline structure of the films. Also, the melting point of the blank PFA, the grafted, and the immobilized films, were determined from the peak in the endotherm at 300, 305, and 310°C, respectively. The increase in the melting point occurred, confirming the

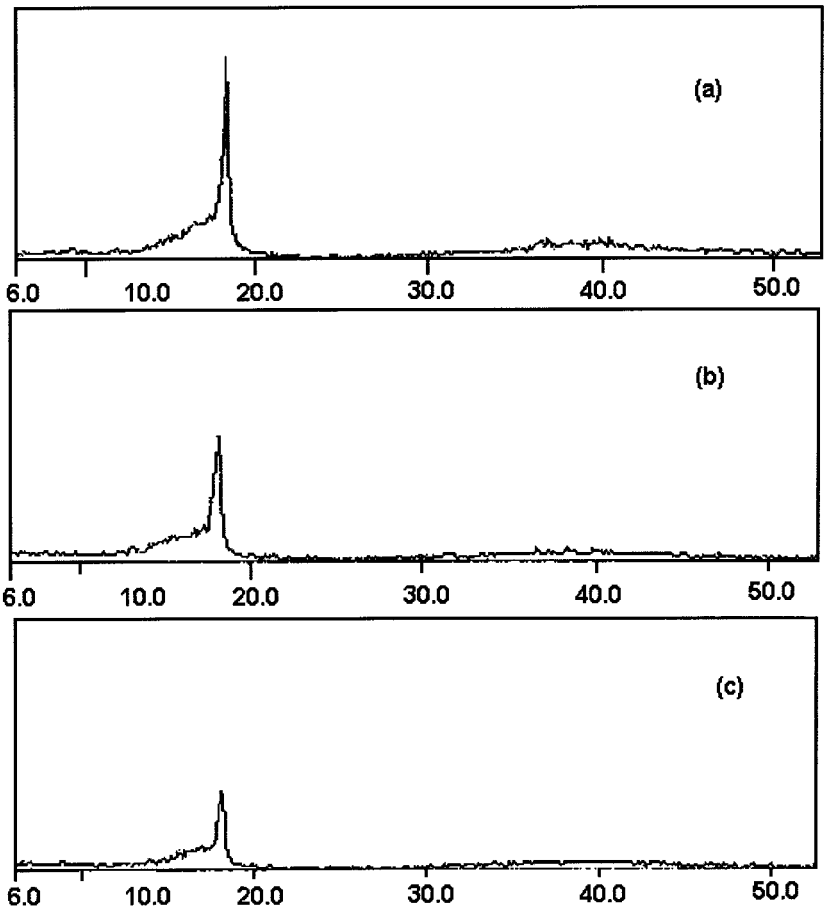

Figure 9 XRD pattern of (a) PFA film (blank), (b) PFA-g-P(MAAc) having *G* % = 6, and (c) PFA-g-P(MAAc)/(enzyme).

stability of the enzyme-containing polymers during the melting process.

X-ray diffraction (XRD)

Figure 8(a–c) of the (XRD) investigates the crystallinity content of the PP film, PP-g-P(MAAc), having *G*% = 178, and the enzyme immobilized on the grafted chains, respectively. It was noted that the grafting process brought about a sudden drop in the degree of ordering of the polymeric material. Also, it led to an effective decrease in the particles size. These findings were evidenced from the observed big drop in the relative intensity of the main diffraction line and to its broadening. The immobilization of the lipase on the PP-g-P(MAAc) was found to increase ordering of the polymeric materials, as indicated from the important increase in the relative intensity of the main diffraction line of such polymeric materials. In fact, the integrated count corresponding to the main line indicated a 610–1577 count due to this treatment. So, the immobilization process increases the rearrangement of the polymeric unit cell components a good deal.

Figure 9(a–c) shows the XRD for the PFA film, PFA-g-P(MAAc), *G*% = 6, and after the immobilization of the enzyme lipase on the grafted chains. It was noted that the grafting and immobilization processes decrease the degree of ordering and the particle size of the grafted membrane by a small amount as the degree of graft value. The degree of grafting is small because the polymer contains fluorine atoms, which form steric hindrance with the monomer (MAAc) and decrease the graft value.

TABLE I
Effect of Graft Yield on the Maximum Lipase Activity

PP		PEA	
Total graft yield (%)	Maximum lipase activity (%)	Total graft yield (%)	Maximum lipase activity (%)
0	94	0	21.2
92.4	100	6.3	92.4
178.0	94.2	7.05	95.6
266.6	78.2	14.1	100
360.0	29.2	—	—

TABLE II
Effect of Lipase Concentration on Enzyme Immobilized onto PP and PFA Grafted MAAc Membranes

Protein added (mg)	Lipase added (Units)	Protein immobilized (mg/cm^3)		Activity immobilized (U/cm^3)		Retention activity (%)	
0.5	10	0.48	0.49	9.6	9.8	96	98
1	20	0.97	0.91	19.4	18.2	97	91
2	40	1.95	1.02	39	20.4	97.5	51
3	60	2.62	1.13	52.4	22.6	87.3	37.7
4	80	3.08	1.20	61.6	24	77	30
5	100	3.42	1.21	68.4	24.2	68.4	24.2

Effect of graft yield on maximum lipase activity

The effect of methacrylic acid graft yield on maximum lipase activity of the PP and PFA membranes are given in Table I. The immobilized lipase activity increased up to 178.5% grafting for PP but decreased at higher graft values. It may be due to the fact that the formation of multibonds between lipase molecule and the PP-g-P(MAAc) membrane when the MAAc concentration used is increased, whereas the immobilized lipase activity on PFA membrane increased with the percentage of grafting. The graft yields above this value might give high densities of MAAc groups, which could have slowed the rate of enzyme penetration into deeper regions of the graft layer, since the mobility of the chains became very limited at high grafting levels.[21]

Effect of enzyme concentration on lipase activity

The maximum amount of lipase was achieved at concentrations 2 and 0.5 mg/cm^3 for preparation I and II, respectively. For preparation I, its was found that a slight increase in immobilized percent was detected with increasing the enzyme concentration up to 2 mg of protein (40 U). However, increasing the amount of added protein resulted in a decrease in retention activity (Table II).

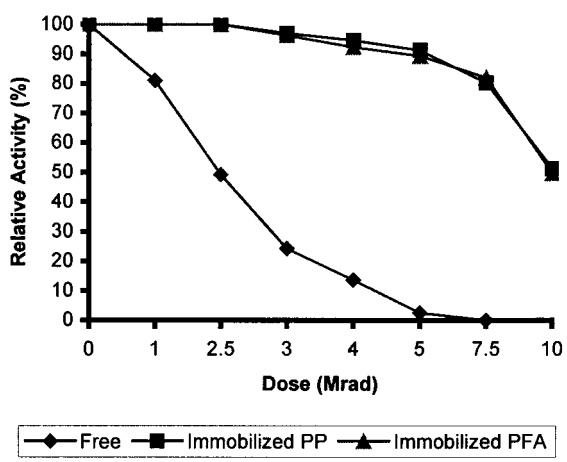

Figure 10 The effect of γ-irradiation on free and immobilized enzymes.

Effect of γ-irradiation

The effect of γ-irradiation on the free and the immobilized lipase was investigated, and the results are shown in Figure 10. The free lipase lost about 50% from its relative activity when exposed to a dose of 2 Mrad and lost the rest of its activity at a dose of about 5 Mrad. The immobilized lipase showed more radiation resistance. The activity of immobilized lipase, irradiated to a dose of 5 Mrad, showed no significant change in its relative activity, while a reduction of 50% was measured at a dose of 10 Mrad. It is obvious that the immobilization of the enzyme onto polymeric support resulted in good radiation resistance of the enzyme. It seems that the polymer stabilized the produced free radicals and protected the enzyme.

Leakage studies

The protein released from the immobilized membrane before and after irradiation was also investigated. The results reveals that the protein released from the preparations III and IV was less than that of nonirradiated membrane (preparations I and II). This may be due to the increase of crosslinking caused by the γ-irradiation (Table III).

TABLE III
Leakage Study Result (% Total Proteins Immobilized Leaked in the Surrounding Medium at 4°C)

	% Total protein leaked			
Time	Prep I	Prep II	Prep III	Prep IV
1	3	2.4	1.2	1
12	3.6	3.2	1.8	1.3
24	4.2	3.8	—	—
48	—	—	—	—

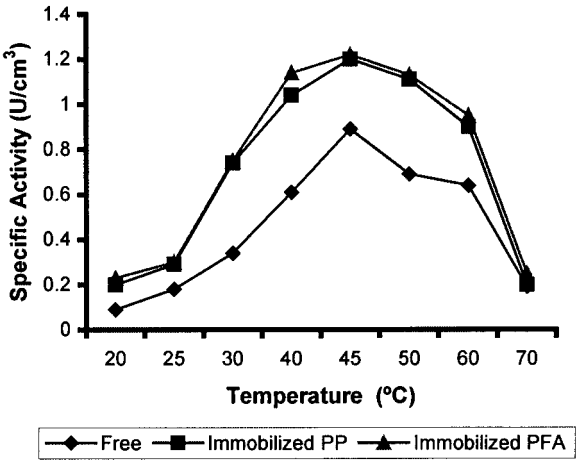

Figure 11 The effect of temperature on both free and immobilized enzymes.

Effect of temperature on lipase activity

The results (Fig. 11) show that, while the specific activity of the immobilized lipase (preparation I and preparation II) was higher than that of the free enzyme, the temperature for optimal activity of the immobilized lipase was found to be 40°C, the same as the free enzyme. The relative activity of the free and immobilized lipase decreased gradually from 45 to 65°C, and a point was reached at which both the free and the immobilized lipase showed the same relative activity. These findings suggest that the increase in the temperature beyond a certain point could cause desorption of the immobilized lipase form its support, resulting in a similar activity as that for the free one.

Several authors have also reported that the immobilization of lipases on a wide range of hydrophobic supports did not alter the optimum temperature (40°C) of the enzymatic reaction; however, beyond this temperature, the activity of the immobilized enzyme decreased to the level of the free one. A similar trend was observed with the immobilization of trypsin by diazo binding with an amino acid copolymer.[22,23]

Effect of pH on lipase activity

Figure 12 shows that the optimum pH for the enzymatic activity of the immobilized lipase was 8. However, the specific activity of the immobilized lipase was higher than that of the free one. Several authors also reported this finding. These results suggest that the immobilization of the enzyme had no effect on the optimum pH for lipase activity. The literature indicated that some immobilized enzymes showed no shift in their pH optimum for activity but exhibited narrower or broader changes in their pH activity profile. A broader profile can be explained by the enzyme's increased resistant to change in pH upon immobilization, while a narrower profile may explained by opposite trend.

However, immobilized lipase (preparation I) was much more stable at the acidic than the alkaline regions. This finding may be due to the fact that immobilization of an enzyme onto any charged support generally causes shifts in pH activity curve; consequently, the change on pH behavior after immobilization onto support can be related to the functional groups of the polymer side chain. On the acidic side, the repulsive forces between the hydroxyl groups of the PP side chain and hydronium ions may be responsible for increase in enzyme stability towards pH.[22–26]

Enzyme kinetics

The kinetics values of K_m and $V_{\max}$ for the activity of the free and immobilized lipase using olive oil as substrate were obtained from Lineweaver–Burke plots of $1/v$ vs $1/[S]$. The results showed a decrease in the K_m value from 10 mmole/dm^{-3} for the free enzyme to 8.6 and 9 mmol/dm^{-3} for immobilized lipase (preparation I and II, respectively). A decrease in K_m value for the immobilized enzyme suggests that a faster rate of reaction can be achieved compared to that with the free one. Kennedy reported a decrease in the K_m value when ficin was immobilized by peptide binding on carboxy methyl celllulose azide in comparison to that observed with the free one. In addition, the hydrolysis of maltose by glucoamylase showed a decrease in the K_m value of the enzyme upon immobilization. The results showed an increase in the $V_{\max}$ value from 22 for the free one to 26 and 29 mmol of olive oil/mg protein^{-1} per for the immobilized lipase (preparation I and II, respectively); this increase may be due to a greater stability of the enzyme, thereby enhancing their interaction at the interface. Kennedy reported

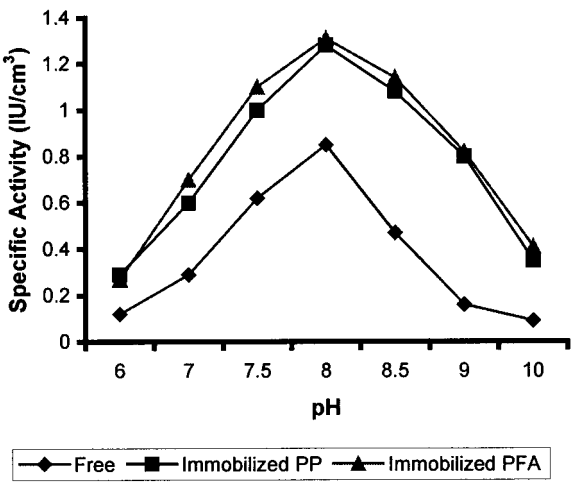

Figure 12 The effect of pH on both free and immobilized enzymes.

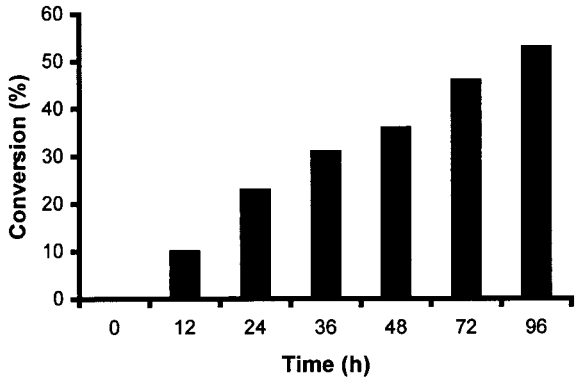

Figure 13 Time course of the esterification reaction of methanol with oleic acid lipase.

that the V_{max} of glucoamylase was about 10 times higher than that of the free one. However, the immobilization of the enzyme β-D-gructofuranosidase by entrapment resulted in a 10-fold decrease in its V_{max} value.[27]

Esterification of fatty acids catalyzed by immobilized lipase

The esterification activity of the different immobilized preparations were investigated in *n*-hexane for the synthesis of methyl fatty acid esters. Figure 13 shows the time course of the esterification of oleic acid with methanol.

Effect of methanol concentration on the methanolysis of olive oil

Figure 14 shows that the relative activity of the free lipase was apparently inactivated when high molar equivalents of methanol were added, while the immobilized one showed an obvious stability towards high molar equivalents of methanol.

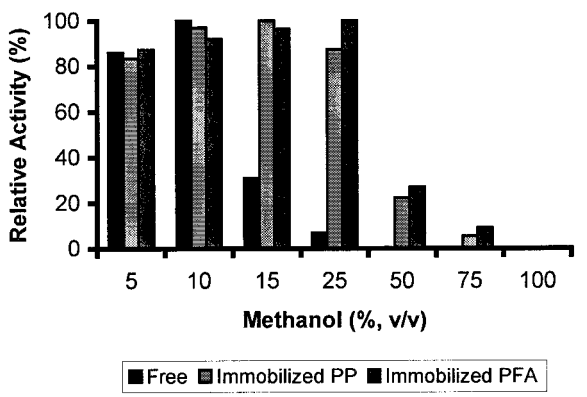

Figure 14 Activities of native and immobilized lipase in various buffer water/methanol mixtures at 25°C with olive oil as substrate.

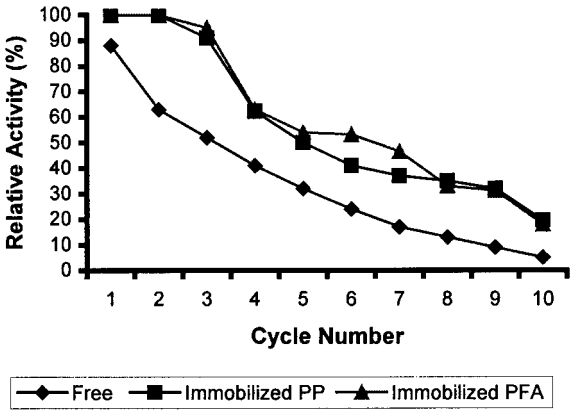

Figure 15 Recyclability of native and immobilized lipase in *n*-hexane at 25°C.

Stability in organic solvents

The recyclability of the biopolymer in organic media has been compared to that for the native and immobilized lipase. The enzyme was allowed to catalyze an esterification of olive oil with methanol (as described in the method section) for 4 h, the reaction mixture was then centrifuged and the supernatant decanted. The enzyme powder was then washed three times with olive oil to remove the remaining reactants and products, dried thoroughly by air blowing, and then recycled into a fresh reaction mixture. As shown in Figure 15, the biopolymer was again the most stable, retaining 32 and 37% of its activity for preparation I and II, respectively, even after ten cycles. Native lipase possessed only 5% activity after ten cycles.[28,29]

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