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Immobilization of porcine pancreatic lipase on glycidyl methacrylate grafted poly vinyl alcohol

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ABSTRACT

Graft copolymerization of glycidyl methacrylate (GMA) on to polyvinyl alcohol (PVA) using benzophenone (BP) as initiator was carried out. Grafted PVA was used as carrier for pancreatic lipase immobilization. The effects of GMA and BP concentrations as well as grafting reaction times on grafting yields and activities of the immobilized lipase were determined. The influence of enzyme concentrations was also studied. The optimal conditions for the grafting reaction were: 1 h at 15 mM BP and 2.3 M GMA, the optimum enzyme concentration for immobilization was 1 mg/ml. After optimization of the immobilization process a physical and chemical characterization of the immobilized enzyme was performed. Furthermore, the thermal, pH, storage and operational stability of the immobilized enzyme in comparison to the free form was tested.

1. Introduction

Lipases are enzymes that catalyze a variety of reactions, such as esterification, interesterification and hydrolysis [1,2]. Because of their selectivities, lipases are important biocatalysts in several applications, such as the synthesis of chiral drug intermediates [3,4] and nutraceutical lipids [4–6]. The broad synthetic potential of lipases is largely due to the fact that they (in contrast to most other enzymes) accept a wide range of substrates and tolerate organic solvents.

Lipase activities are greatly enhanced by water/lipid interfaces, a phenomenon known as interfacial activation [7,8]. Interfacial activation has been related to the presence of an amphiphilic peptide loop (lid) that covers the active site. When dissolved in water the lid moves toward to the solvent, possibly in order to prevent aggregation of the enzyme. Upon adsorption of the lipase onto a water/lipid interface the lid opens and a hydrophobic side is exposed toward to the interface. Lipases from *Candida rugosa* [9,10], *Mucor miehei* [11,12], *Geotrichum candidum* [13,14], *Humicola lanuginosa* [15] and pancreas [16,17] have been crystallized both in the absence and presence of a substrate analogue or inhibitor demonstrating that the lid undergoes a rearrangement rendering the active site accessible for the substrate.

The price of lipases is relatively high so in practical applications reuse of the enzymes is of importance [18]. In order to expand their synthetic utility further efficient methods of lipase immobilization are required because immobilization promotes enzyme reuse and thus reduces overall process costs [4]. Moreover, enzyme stability can often be improved by immobilization. The enzyme–support interactions formed during the immobilization process can, furthermore, modify the conformational structure of the protein resulting in a modulation of the catalytic properties of enzyme itself. The extent of stabilization depends largely on the enzyme structure, the immobilization method applied and the type of support [19]. Lipases show two different conformations: the closed form considered as inactive when the active site is excluded from the solvent by the lid loop, and the open form, where this lid is displaced and the active site is fully exposed to the reaction medium [20–23]. Both forms of lipases exist in an equilibrium which, on the other hand, is controlled by the experimental conditions. Regarding these features, different strategies have been developed to generate immobilized lipase molecules with improved activity [24]. Special attention has been paid to the selective adsorption of lipases to tailor-made supports possessing strongly hydrophobic surfaces. This new immobilization procedure is based on the assumption that the large hydrophobic area surrounding the active site of lipases is also involved in their adsorption to strongly hydrophobic solid surfaces. Immobilization by adsorption is the easiest and the cheapest technique to prepare solid-support biocatalysts. However, since the interacting bonds are weak the enzyme can be easily desorbed from

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the carrier. Adsorption should not be used if the presence of the enzyme in the product cannot be tolerated [25].

Several studies have been reported on the use of polyvinyl alcohol (PVA) as support for biotechnological applications [26]. It is used in various crosslinked forms giving hydrogels or membranes which represent novel matrices for protein purification and immobilization studies [27–29]. It is well suited for these purposes because of its hydrophilicity and is, in addition, commercially available in a wide range of molecular weights at low price and it can be easily modified through its hydroxylic groups [30].

Modification of polymers has received much attention recently. Among the methods of chemical polymer modification grafting represents one of the most promising approaches since graft copolymerization will impart a variety of new functional groups to a polymer [31]. Modification of hydrophobic polymers into a hydrophilic or vice versa can be achieved by wet (acid, alkali), dry (plasma) and radiation treatments (ultraviolet radiation and laser) without affecting the bulk properties of the polymer [32]. In order to modify the properties of a polymer in a desired way the so-called photografting is performed by irradiating it in a solvent containing selected monomers with appropriate radiation sources. The energy sources commonly used are high-energy electrons, X-rays, UV and visible light. Many different routes can be employed to introduce graft chains onto the surface of polymers depending on the system on the goal of later application [33–35]. Ultraviolet energy (UV) has been extensively applied for surface graft polymerization of polymers with the aid of a photoinitiator or photosensitizer, such as benzophenone (BP). Earlier reports concerned with UV irradiation at the vapor phase of monomer and sensitizer under a reduced pressure [36] or in the presence of inert gas [37]. After proper modification, polymeric membrane can become a good carrier for binding enzyme [38]. Glycidyl methacrylate (GMA) is an attractive monomer and BP is an efficient initiator for the photochemical grafting [39].

Glycidyl methacrylate, the ester of methacrylic acid and 2,3-epoxy-propanol, bears a reactive epoxide group which reacts with sulfhydryl- [40,41], amino- [42,43], carboxyl- [44,45] or hydroxylgroups [46] to form stable covalent bonds with biomolecules without any linker. Such epoxy-modified polymer-surfaces are stable during long storage periods and relatively resistant against hydrolysis. Biomolecules like proteins become covalently coupled by opening the epoxide ring.

The aim of this study is to improve the lipase activity by opening the active site closing lid structure during immobilization. For this purpose the hydrophilic character of PVA was changed by photografting it with hydrophobic GMA groups thus mimicking a chemical interface which should be suitable for interfacial activation of lipases. Porcine pancreatic lipase was covalently immobilized on glycidyl methacrylate grafted polyvinyl alcohol by means of carbonyl diimidazole (CDI). The catalytic activity of the grafted polymer was characterized as a function of grafting time, GMA concentration, BP concentration and enzyme concentration. Characterization of free and immobilized enzyme was also carried out.

2. Materials and methods

2.1. Materials

Lipase (EC 3.1.1.3 Type II, crude; from porcine pancreas), polyvinyl alcohol (PVA Type III), carbonyl diimidazole and glycidyl methacrylate were purchased from Sigma Chemical Co., benzophenone was obtained from Merck, dioxane was obtained from Carlo Erba. All other chemicals were analytical reagent grade.

2.2. Activity measurements

The hydrolytic activities of free and immobilized lipases were determined at 30 °C and pH 8.0 by pH-stat titration using an automatic titrator (718 Stat Titrino, Metrohm Ltd., Switzerland). The released free fatty acids from tributyrin were titrated with 0.01 M NaOH [47]. One unit of lipase activity was defined as 1 μmol of fatty acid released per minute.

2.3. Protein determination

Protein concentrations were determined by the method of Lowry et al. [48] using bovine serum albumin as the standard. The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

2.4. Support preparation and activation

2.4.1. Glycidyl methacrylate grafting on PVA

The PVA powder, BP as photoinitiator and GMA were added to water:ethanol (1:3) in a glass reaction vessel. The reaction mixture was stirred at a constant rate and degassed with nitrogen gas for 5 min at 25 °C. A nitrogen atmosphere was maintained throughout the reaction period. Then the reaction mixture was exposed to two UV irradiation bulbs (each bulb is 4 W, complete spectrum emission from 365 nm to visible light) for 1 h at 25 °C. The PVA-g-GMA (GMA-grafted PVA) polymer was washed with ethanol three times to remove homopolymers, unreacted monomers and benzophenone in the reaction vessel and dried until constant weight. The grafting percentage was measured by titration [49].

2.4.2. Activation of grafted PVA with CDI for enzyme immobilization

Grafted PVA was activated with CDI for covalent attachment of the enzyme on PVA via amino groups. 7 ml dioxane and 24 mg CDI were added to 1 g of grafted PVA and the reaction mixture was incubated in an orbital shaker for 30 min at 25 °C. Then the mixture was filtered and washed with 25 ml of dioxane and dried.

2.5. Enzyme immobilization

The PVA-g-GMA that synthesized at optimum conditions for graft polymerization was used for all immobilization procedures. Immobilization process was performed using two different methods. Pancreatic lipase was directly immobilized on grafted PVA and also immobilization process was carried out after activation of grafted PVA with CDI. 10 ml enzyme solution in 0.05 M pH 7.5 phosphate buffer was added to 1 g PVA and the reaction mixture was incubated for 16 h under mild shaking at 4 °C. Thereafter the carrier was separated by filtration and washed with 0.05 M pH 7.5 phosphate buffer to removed the unbound enzyme. When not in use, immobilized enzymes were stored at 4 °C.

2.6. Physico-chemical properties of free and immobilized lipase

2.6.1. Effect of pH on activity

The effect of pH on the activity of free and immobilized lipase was assayed by adjusting pH values ranging from 7.0 to 10.0 by using the standard activity assay procedure as mentioned above.

2.6.2. Effect of temperature on activity

The temperature dependence of the enzyme activity was investigated between 20 and 45 °C and the activities of both enzymes were assayed under the standard assay conditions.

2.6.3. pH stability

The pH stabilities of free and immobilized enzymes were compared in the phosphate buffer (50 mM) between pH 6.5 and 8.5. Both forms of enzymes were incubated in buffer for 20 min at 4 °C and the remaining activities were assayed using the standard assay conditions.

2.6.4. Thermal stability

The rates of thermal inactivation of the free and immobilized enzymes were studied in the temperature range 4–55 °C. Both forms of enzyme were incubated in 0.1 M NaCl for 20 min at different temperatures and after cooling, the remaining activities were assayed under the standard assay conditions.

2.6.5. Storage stability

Free and immobilized enzymes were stored at 4 °C. The storage stability of enzymes was determined by measurement of the activities of samples taken at regular time intervals and compared.

2.6.6. Operational stability

The reaction of tributyrin hydrolysis catalyzed by the immobilized enzyme was carried out continuously for 12 h at 30 °C in a continuous stirred tank reactor measuring of the activity of samples taken at regular time intervals. The half-life ($t_{1/2}$) of the biocatalyst was determined by

$$t_{1/2} = \frac{0.693}{K_D}, \quad K_D = \frac{2.303}{t \log(A_0/A)} \tag{1}$$

3. Results and discussion

To investigate the effect of GMA grafting on the activity of lipase that immobilized on PVA, immobilization was performed with PVA-g-GMA and non-grafted PVA. The results are summarized in Table 1. As shown in the table, GMA grafting causes a relatively high increase of immobilized enzyme activity whereas it has no apparent effect on protein binding. Presumably the activity difference between the immobilized forms is caused by a conformational conversion of the enzyme to a more active form as a consequence of interaction of the lid structure with the hydrophobic GMA side chains during the immobilization as outlined in Section 1. Obviously the conformational conversion relates directly to the hydrophobic contribution of GMA copolymer to the PVA.

In order to test the effect of CDI activation on the immobilized enzyme activity an enzyme solution was added to CDI activated and non-activated PVA-g-GMA. The activities of enzymes that immobilized on CDI activated PVA-g-GMA and non-activated PVA-g-GMA were 80 and 57 U/g, respectively, although the bound protein amounts showed no significant difference (data not shown). Without CDI activation of PVA-g-GMA chemical immobilization through the epoxide groups on the graft polymer occurred predominantly via amino groups of the lipase molecule whereas in the case CDI activation PVA hydroxyls obviously contribute to enzyme binding. This contribution of OH groups to the fixation process possibly yields an enzyme immobilization with a more favourite active site orientation.

3.1. IR-spectroscopy of PVA-g-GMA

PVA-g-GMA was analyzed by IR-spectroscopy (PerkinElmer Spectrum 100 Series). The characteristic peaks assignments for PVA are as follows: 3405 cm^{-1} (—OH), 1097 cm^{-1} (—C—O), 2936 cm^{-1} (—CH). The vibration band at 1728 cm^{-1} represents the ester configuration (Fig. 1) confirming a successful graft polymerization of GMA onto PVA.

3.2. Influence of grafting reaction conditions on immobilized enzyme activity

The catalytic activity of lipase that immobilized on PVA-g-GMA was tested as a function of grafting reaction conditions. Relative activities of immobilized enzyme and grafting percentages of PVA-g-GMA were plotted versus different quantities of each grafting reaction parameter.

3.2.1. Effect of grafting time

The grafting reactions were carried out for different time periods (0.5–3 h). After activation of PVA-g-GMA with CDI, immobilization of the enzyme was performed at standard conditions. Immobilized enzyme activities were measured and relative activities were estimated. As shown in Fig. 2 the optimum grafting time is determined as 1 h.

3.2.2. Effect of initiator (BP) concentration

The grafting reactions were carried out with different BP concentrations (7.7–30 mM). Fig. 3 shows the effect of BP concentration on immobilized enzyme activity and grafting percentage. The optimum BP concentration was determined as 15.2 mM. Higher levels of initiator, on the other hand, decrease the conversion of grafted monomer. The enhancement of grafting by increasing the BP concentration to a level 15.2 mM implies that as a direct effect a higher BP concentration is to increases the reaction rate, beyond the optimal value, however, light absorption is increased, thus lowering the effective UV intensity at the interface of the BP solution and the PVA.

3.2.3. Effect of monomer (GMA) concentration

Fig. 4 shows the effect of GMA concentration on graft copolymerization and the activity of the immobilized enzyme. The grafting percentage increased continuously with the GMA concentration but the activity of immobilized enzyme increased reached a maximum value at 2.3 M GMA, thereafter the activity decreased. As mentioned in Section 1 activation in the presence of hydrophobic interfaces is a characteristic property of lipases, in the absence of these interfaces, lipases have a secondary structure (termed the 'lid') occluding their active sites, thus rendering them inaccessible to substrates. In the presence of hydrophobic interfaces important conformational changes take place yielding the active 'open structure' of lipases [50]. It is presumed that GMA grafted onto PVA induces this interaction with the hydrophobic areas covering the

Table 1
Effect of GMA grafting on activity of immobilized enzyme

	Free enzyme total activity (U)	Free enzyme protein (mg)	Free enzyme spes. activity (U/mg)	Immobilized enzyme activity (U)	Bounded protein (mg)	Immobilized enzyme spes. activity (U/mg)	Activity yield (%)
Control PVA	749	6.55	114	40	1.57	25	22
Grafted PVA	749	6.55	114	80	1.21	66	58

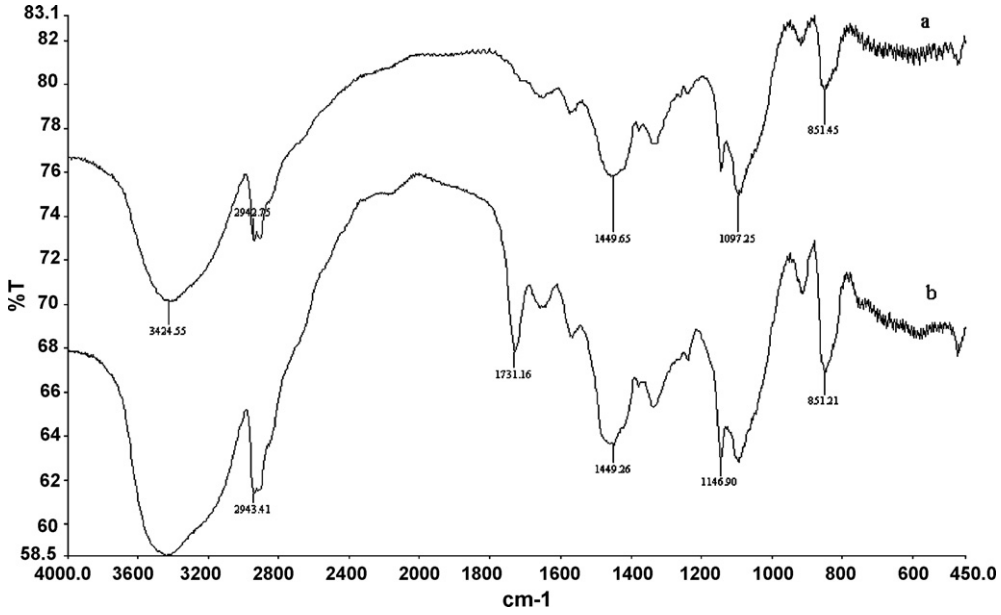

Fig. 1. FTIR spectrum of PVA (a) and PVA-g-GMA (b).

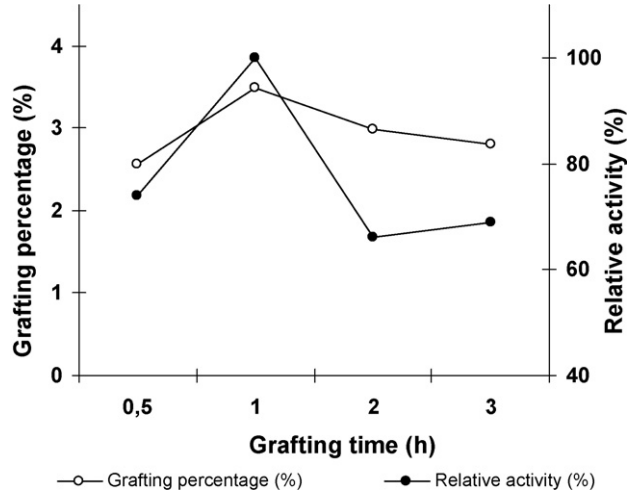

Fig. 2. Effect of grafting time onto graft copolymerization.

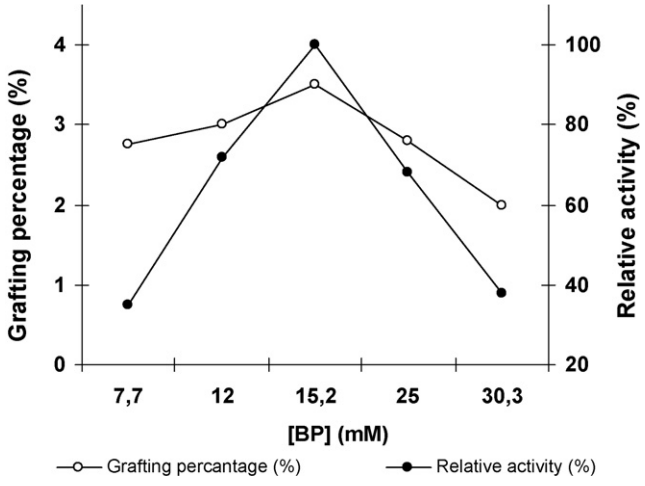

Fig. 3. Effect of initiator concentration onto graft copolymerization.

active center of the lipase and leading thus to an immobilization of the enzyme in an open form. Beyond the 2.3 M GMA concentration, copolymer extent may cause a steric hindrance reducing the activity of the immobilized enzyme again.

3.3. Effect of enzyme concentration

PVA-g-GMA synthesized at optimum conditions was used for the following immobilization studies. Fig. 5 shows the effect of enzyme concentration on the activity of the immobilized enzyme. An increase in enzyme concentration from 0.5 to 1 mg/ml led to an activity rise, further increase of enzyme concentration reduced it.

3.4. Physico-chemical properties of free and immobilized lipase

The optimum pH values of free and immobilized enzymes were determined from the graph of pH plotted against the percentage of relative activity (Fig. 6). The optimum pH value of the immobilized enzyme was shifted to the 9.5 from 8.5. Lipase is linked to

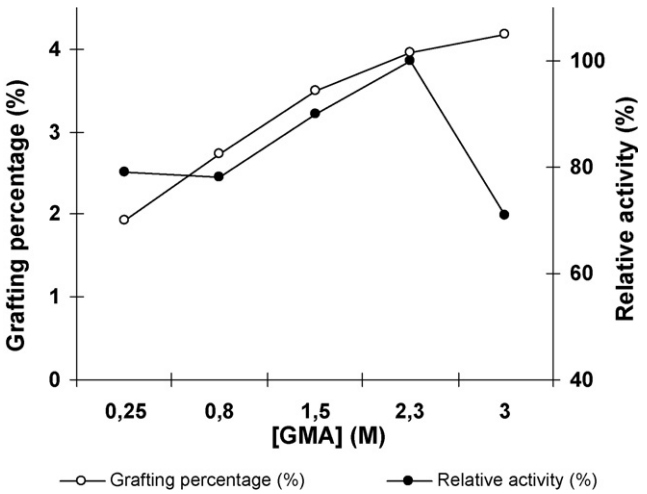

Fig. 4. Effect of monomer concentration onto graft copolymerization.

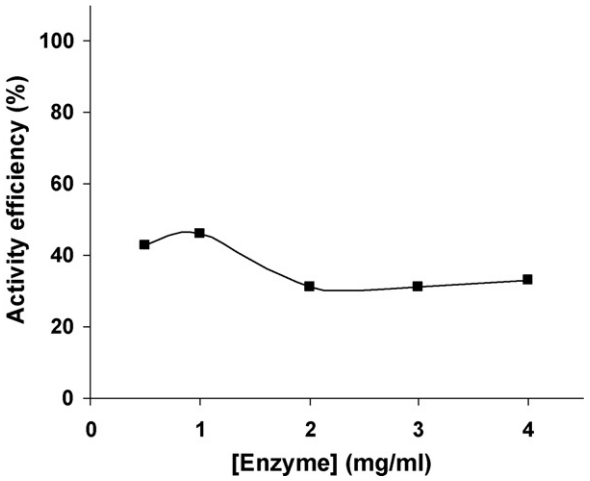

Fig. 5. Effect of enzyme concentration on immobilization.

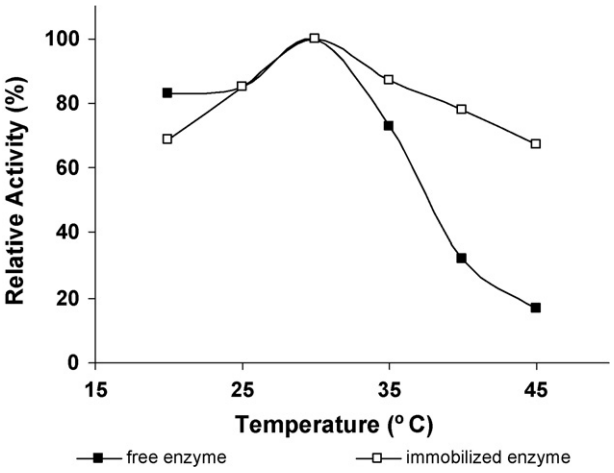

Fig. 7. Effect of temperature on enzyme activity.

the activated matrix via its free amino groups, after the immobilization acidic groups prevail providing the enzyme with a more polyanionic character. During enzymatic hydrolysis a pH gradient is formed between the domain of immobilized lipase particles and the external solution resulting in the observed shift of the pH optimum to the alkaline region as expected [51]. Similar results have been reported for porcine pancreatic lipase immobilized on aminated silica gel and a polysuccinimide derivative. The immobilized PPL retained high-relative activity within a pH range from 7.0 to 12.0. In contrast, the relative activity of the free PPL fluctuated dramatically within a narrow pH range. Similar results have been reported porcine pancreatic lipase covalently immobilized on support derived from aminated silica gel and polysuccinimide [52].

Fig. 7 shows the temperature profiles for the free and immobilized lipase preparations. Their responses to temperature were similar with maximal activities at 30 °C. However, immobilized lipase maintained a higher relative activity than free lipase at temperatures higher than 30 °C indicating that immobilized lipase compared to the free enzyme was less sensitive to temperature changes. This is in accordance with many reports that optimum temperature values of immobilized enzyme are higher than those of free enzymes [53–58], the catalytic activity of enzymes is related to their conformation and in general the activity value increases with a rise in temperature as is usually observed for chemical catalysts. But enzymes are unstable under high temperatures because increase in environmental temperature results the deformation of enzyme catalytically active globular conformation. Since immobilization stabilizes the enzyme structure the optimum temperatures for activity are consequentially expected to increase. Unfortunately, this principle does not apply in general, sometimes optimum pH and optimum temperature values remain unchanged after immobilization despite relative activities might be quite different for immobilized and free enzyme [59–61].

The pH stabilities of the both forms of the lipase were compared in 50 mM phosphate buffer in the pH range 6.5–8.5. The immobilized enzyme displayed a better stability than its free form for this pH range (Fig. 8).

The thermal stabilities of the immobilized and free lipase were investigated in the temperature range 4–55 °C. The results showed that the immobilized lipase was more stable (Fig. 9) which complies with the observation that thermal stability of an enzyme is improved when coupled to a polyanionic carrier [62,63].

The storage stability of the immobilized enzyme was clearly better than the free enzyme (Fig. 10). The free enzyme rapidly lost its activity with a residual value of 8% after 22 days, the activity decrease occurred more slowly with the immobilized enzyme, and about 50% of its initial activity was recovered after the same period.

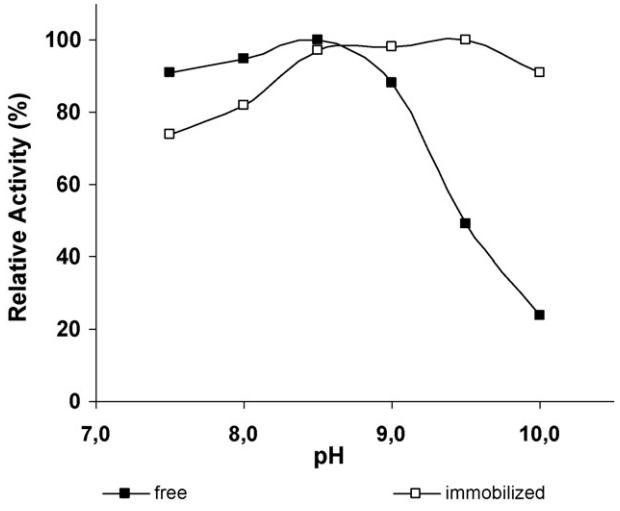

Fig. 6. Effect of pH on enzyme activity.

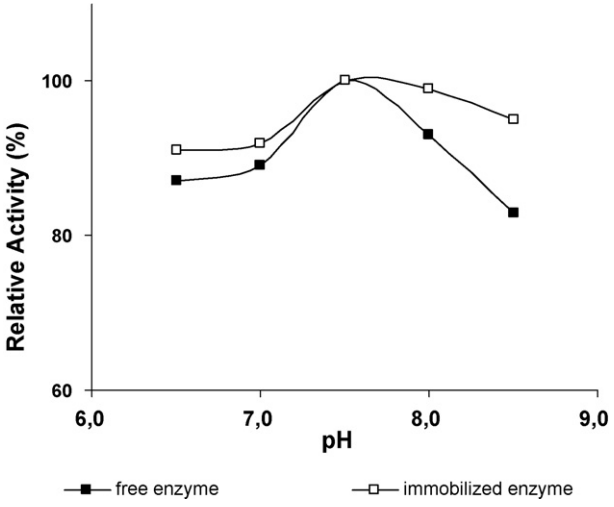

Fig. 8. pH stability of enzymes.

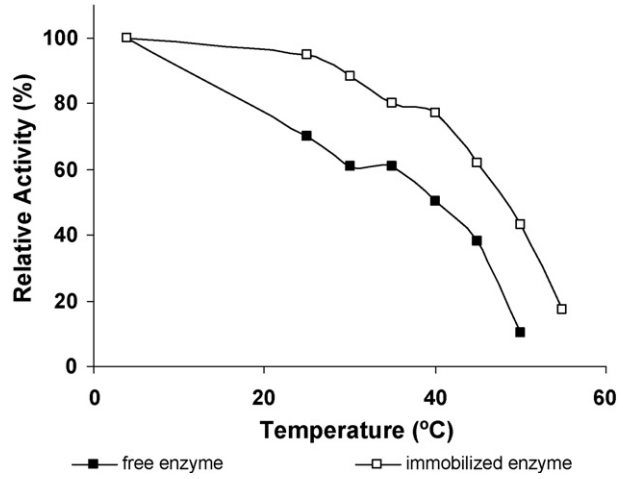

Fig. 9. Thermal stability of enzymes.

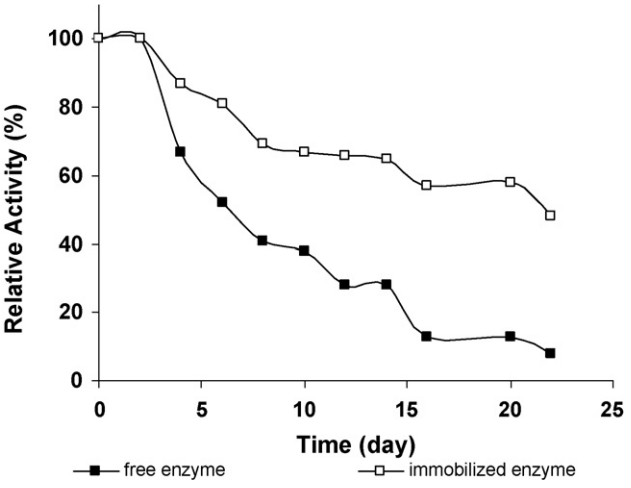

Fig. 10. Storage stability of enzymes.

The operational activity of the immobilized lipase was studied by tributyrin hydrolysis carried out continuously for 12 h. The immobilized enzyme lost 50% of its activity within 6.2 h this value thus represents its half-life ($t_{1/2}$) under the conditions of this experiment.

The aim of this work was modelling the carrier PVA in such a way that a suitable microenvironment could be created for the immobilization of lipase in a more active form. For this purpose GMA grafting on PVA was performed in order to change the hydrophilic character of the carrier to a partially hydrophobic one. The effect of grafting reaction conditions (monomer concentration, initiator concentration, reaction time) on the hydrophilic/hydrophobic balance of PVA was investigated and the effect of this balance on the activity of the immobilized enzyme was studied. The results show that GMA grafting caused an activity increase of the immobilized enzyme in comparison with an enzyme that had been immobilized on non-grafted PVA. Immobilized enzymes had clearly better properties and stabilities when compared to the free form.

4. Conclusion

The pancreatic lipase immobilization was performed on a PVA carrier with an increased hydrophobic characteristic obtained by GMA grafting. The grafted PVA was characterized by FTIR. The optimum conditions for grafting reaction were 1-h reaction time in the presence of 15 mM BP and 2.3 M GMA, for immobilization the optimal enzyme concentration was 1 mg/ml. Characterization of physico-chemical properties of both the immobilized and free enzyme was carried out. The esterification activity of immobilized lipase will be the subject of our future research regarding the synthesis of flavour esters in organic media.

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