Alginate–Poly(vinyl alcohol) Core–Shell Microspheres for Lipase Immobilization

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ABSTRACT: A lipase [triacylglycerol ester hydrolase (EC 3.1.1.3)] was encapsulated in sodium alginate (AlgNa)/poly(vinyl alcohol) (PVA) microspheres. Spherical AlgNa/PVA beads were prepared by the ionotropic gelation of an AlgNa/PVA blend in the presence of calcium tetraborate (CaB_4O_7). The particles were spherical and had an average diameter of 400 μm. The microspheres were studied with differential scanning calorimetry, Fourier transform infrared (FTIR) spectroscopy, and water transport by the equilibrium degree of swelling. The elevation of the glass-transition temperature of the microspheres indicated specific crosslinking reactions of the component polymers (AlgNa/PVA). FTIR spectra showed no evidence of a strong chemical interaction changing the nature of the functional groups of both AlgNa and PVA in the AlgNa/PVA blends. The water diffusion coefficients increased with increasing PVA content in the microspheres, indicating a decrease in the resistance to mass transfer through the AlgNa/PVA microsphere wall. The AlgNa/PVA microspheres were characterized by the Michaelis constant (K_M) and the maximum reaction velocity (V_{max}), which were determined for both free and immobilized lipases. The enzyme affinity for the substrate (K_M/V_{max}) remained quite good after immobilization. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 1553–1560, 2006

Key words: core-shell polymers; enzymes; hydrogels; water-soluble polymers

INTRODUCTION

Enzyme microencapsulation is a very useful physical entrapment of biocatalysts inside a polymeric membrane by the phase transition of a polymer solution containing the biocatalyst. Polymer microspheres can be prepared with several processes, such as coacervation phase separation, interfacial polymerization, solvent evaporation, spray coating, multiorifice centrifugation, and air suspension.[1–3] The selected enzyme immobilization process depends on the desired size of the encapsulated products and the physicochemical properties of both the enzyme to be encapsulated and the polymer microsphere.

Physical entrapment in a polymeric gel microsphere is by far the most commonly used technique for enzyme immobilization. However, true success may be limited by the problems associated with the mass-transfer resistance imposed by the fact that the substrate has to diffuse to the reaction site and with the mechanical stability of the microspheres in bioreactors.

Alginate, commercially available as alginate sodium salt and commonly called sodium alginate (AlgNa), is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae. This polysaccharide forms a family of linear natural copolymers of β-D-mannuropyranosyluronic acid (M) and α-L-gulopyranosyluronic acid (G) units linked together by β-1,4- and α-1,4-glycosidic bonds, with various proportions and segmental arrangements of M and G units along the chain.

Alginate has the ability to bind multivalent cations, leading to the formation of covalent bonds and insoluble hydrogels. This anionic polysaccharide forms strong gels with Ca^{+2}, giving microspheres with good strength and flexibility. Such crosslinking stiffens and roughens the polymer, reducing swelling in water and organic solvents.[4–6]

Poly(vinyl alcohol) (PVA) is a synthetic, nontoxic, high-strength polymer that has been used extensively in biotechnology for enzyme and cell immobilization

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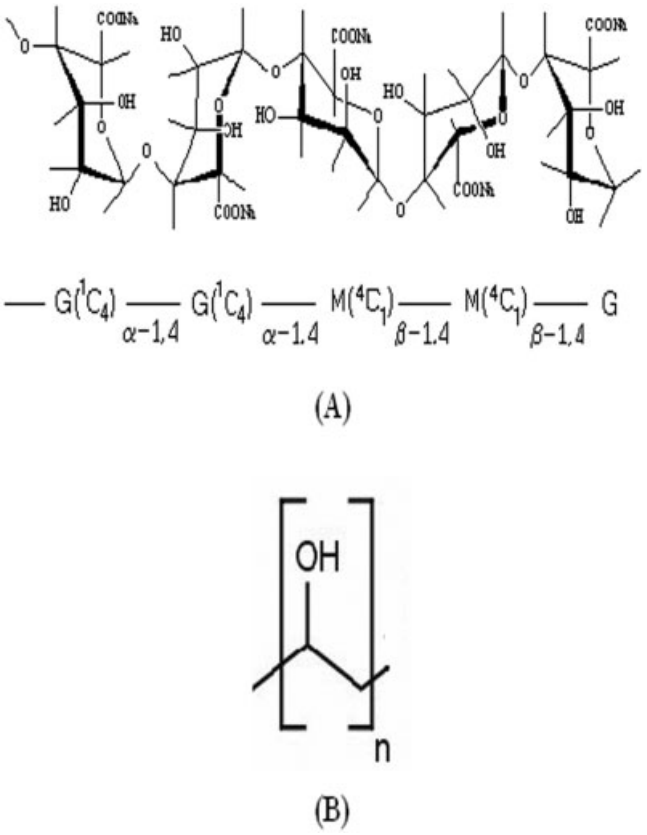


Scheme 1 Structures of the polymer systems used in this work: (A) AlgNa and (B) PVA.

because of its properties of protein stabilization and preservation of biological activity.[7–9]

Hydrogels of PVA and AlgNa have found extensive applications as carrier materials for immobilizing enzymes and cells, but their physicochemical properties need to be improved further for industrial applications.[10] The instability of alginate microspheres in citrate or phosphate pH buffers and the low mass transfer in PVA beads have limited the application of these carriers in batch or continuous bioreactors.[11–13] Scheme 1 shows the chemical structures of AlgNa and PVA polymers used in this work.

In contrast to the numerous studies performed on the physical properties and biotechnological applications of alginate gels as matrices for enzyme immobilization, there have been only a few examples of studies dealing with this polysaccharide and synthetic polymer blends.[14–17]

Polymer blending is an important method for the modification or improvement of the physical properties of polymeric materials. The hybridization of natural polymers with synthetic macromolecules may be of great significance for attaining desirable physical properties for industrial applications. Natural gel matrices are biodegradable and subject to abrasion, whereas synthetic hydrogels such as PVA have better mechanical properties.

The lower mass-transfer resistance of PVA in comparison with alginate beads suggests that the use of AlgNa/PVA blends could be an interesting approach to obtaining stable microspheres for use in biotechnological processes.

To improve the dimensional stability (mechanical strength) of alginate microspheres and to obtain a high-performance capsule, microspheres consisting of a PVA/AlgNa blend crosslinked with calcium tetraborate (CaB_4O_7) were prepared to investigate its application in lipase immobilization. The biochemical properties of the immobilized lipase, such as the hydrolytic activity and kinetic behavior thermal stability, and the recycled use of the biocatalyst were investigated.

Lipases are used frequently as chiral catalysts in the synthesis of various fine chemicals and intermediates.[18] Lipase-catalyzed chemical transformations are now widely recognized as practical alternatives to traditional (nonbiological) organic synthesis and as convenient solutions to certain intractable synthetic problems.

EXPERIMENTAL

Materials

Lipase from *Candida cylindracea* (EC 3.1.1.3; type VII containing 3000 U/mg of the solid with olive oil as the substrate) was obtained from SPfarma. One unit of lipase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fatty acid from olive oil as a substrate per minute at pH 8.9 and 37°C. PVA was purchased from Sigma. It had a polymerization degree of 2000 and was fully saponified (ca. 99.9%). AlgNa (average molecular weight = 60,000) was purchased from Sanofi Co.

Fabrication of the PVA/AlgNa microspheres

The AlgNa/PVA microspheres were prepared through the interfacial insolubilization reaction. Aqueous solutions of AlgNa and PVA were prepared separately at a concentration of 2.0 wt %. The powdered materials were dissolved in water at room temperature (25°C) for AlgNa and at 75°C for PVA, with continuous stirring. The AlgNa and PVA solutions were mixed in the desired proportions at room temperature and then stirred overnight. Then, the lipase powder was introduced into the AlgNa/PVA solution, and the mixture was stirred at 150 rpm at 4°C for 1 h. The resultant solution was optically clear and showed no visible phase separation. The relative composition of the two polymers in the mixed solutions ranged from 10/90 to 30/70 (wt %) PVA/AlgNa. The polymer solution was then added dropwise to the gelation medium of 250 mL of a 6.0 wt % CaB_4O_7 aqueous solution with a 25-mL hypodermic syringe

through a #21 needle under constant stirring at room temperature. After the completion of the reaction of crosslinking, the microspheres were collected by filtration. frozen for 30 min at −40°C, and lyophilized for 7 h.

The amounts of protein in the enzyme solution and in the wash solutions were determined with Coomassie Brilliant Blue G250, as described by Bradford,[19] with bovine serum albumin as a standard. The amount of the bound enzyme was calculated as follows:

$$q = \frac{(C_i - C_f)V}{W} \quad (1)$$

where q is the amount of the encapsulated enzyme in PVA/AlgNa microspheres (mg/g); C_i and C_f are the initial and final concentrations (mg/mL) of the enzyme in the medium, respectively; V is the volume of the medium (mL); and W is the weight (g) of the microspheres.

Analysis of the PVA/AlgNa morphology and microsphere morphology

The mean size of the capsules was determined from the average for 100 particles measured with a light microscope connected to a digital video camera (ML-2300, Sony, Japan) and digitalized with computerized image analysis.[20]

The morphology of the microspheres was analyzed with scanning electron microscopy (SEM; JEOL XL 30, Phillips).

The chemical structure of the AlgNa/PVA microspheres was analyzed with Fourier transform infrared (FTIR) spectroscopy (Nicolet, Madison, WI). FTIR was performed on KBr pellets at a resolution of 4 cm^{-1}.

The glass-transition temperatures (T_g's) of the AlgNa/PVA microspheres were measured with differential scanning calorimetry (DSC). DSC was carried out on approximately 6-mg samples with a Mettler TA 4000 thermal analysis unit with a DSC cell. Temperature readings were calibrated with an indium standard. The samples were first heated to 245°C and subsequently quenched to −20°C. The second heating scans were run from this temperature to 250°C to record stable thermograms.

All the measurements were performed at a heating rate of 10°C/min under a nitrogen atmosphere.

Equilibrium swelling experiments

Known weights of the dry microspheres were immersed in distilled water at 25°C until equilibrium was reached (24 h). Then, the microspheres were removed, blotted quickly with absorbent paper to remove the water attached to their surface, and weighed.

The water uptake was calculated as follows:

$$\text{Water uptake (wt\%)} = \frac{W_{wet} - W_{dry}}{W_{wet}} \times 100 \quad (2)$$

where W_{wet} is the weight of the swollen microspheres and W_{dry} is the weight of the dry microspheres.

Assay of the lipase activity

The substrate for the catalytic tests was olive oil.[21–23] This substrate was dissolved in water-saturated isooctane (10 mL), to which either industrial enzyme powder or the AlgNa/PVA microspheres (0.2 g) containing the enzyme (0.102 mg) were added, inside a 50-mL, glass bottle considered a batch reactor. The glass bottle was placed in a shaking bath (Bioblock) operating at 180 rpm and 37°C. At various times, aliquots of 100 μL were taken and analyzed after dilution in ethanol. The released fatty acids were determined by titration with 5 mM NaOH with an automatic titrator. The specific activities of both the free and immobilized lipases were determined by the measurement of the amount of the enzyme that catalyzed the formation of 1 μmol of fatty acid from olive oil as the substrate per minute under the assay conditions.

RESULTS AND DISCUSSION

Many researchers have extensively studied the structures of microspheres prepared by physical methods. Previous work indicates that it is possible to synthesize monodisperse core–shell microspheres through the crosslinking of the spherical microdomains of microphase-separated PVA blends with a sphere–matrix morphology.[24,25]

Figure 1 shows SEM micrographs of PVA/AlgNa microspheres produced by the interfacial insolubilization method. Microspheres with a good spherical geometry were obtained. The SEM micrographs show a core–shell structure after crosslinking with CaB_4O_7. The mechanism of ion-assisted crosslinks of PVA is believed to be a so-called didiol complexation, which is formed between the two-diol unit and one borate ion. Thus, the core–shell formation can be attributed to the fact that the Ca^{+2} ions diffused into the core and extracted the $B_4O_7^{-2}$ ions, resulting in a liquid-core system. One the other hand, Ca^{+2} ions were not extracted by the borate ions, and so a solid wall of alginate was formed.

The surface morphology of the AlgNa/PVA microspheres [Fig. 1(B,C)] shows irregular pores of various dimensions and, at the same time, a high internal surface area [Fig. 1(D)]. In the absence of PVA, al-

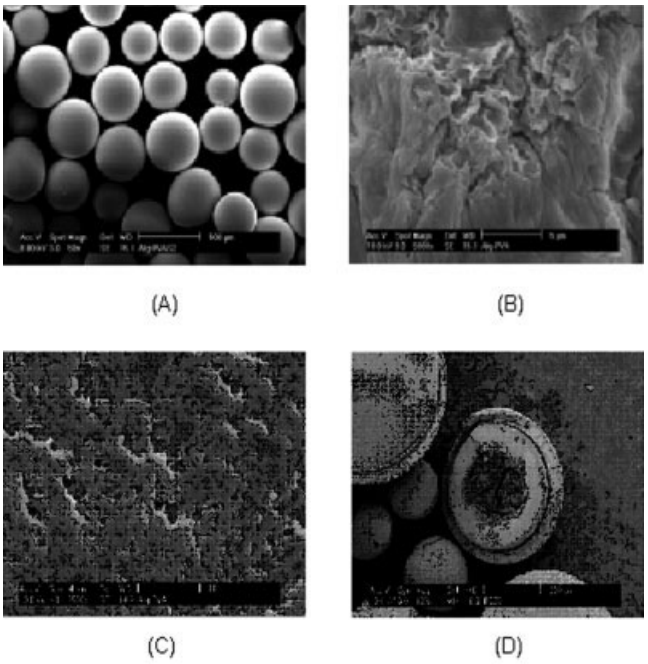


Figure 1 SEM micrographs of (A) the microspheres obtained by the coacervation method, (B,C) the surface of a microsphere, and (D) the internal morphology (cross section) of the AlgNa/PVA microspheres. The bars correspond to (A) 500, (B) 5, (C) 10, and (D) 200 μm. The PVA concentration in the AlgNa/PVA blend was 20 wt %.

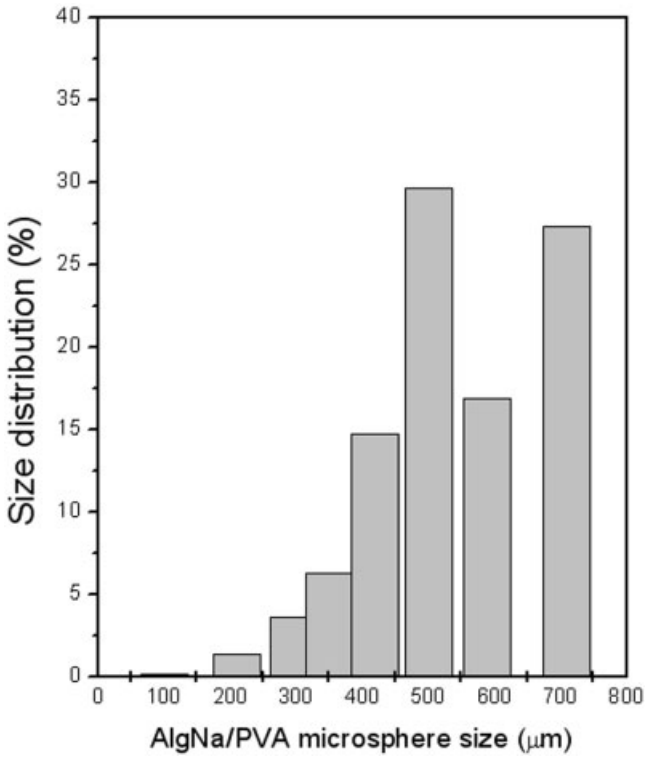


Figure 2 Particle size distribution of the AlgNa/PVA microspheres determined by image analysis.

though the air surface was very rough, no pore on the AlgNa surface or hollow microspheres was found. In the aqueous medium, the tetraborate anion was a weak base. It reacted with water (hydrolyzes) to form the hydroxide ion (OH^-), a strong base resulting in the formation of $Ca(OH)_2$ in the microenvironment of the microsphere surface. The $Ca(OH)_2$ formed *in situ* could play an important role in the microsphere morphology. Its relatively low solubility in water could in fact lead to additional crosslinking of the alginate microspheres, whereas the removal of precipitated $Ca(OH)_2$ could account for the uniform microporosity within the particle matrix.

The enzyme encapsulation technique may be applicable because the size of the substrate molecule is less than that of the lipase.[26] Figure 2 shows the particle size distribution of the AlgNa/PVA microspheres. The mean particle size of the AlgNa/PVA microspheres was 400 μm.

The FTIR spectrum of pure PVA [Fig. 3(A)] shows absorption peaks at about 3256 cm^{-1} (—OH) and at about 1086 and 1415 cm^{-1} for the —C—O group. Peaks of AlgNa [Fig. 3(B)] at 3440, 1610, and 1089 cm^{-1} were attributed to hydroxyl, C—O—O—, and C—O— groups, respectively. It is clear from the FTIR spectra that there were no peaks in the spectrum of the blend other than peaks corresponding to its individual components, and so FTIR shows no evidence of a strong chemical interaction changing the nature of the functional groups in the AlgNa/PVA blends.

The compatibility of AlgNa/PVA blends is critical for controlling the resulting phase behavior. In turn, the phase behavior may affect the physical chemistry. T_g can be used as an important criterion in determining the boundary for the mobility of mixed biopolymer systems. Figure 4 shows the T_g data obtained from DSC for different crosslinker (CaB_4O_7) concentrations. The elevation in T_g with an increase in the CaB_4O_7 concentration is evident. These changes in T_g

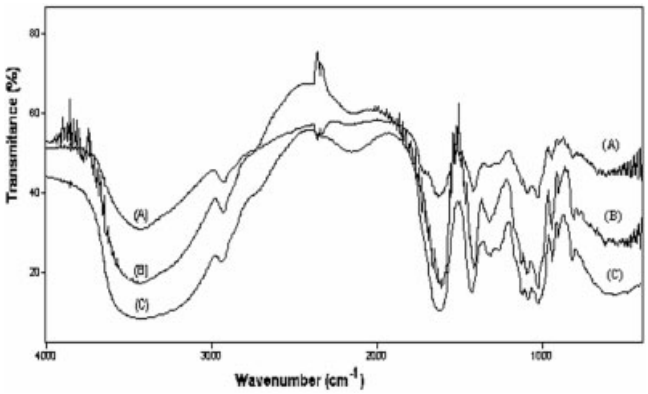


Figure 3 FTIR spectra of (A) AlgNa, (B) PVA, and (C) the AlgNa/PVA blend. The PVA concentration in the AlgNa/PVA blend was 20 wt %.

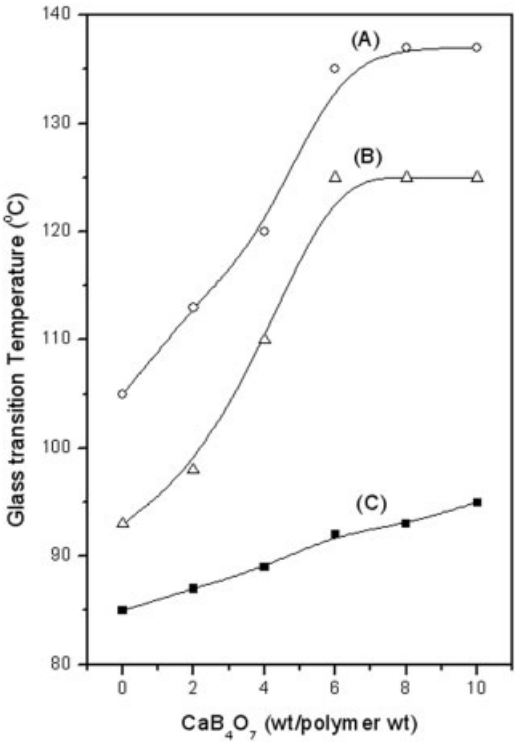


Figure 4 Effect of the CaB_4O_7 concentration on T_g of the AlgNa/PVA microspheres: (A) AlgNa, (B) AlgNa/PVA, and (C) PVA. The PVA concentration in the AlgNa/PVA blend was 20 wt %.

may be associated with the crosslinking reactions of AlgNa caused by CaB_4O_7. It is well known that in the presence of Ca^{+2}, alginate can be readily crosslinked by complexation between carboxylate anions of this polysaccharide and calcium cations. The fact that free carboxylic groups are repeated in the macromolecule makes them accessible to divalent Ca^{+2}, and the formation of coordination complexes occurs in the jellification process.

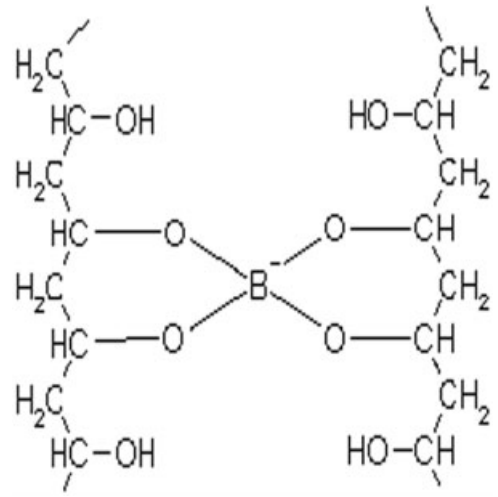
In the presence of borate compounds, PVA chains with abundant hydroxyl groups can also form a network through a borate-ion-aided complexing reaction according to Scheme 2.

As a result of the two kinds of crosslinks in both polymer components, phase segregation may be inhibited when a low-concentration CaB_4O_7 solution is used.

The water diffusion coefficients calculated from gravimetric results can be calculated according to the following equation:[27]

$$\ln\left(1 - \frac{M_t}{M_{170}}\right) = \ln\frac{6}{\pi_2} - D\left[\frac{\pi}{r}\right]^2 \cdot t \qquad (3)$$

where M_t/M_∞ is the fractional water uptake by the polymer at immersion time t, r is the radius of the microsphere, and D is the water diffusion coefficient.



Scheme 2 Crosslinking of PVA by boric acid.

Figure 5 shows the water diffusion coefficients for AlgNa/PVA for various PVA contents of the microspheres. As evident from the data in Figure 5, the water diffusion coefficients increase with increasing PVA content, indicating decreasing resistance to mass transfer through the AlgNa/PVA microsphere wall.

Because of their high activity and selectivity, lipases (EC 3.1.1.3) have great potential for use as biocatalysts in industrial applications.[28–30] A so-called lid may

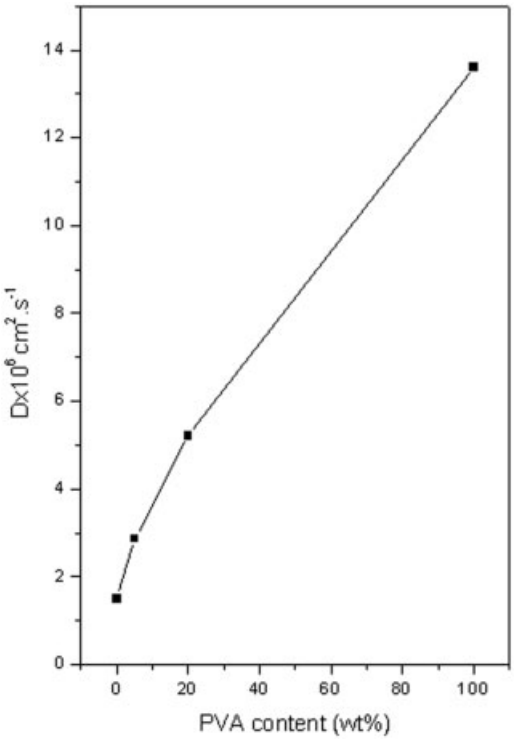


Figure 5 Dependence of the water diffusion coefficient (D) of the PVA composition on the AlgNa/PVA microspheres (temperature = 298 K).

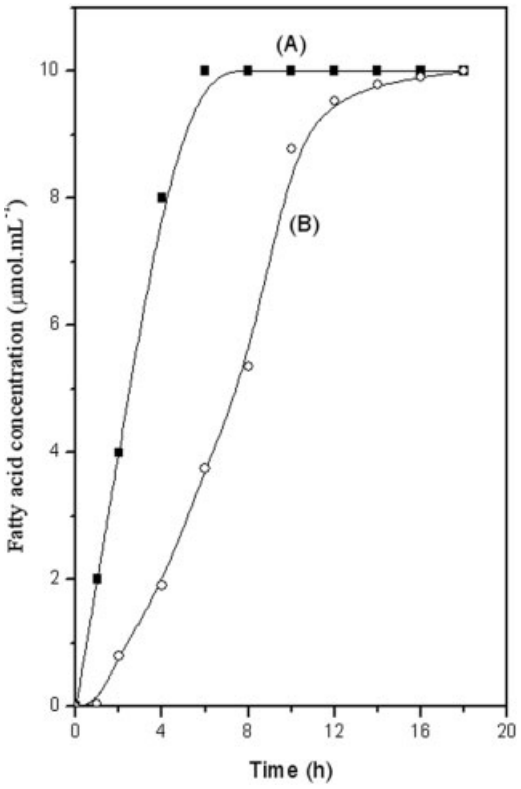


Figure 6 Hydrolysis of olive oil with AlgNa/PVA microspheres: (A) immobilized and (B) soluble lipase. The PVA concentration in the AlgNa/PVA blend was 20 wt %.

structurally characterize the molecular structure of lipases. When hydrophobic substrates interact with the lipase, the lid opens and thus exposes the active site in a process called interfacial activation.[31,32]

Most reactions catalyzed by lipases are carried out in biphasic media because of the low solubilities of the reactants and products in conventional aqueous media. The encapsulation through the confinement of the lipase solution within polymer microspheres provides an aqueous environment for the lipase, thus reducing the problem of interfacial denaturation characteristics of two-phase systems.[18]

In recent years, the use of lipases to cleave carboxylic ester bonds in tri-, di-, and monoacylglycerols and to catalyze transesterification reactions has become an attractive alternative to classical methodologies of chemical synthesis.

The production of fatty acid from olive oil with the immobilized lipase on AlgNa/PVA microspheres is shown in Figure 6. With the immobilized form of the enzyme, the maximum fatty acid concentration in the reaction media was achieved after 4 h, whereas for the free lipase; about 12 h of incubation was needed. The immobilized lipase appeared to be more favorable for the hydrolysis of olive oil than the free enzyme, probably because of the better interfacial conditions prevailing in the AlgNa/PVA microspheres. Similar results have been reported for other forms of immobilized lipases.[33]

The initial hydrolytic reaction rates of olive oil were measured at different substrate concentrations, as shown in Figure 7. When the activities of the free and immobilized lipases (with the same amount of the free enzyme) were compared, we observed that the lipase immobilized in the AlgNa/PVA microspheres was more active than the free enzyme. In this case, the difference in the enzymatic activities may be related to the high hydration of the microenvironment of the lipase immobilized on the AlgNa/PVA microspheres.

Assuming that the hydrolysis reaction of olive oil by the encapsulated lipase obeyed Michaelis–Menten kinetics, we calculated the apparent Michaelis constant (K_M) and the maximum velocity (V_{max}) by linear regression. The straight lines of the Lineweaver–Burk plot (Fig. 8) gave an apparent K_M value of 1.41 mol/dm^3 for the free lipase and 0.33 mol/dm^3 for the immobilized enzyme. The low K_M value in the encapsulated system with respect to that of the free lipase may be due to the mass-transport resistance to the substrate into the porous AlgNa/PVA microspheres.

The effect of temperature on the free and the immobilized lipase activity was investigated with olive oil for 10 min at the indicated temperatures, and the

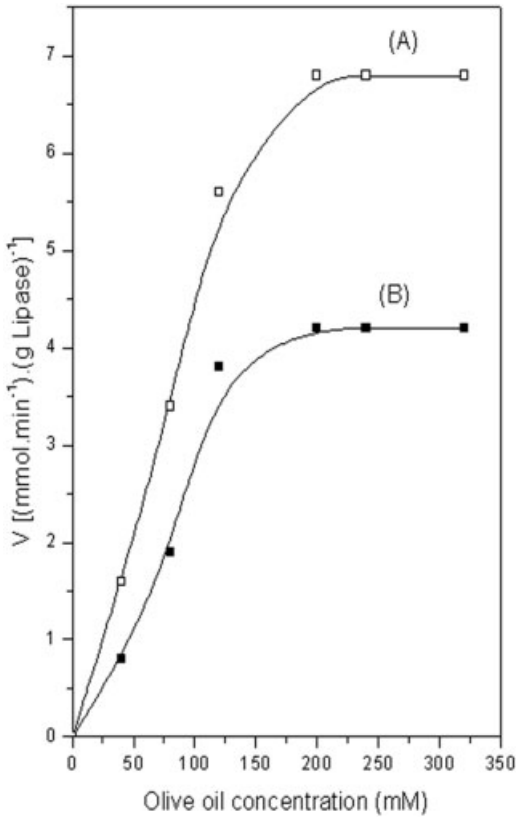


Figure 7 Effect of the substrate concentration on the hydrolytic rate of olive oil by (A) immobilized and (B) free lipase.

results are shown in Figure 9. The maximal activity of the free enzyme appeared at 35°C, but the optimum temperature of the immobilized lipase was 50°C. This increases the potential of the immobilized lipase as a practical catalyst on an industrial scale because higher temperatures reduce the viscosity of the reaction medium, minimizing the mechanical energy in agitation and other industrial operations.

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. Thus, an important parameter for an immobilized lipase is the storage stability of the AlgNa/PVA microspheres. To determine the impact of storage on the lipase activity, after synthesis, the microspheres were stored for 60 days in a phosphate buffer solution (PBS; 50 m*M*, pH 7,0) at 4°C and then were characterized according to the methodology previously described. After each use, the microspheres were rinsed and stored in a PBS solution until the next reuse. To evaluate the enzyme stability during successive reutilization cycles, the microsphere activities were measured for 12 cycles.

Figure 10 shows that, under the same storage conditions, the activity of the immobilized lipase decreased more slowly than that of the free lipase. The free enzyme lost all its activity within a 4-week period. The immobilized lipase preserved about 85% of its initial activity during a 12-week storage period (Fig. 10). The higher stability of the immobilized lipase could be attributed to the protective microenvironment provided by the AlgNa/PVA microspheres for lipase.

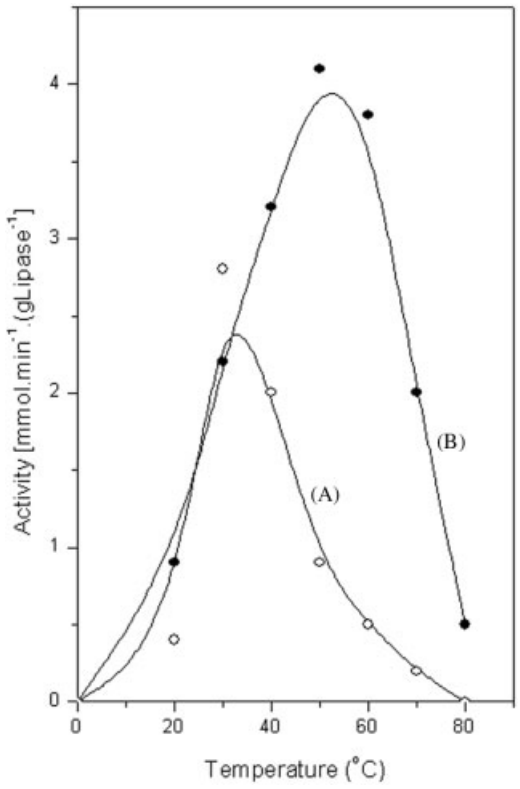


Figure 9 Effect of temperature on the activities of (A) free or (B) immobilized lipase in a PBS buffer and 0.1*M* olive oil emulsion (pH 7.0) for 10 min at the indicated temperatures. The assay was performed with 5 mg/mL free lipase or lipase immobilized on the microspheres and a 0.10*M* olive oil emulsion at 37°C.

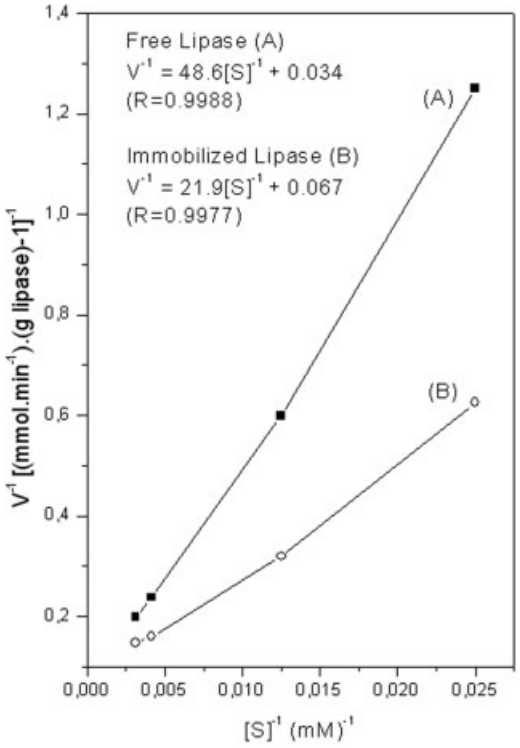


Figure 8 Lineweaver–Burk plot for (A) free and (B) immobilized lipase.

CONCLUSIONS

In this work, PVA was blended with AlgNa to form porous microspheres for lipase immobilization. The feasibility of preparing mechanically more stable AlgNa/PVA microspheres for use in bioreactors was demonstrated. Olive oil was more quickly hydrolyzed by the immobilized enzyme than by the free lipase. The low K_M/V_{max} ratio observed for the immobilized lipase suggested that the encapsulation of lipase by an AlgNa/PVA porous wall may be suitable as an immobilization method. Because of the low-temperature process, high porosity, large surface area, and low density, AlgNa/PVA porous microspheres appear to be an interesting alternative for heterogeneous catalysis and especially biocatalysis. The use of an immobilized lipase with AlgNa/PVA microspheres can reduce the capital and operation costs in industrial processes.

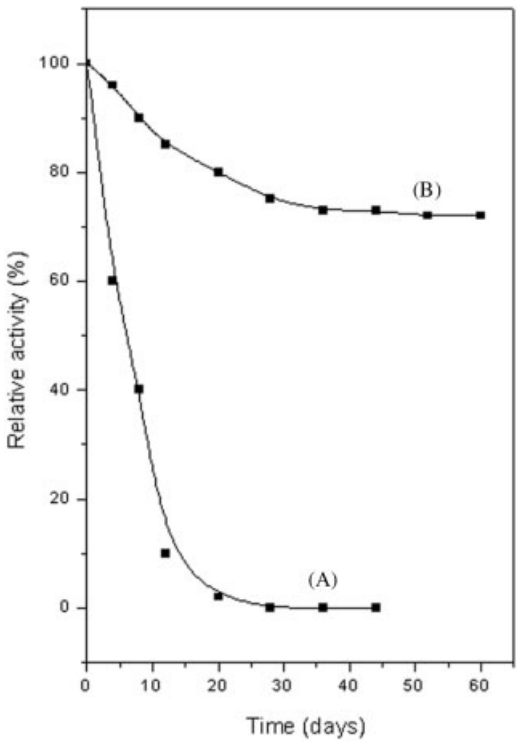


Figure 10 Stability of (A) free and (B) immobilized lipase in AlgNa/PVA microspheres in a phosphate buffer at pH 7.0 and 50°C.

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