

ALGINATE-POLY(VINYL ALCOHOL) CORE-SHELL MICROSPHERES FOR LIPASE IMMOBILIZATION

Saraiva Silva, G.¹, Fernandez, L.R.V.¹, Higa, O.Z.², Vítolo, M.³, De Queiroz, A.A.⁴

¹Universidade de Guarulhos (UnG).

²Laboratório de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares
(IPEN-USP). São Paulo-SP.

³Departamento de Tecnologia Bioquímico-Farmacêutica/Faculdade de Ciências Farmacêuticas da
Universidade de São Paulo (FCF-USP). São Paulo-SP.

⁴Departamento de Física e Química/Instituto de Ciências Exatas-Universidade Federal de Itajubá
(UNIFEI). Itajubá-MG, Brasil. E-mail: Alencar@efei.br

ABSTRACT

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) was encapsulated in sodium alginate/poly(vinyl alcohol) (AlgNa/PVA) microspheres. Spherical AlgNa/PVA beads have been prepared by ionotropic gelation of the AlgNa/PVA blend in the presence of calcium tetraborate (CaB₄O₇). The produced particles have a spherical shape with an average diameter of 400 μm. The microspheres were studied by differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and water transport by equilibrium degree of swelling. The glass transition temperature (T_g) of the AlgNa/PVA was depressed systematically with increasing PVA content in the microspheres. FTIR spectra have shown no evidence of strong chemical interaction changing the nature of the functional groups of both, AlgNa and PVA on AlgNa/PVA blends. The water diffusion coefficients increases with increasing PVA content on the microspheres indicating a decrease in the resistance to mass transfers 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}, were determined for yhe free lipase and for the immobilized lipase. The enzyme affinity for the substrate (K_M/V_{max}) remains quite good after immobilization.

Keywords: Lipase, Poly(vinyl alcohol), Sodium alginate.

INTRODUCTION

Microencapsulation is a very useful physical entrapment of enzymes inside a polymeric skin via a phase separation process in solutions containing the bioactive protein. Polymer microcapsules are prepared employing several processes such as, coacervation phase separation, interfacial polymerization, solvent evaporation, spray coating, multiorifice centrifugal process and air suspension (Pavanetto et al., 1992; Magdassi et al., 1996; Wen et al., 1991). Because each of those processes has its advantages and disadvantages, no one process is suitable for all substances. Therefore, the process selected depends on the desired size of the encapsulated products and the physicochemical properties of both the bioactive substance to be encapsulated and the coating materials.

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

Alginate, commercially available as alginic acid, sodium salt, commonly denominated sodium alginate, is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae. This polysaccharide form a family of linear natural copolymers of β -D-mannuronypyranosyluronic acid (M) and α -L-gulopyranosyluronic acid (G) units linked together by β -1,4 and α -1,4 glycosidic bonds, with varying proportions and segmental arrangements of M and G units along the chain. Segments containing only D-mannuronypyranosyl units are in the 4C_1 conformation, while L-gulopyranosyl units are in the 1C_4 conformations, which give the different blocks quite different chain conformations. M-block regions are flat and ribbon-like, similar to the conformation of cellulose, because of the equatorial-equatorial bonding. G-block regions have a pleated (corrugated) conformation as a result of axial-axial glycosidic bonds.

Polyvalent cations bind to the biopolymer whenever there are two neighboring guluronic acid residues producing a ionotropic gelation of alginate. The calcium salt of alginates is insoluble as result from the autocoperative reaction between calcium ions and the G-block regions of the chain. The holes formed between two G-block chains are cavities that bind calcium ions. The result is a junction zone that has been called "egg box" arrangement with the calcium ions being likened to eggs in the pockets of an egg carton. The strength of the gel depends on the content of G blocks in the alginate and the concentration of calcium ions (King, 1983; Moe et al., 1995; Grasdalen et al., 1981).

Poly(vinyl alcohol) (PVA) is a synthetically nontoxic, high-strength polymer that have been used extensively as matrix for the enzymes and cells immobilization due to their property of protein stabilization and preservation of the biological activity (Arakawa, et al., 1982; Uhlich et al., 1999; Imai et al., 1986). Figure 1 shows the chemical structures of AlgNa and PVA polymers used in this work.

Hydrogels of poly(vinyl alcohol) (PVA) and sodium alginate (Alg) have found extensive applications as a carrier materials to immobilize enzymes and cells but their properties need to be improved further for industrial applications (Hashimoto et al., 1987).

However, 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 (Wu et al., 1992; Khoo et al., 2001; Liu et al., 1997).

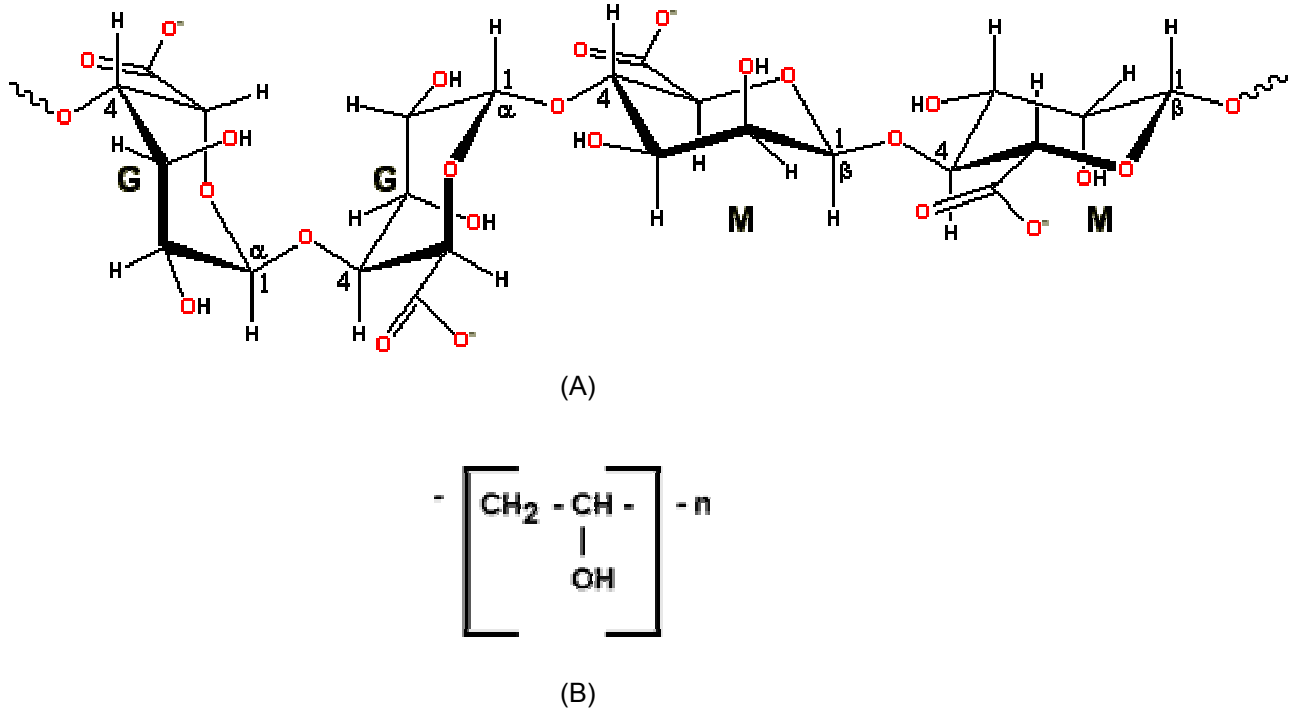


Figure 1- Structures of the polymer systems used in this work: sodium alginate (AlgNa) (A) and poly(vinyl alcohol) (PVA) (B).

In contrast to the numerous studies performed on the physical properties and biotechnological applications of alginate gels as matrix for enzyme immobilization, there have been only a few examples of studies dealing this polysaccharide and synthetic polymer blends (Leenen et al., 1996; Shindo et al., 1990; Yonese et al., 1992; Miura et al., 1999). Polymer blending is an important method for modification or improvement of the physical properties of polymeric materials. The hybridization of natural polymers with synthetic macromolecules may be of great significance to attain the desirable physical properties to industrial applications.

The lower mass transfer resistance in PVA compared with alginate beads suggests that the use of calcium alginate (AlgNa) /poly(vinyl alcohol) (PVA) blends appears to be an interesting methodology to obtain stable microspheres for use in biotechnological processes. Natural gel matrices are biodegradable and subject to abrasion whereas synthetic gels have better mechanical properties and are not biodegradable.

In order to improve dimensional stability (mechanical strength) of alginate microspheres and obtain a high performance capsule, microspheres consisting of PVA and AlgNa blend crosslinked with calcium tetraborate (CaB_4O_7) was prepared to investigate its application in biotechnology processes.

The interest of lipases is directed at their widespread application in the resolution of racemic mixtures through esterification reactions in organic solvents with low amount of water (Santaniello et al., 1992).

MATERIALS AND METHODS

Materials

Lipase from *Candida cylindracea* (EC 3.1.1.3; type VII containing 3000 U.mg⁻¹ solid, using olive oil as substrate) was obtained from SPfarma. One unit (1U) of lipase activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of fatty acid from olive oil as substrate.min⁻¹ at pH 8.9 and 37 °C. Poly(vinyl alcohol) (PVA) was purchased from Sigma. It had a polymerization degree of 2,000 and was fully saponified (about 99,9%). Sodium alginate (AlgNa, average molecular weight of 60,000 was purchased by Sanofi Co.

Fabrication of PVA/AlgNa microspheres

Hydrogel type AlgNa/PVA microspheres was prepared using the interfacial insolubilization reaction. Aqueous solutions of sodium alginate and PVA were prepared separately at concentration of 2.0 wt%. The powder materials were dissolved in water at room temperature (25 °C) for alginate and at 75 °C for PVA, with continuous stirring. The two polymer solutions were mixed in the desired proportions at room temperature and then stirred overnight. Then lipase powder was introduced to 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 was ranged from 10/90-30/70 in PVA/AlgNa weight percent ratio. The polymer solution was then added dropwise into gelation media of 250 mL of 0.4 wt% CaB₄O₇ aqueous solution using a 25 mL hypodermic syringe through a needle #21 under constant stirring at room temperature. After the reaction of crosslinking was completed, the microspheres were collected by filtration, frozen for 30 min at -40 °C followed by lyophilization for 7 h. The amounts of protein in the enzyme solution and in the wash solutions were determined by using Coomassie Brilliant Blue G250, as described by Bradford with bovine serum albumin (BSA) as a standard Bradford, 1976). The amount of bound enzyme was calculated as:

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

where q is the amount of encapsulated enzyme in PVA/AlgNa microspheres (mg.g⁻¹), C_i and C_f are the initial and final concentrations (mg.mL⁻¹) of the enzyme in the medium, respectively, V is the volume of the medium (mL), W is the weight of the microspheres (g).

Analysis of PVA/AlgNa microstructure and microspheres morphology

The mean size of the capsules was determined from the average for 100 particles measured using a light microscope connected with a digital video camera (ML-2300, Sony, Japan) and digitalized using computerized image analysis Alves et al., 2003).

The morphology of the microspheres was analyzed using scanning electron microscopy (SEM, JOEL6300).

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

Glass transition temperatures of the AlgNa/PVA microspheres were measured by differential scanning calorimetry (DSC). Differential scanning calorimetry (DSC) was carried out on ca. 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 up to $245\text{ }^{\circ}\text{C}$ and subsequently quenched to $-20\text{ }^{\circ}\text{C}$. The second heating scans were run from this temperature to $250\text{ }^{\circ}\text{C}$, to record stable thermograms. All the measurements were performed at a heating rate of $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ under a nitrogen atmosphere.

Equilibrium swelling experiments

Water sorption was measured gravimetrically at $25\text{ }^{\circ}\text{C}$. Samples were immersed in distilled water and its weight was measured as a function of immersion time over a 24 hours period. The pre-weighed dry AlgNa/PVA microspheres were immersed in distilled water and the weight change was monitored at different time intervals till the beads showed saturation in the water sorption.

Assay of lipase activity

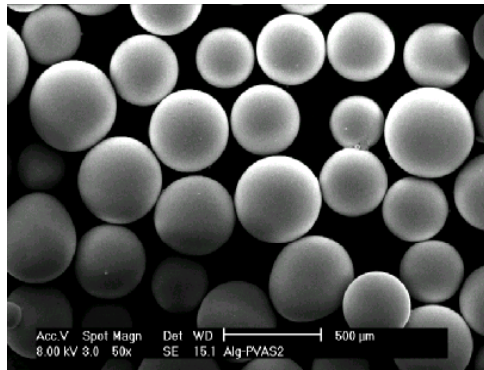
The substrate for the catalytic tests was olive oil. This substrate was dissolved in water saturated isooctane (10 mL) to which either industrial enzyme powder or the AlgNa/PVA microspheres (0.2 g) containing the enzyme (0.102 mg) were added, inside a 50 mL glass bottle considered as a batch reactor. The glass bottle was placed in a shaking bath (Bioblock) operating at 180 rpm and $37\text{ }^{\circ}\text{C}$. At various time intervals, aliquots of $100\text{ }\mu\text{L}$ were taken and analyzed after dilution in ethanol. The released fatty acids were determined by titration with 5 mM NaOH using an automatic titrator. The specific activities of both the free and the immobilized lipase were determined by measuring the amount of enzyme that catalyzes the formation of one μmol of fatty acid from olive oil as substrate per minute under the assay condition (Cho et al., 1993; Guo et al., 2003; Valivety et al., 1992).

RESULTS AND DISCUSSION

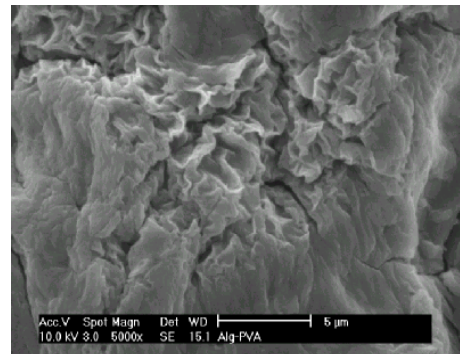
Many researchers have extensively studied the structure of microspheres prepared by physical methods. Previous work indicates that it is possible to synthesize monodisperse core-shell type microspheres by crosslinking the spherical microdomains of microphase-separated PVA blends with sphere-matrix morphology (Ishizu, 1998; Saito et al., 1995).

Figure 2 shows the SEM micrographs of AlgNa/PVA microspheres produced by the interfacial insolubilization method. Microspheres with a good spherical geometry was obtained. In addition, our preparation procedure allows obtaining a high microsphere recovery (approximately 90%) with respect to the starting amount of the PVA and alginate used. The surface morphology of the

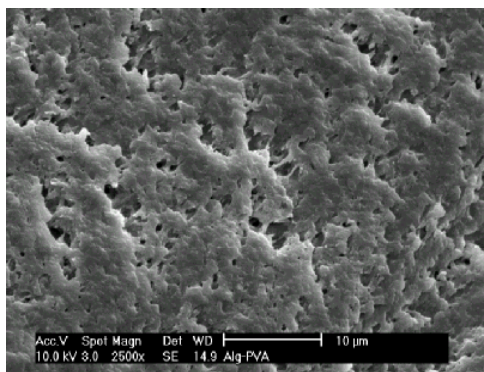
AlgNa/PVA microspheres (Fig. 2-B,C) shows irregular pores of varying dimensions, which may lead to high internal surface area (Fig. 2-D) or the reaction of substrate with the immobilized enzyme molecule. In the absence of PVA, although the air surface is very rough, no pore on the AlgNa surface is found.



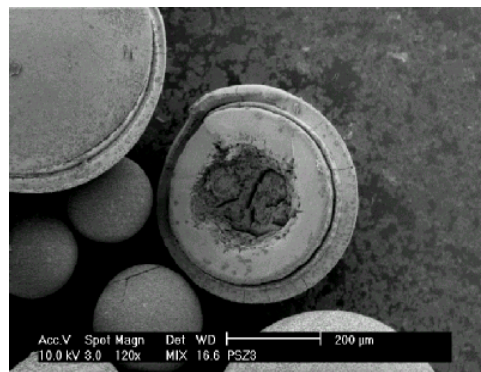
(A)



(B)



(C)



(D)

Figure 2- Scanning electron micrographs showing the microspheres obtained by the coacervation method (A), the surface of a microsphere (B,C) and internal morphology (cross-section) (D) of AlgNa/PVA microspheres. The bar corresponds to 500 μm (A), 5 μm (B), 10 μm and 200 μm (D). PVA content on the AlgNa/PVA blend: 20% (w/w).

The enzyme encapsulation technique may be applicable since the size of the substrate molecule is minor than that of the lipase (Paiva et al., 1997). Figure 3A,B show the particle size distribution of the microcapsules. The mean particle sizes of the AlgNa/PVA microspheres were 400 μm .

The FTIR spectrum of the alginate (Figure 4) shows the characteristic peaks at 1607 cm^{-1} (COOH stretching) and 1036 cm^{-1} (C-O-C stretching). The FTIR spectrum of pure PVA (Fig. 4) showed the absorption peaks at about 1086 and 1415 cm^{-1} for the -C-O group. It is clear from FTIR spectra that there are no peaks in the spectrum of the blend other than peaks corresponding

to its individual components and so the FTIR has shown no evidence of strong chemical interaction changing the nature of the functional groups on the microspheres.

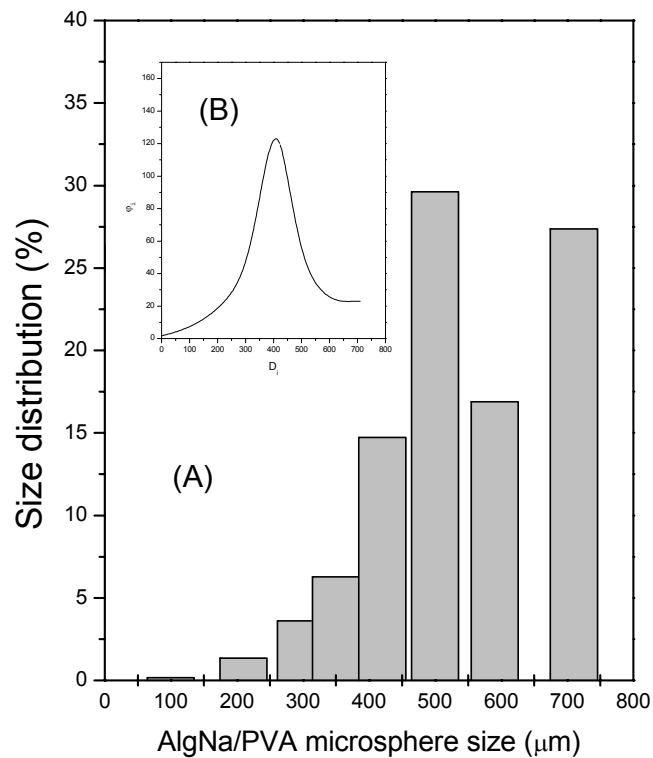


Figure 3- Particle size distribution of AlgNa/PVA microspheres determined by image analysis: size distribution (A) and derivative curve (B).

One of the most commonly used methods to estimate polymer-polymer miscibility is the determination of the T_g of the blend compared with the T_g 's of the two components separately. Figure 5 shows the T_g data obtained by DSC for different polymer compositions. As can be seen from Figure 5, the glass transition temperature (T_g) of the blend was depressed systematically to lower temperatures with increasing PVA content. The observed depression in T_g of AlgNa/PVA blends may be associated to the cross-linking reactions of the component polymers caused by the CaB_4O_7 . It is well known that in the presence of Ca^{+2} alginate may be readily cross-linked by complexation between carboxylate anions of this polysaccharide and the metallic cations. In the presence of borate compounds, PVA chains having abundant hydroxyl groups can also form a network through a borate-ion-aided complexing reaction. As consequence of the two kinds of cross-links in both polymer components, phase segregation is inhibited when a low concentration of CaB_4O_7 solution is used.

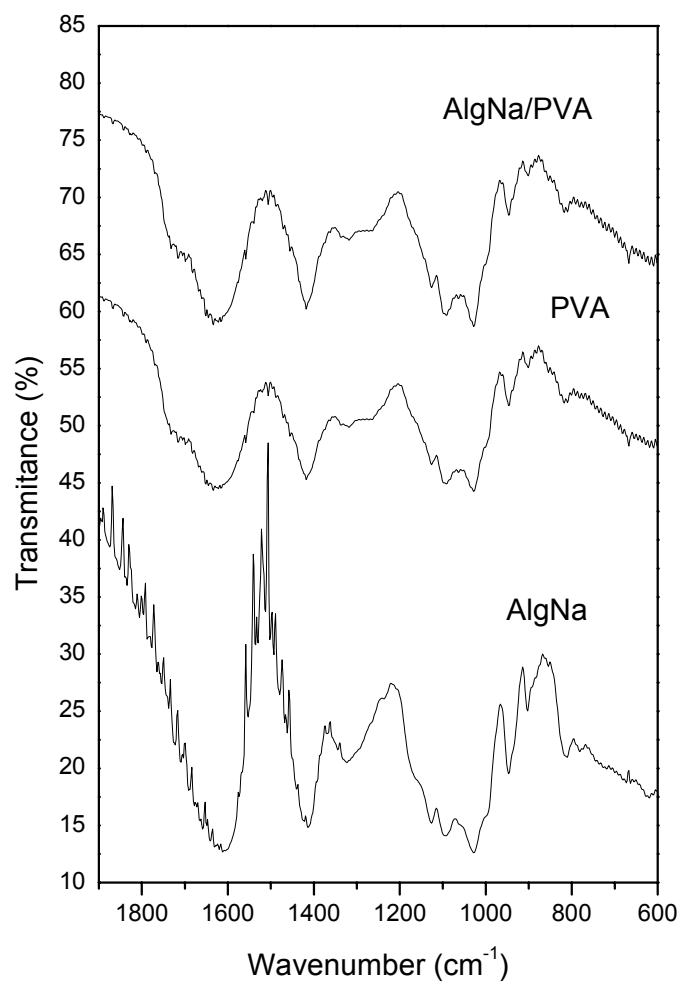


Figure 4- FTIR spectra of the AlgNa, PVA and the AlgNa/PVA blend (PVA content: 20 wt%).

The water diffusion coefficients calculated from gravimetric results can be calculated according to the following equation (Crank, 1975):

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\frac{6}{\pi^2} - D\left[\frac{\pi}{r}\right]^2 t \quad (2)$$

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 the water diffusion coefficient.

Figure 6 shows the water diffusion coefficients into AlgNa/PVA for various PVA content of the microspheres. As is evident from the data in Figure 6, the water diffusion coefficients increase with increasing PVA content indicating a decrease in the resistance to mass transfer through the AlgNa/PVA microsphere wall.

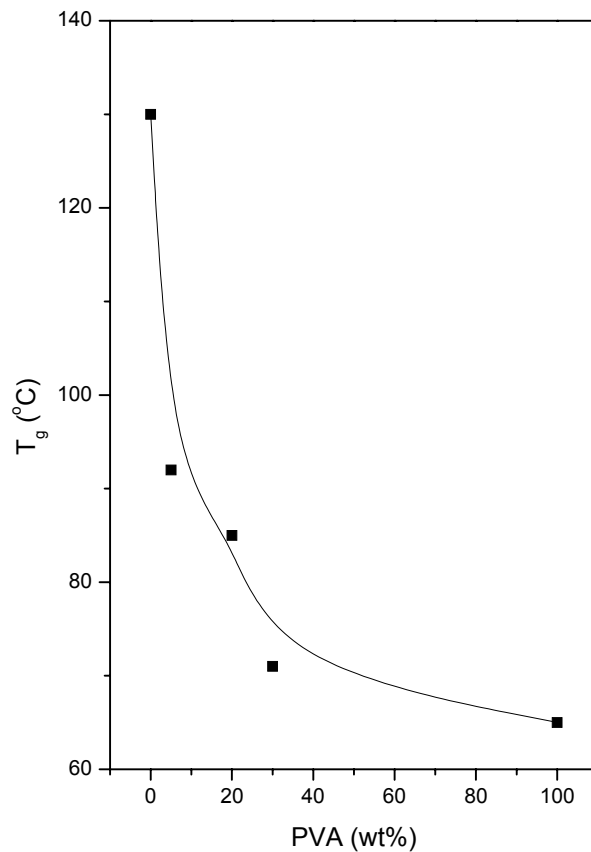


Figure 5- Effect of PVA content on glass transition temperature (T_g) of AlgNa/PVA microspheres.

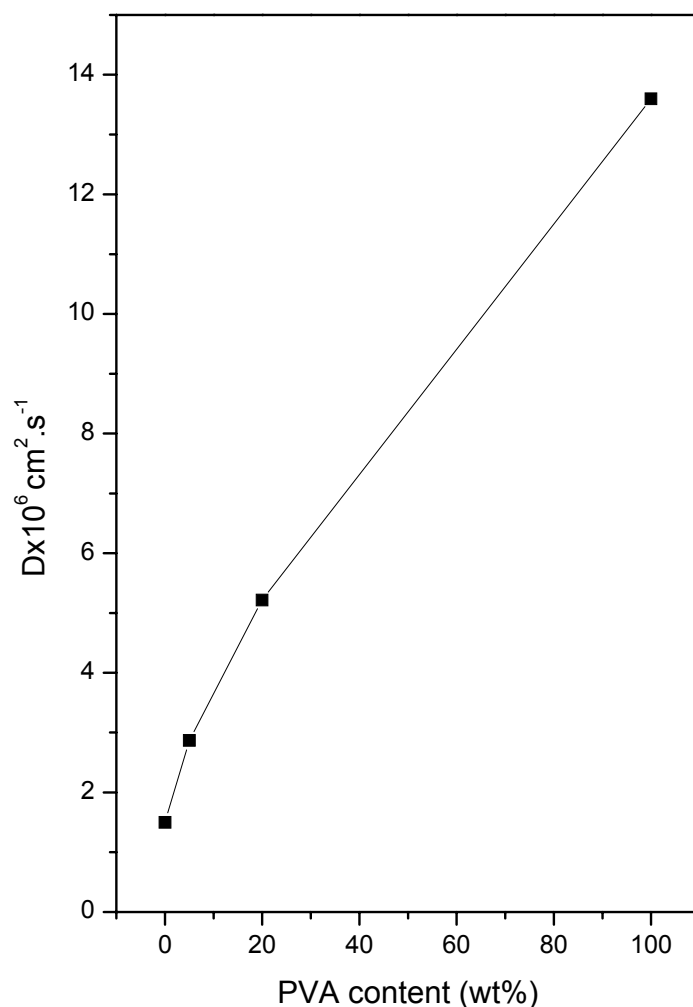


Figure 6- Dependence of water diffusion coefficients of the PVA composition on the AlgNa/PVA microspheres (T = 298 K).

Because of their high activity and selectivity lipases (E.C.3.1.1.3) have a great potential for the use as biocatalysts in industrial applications (Gotor, 1999; Yahya et al., 1998; Paiva et al., 1997; Villeneuve et al., 2000). A so-called lid may 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 (Brzozowski et al., 1991; Balashev et al., 2001).

Most reactions catalyzed by lipases are carried out in biphasic media due to the low solubilities of 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 the two-phase systems (Santaniello et al., 1993).

The fatty acid production from olive oil using the immobilized lipase on AlgNa/PVA microspheres is shown in Figure 7. With the immobilized form of the enzyme, the maximum fatty acid concentration in the reaction media was achieved after 4 h while for the free lipase, about

about 12 h of incubation was needed. The immobilized lipase appear to be more favorable form for the hydrolysis of olive oil than the free enzyme probably due to a better interfacial conditions prevailing in the AlgNa/PVA microspheres. Similar results have been reported for other forms of immobilized lipase (Kazlauskas, 1994).

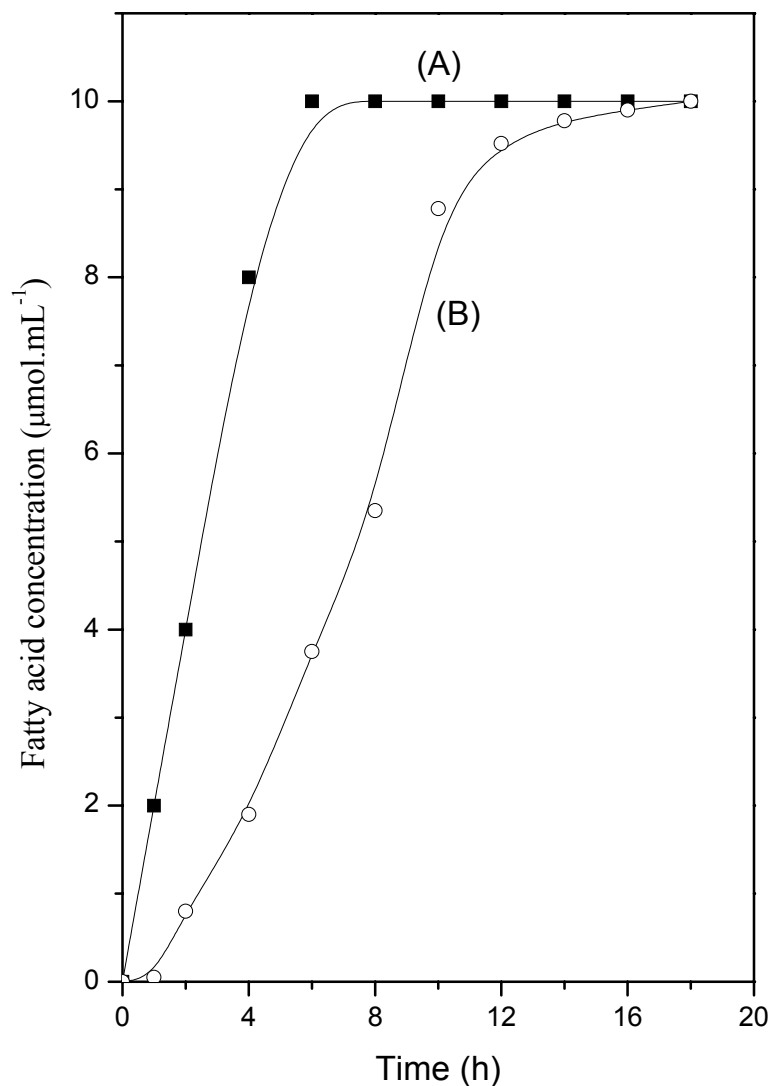


Figure 8- Hydrolysis of olive oil with AlgNa/PVA microspheres: immobilized (A) and soluble lipase (B).

The initial hydrolytic reaction rates of olive oil were measured at different substrate concentrations as shown in Figure 8. When the activities of the free and immobilized lipase (which the same amount of free enzyme) are compared, it was observed that the lipase immobilized in the AlgNa/PVA microspheres is more active than the free enzyme. In this case, the difference in the enzymatic activities may be related to a high hydration of the microenvironment of the lipase immobilized on AlgNa/PVA microspheres.

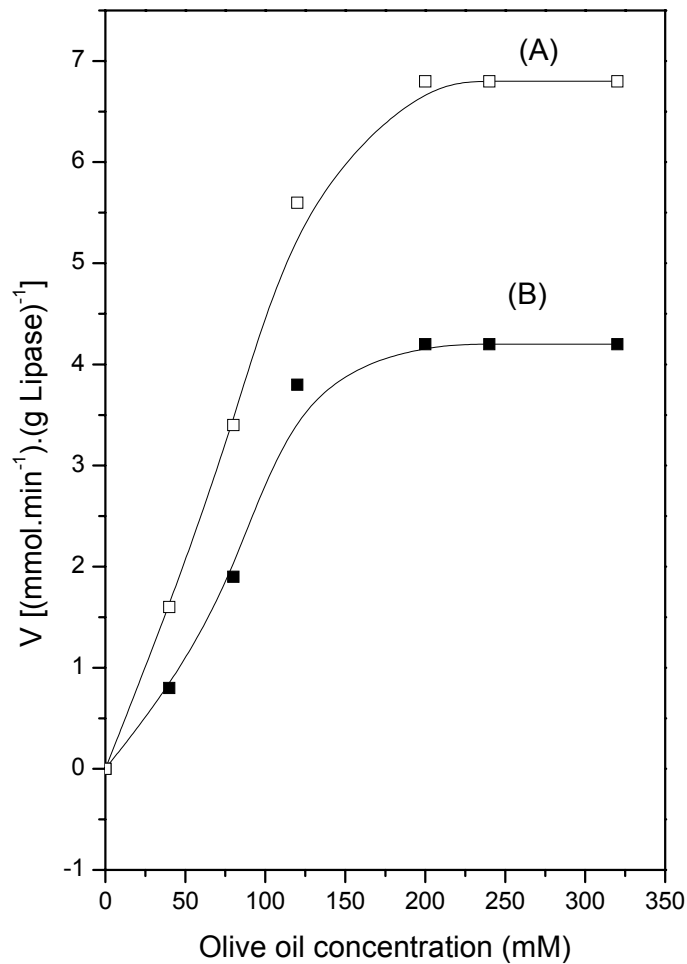


Figure 9- Effect of substrate concentration on the hydrolytic rate of olive oil by immobilized (A) and free (B) lipase.

Assuming that the hydrolysis reaction of olive oil by the encapsulated lipase obey the Michaelis-Menten kinetics and the apparent Michaelis constant, K_m , and the maximum velocity, V_{max} , were calculated by linear regression. The straight lines of the Lineweaver-Burk plot (Figure 9) give the apparent Michaelis constants (K_m) as 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 relatively to the free lipase may be due to the mass transport resistance to the substrate into the porous AlgNa/PVA microspheres.

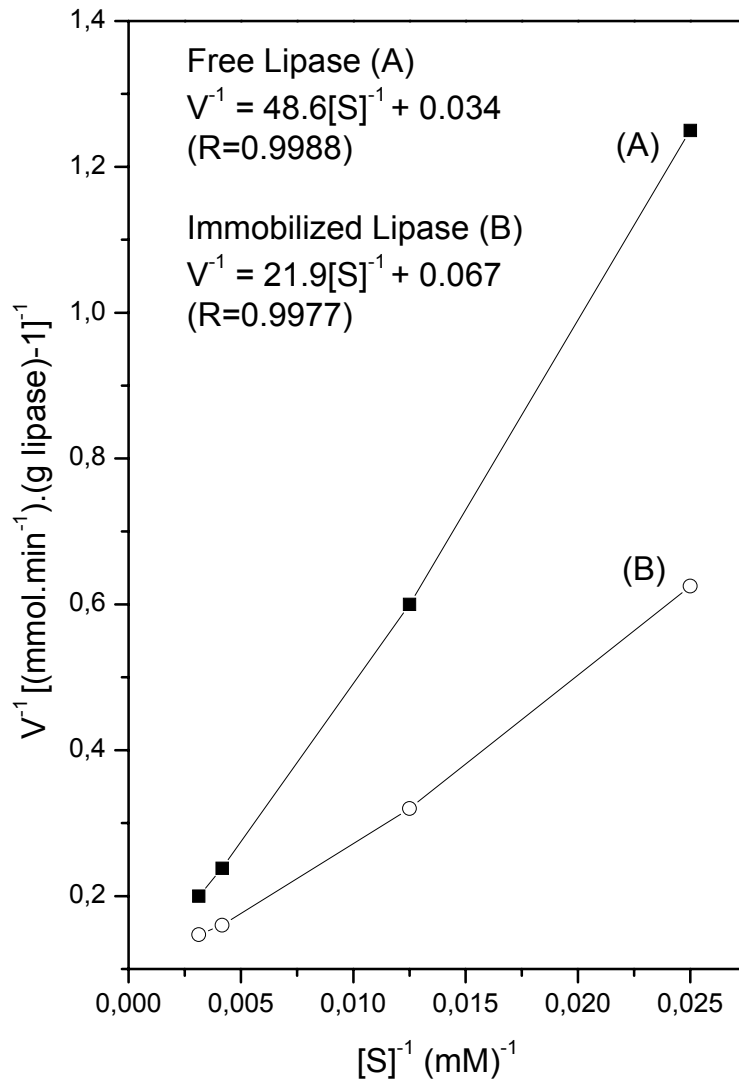


Figure 10- Lineweaver-Burk plot for free (A) and immobilized lipase (B).

CONCLUSION

In this work PVA has been blended with sodium alginate to form porous microspheres for lipase immobilization. The feasibility of preparing a more stable mechanically AlgNa/PVA microspheres for use in bioreactors was demonstrated. Olive oil was more quickly hydrolyzed by immobilized enzyme than by free lipase. The low K_m/V_{max} ratio observed for the immobilized lipase suggests that the encapsulation of lipase by a AlgNa/PVA porous wall may be suitable as an immobilization method. Because of their low temperature process, high porosity, large surface area and low density, the AlgNa/PVA porous microspheres appears to be an interesting alternative for applied in heterogeneous catalysis and specially biocatalysis.

REFERENCES

1. Arakawa T., Timasheff S.N. Stabilization of protein structure by sugars. *Biochemistry* **21**: 6536-44, 1982.
2. Alves S.B., De Queiroz A.A.A., Higa O.Z. Digital image processing for biocompatibility studies of clinical implant materials. *Artificial organs* **27**(5): 444-6.
3. Balashev K., Jensen T.R., Kjaer K., Bjørnholm T. Novel methods for studying lipids and lipases and their mutual interaction at interfaces. Part I. Atomic force microscopy. *Biochim.* **83**: 387-97, 2001.
4. Bradford M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt.Biochem.* **72**: 248-54, 1976.
5. Brzozowski A.M., Derewenda U., Derewenda Z.S., Dodson G.G., Lawson D.M., Turkenburg J.P., Bjorkling F., Huge-Jensen B., Patkar S.A., Thin L. *Nature* **351**: 491, 1991.
6. Cho S.W., Rhee S.J. Immobilization of lipase for effective interesterification of fats and oils in organic solvent. *Biotechnol.Bioeng.* **41**: 204-10, 1993.
7. Crank J. *The mathematics of diffusion*. Oxford: Oxford University Press, 1975.
8. Guo Z., Bai S., Sun Y. Preparation and characterization of immobilized lipase on magnetic hydrophobic microspheres. *Enz.Microb.Technol.* **32**: 776-82, 2003.
9. Grasdalen H., Larsen B., Smidsrod O. ¹³C-NMR studies of monomeric composition and sequence in alginate. *Carbohydr.Res.* **89**: 179-01, 1981.
10. Gotor V. Non-conventional hydrolase chemistry: amide and carbamate bond formation catalyzed by lipases. *Bioorg.Med.Chem.* **7**: 2189-97, 1999.
11. Hashimoto S., Furukawa K. Immobilization of activated sludge by PVA-boric acid method. *Biotechnol.Bioeng.* **30**: 52-9, 1987.
12. Imai K., Shiomi T., Uchida K., Miya M. Immobilization of enzyme into poly(vinyl alcohol) membrane. *Biotechnol.Bioeng.* **28**: 1721-26, 1986.
13. Ishizu K. Synthesis and structural ordering of core-shell polymer microspheres. *Prog.Polym.Sci.* **23**: 1383-1408, 1998.
14. Kazlauskas R.J. Elucidating structure-mechanism relationships in lipases: Prospects for predicting and engineering catalytic properties. *TIBTECH* **12**: 464-72, 1994.
15. King A .H. Brown seaweed extracts (alginates). In: *Food hydrocolloids*. Glicksman M. (Ed.). Boca Raton (Florida), CRC Press, 1983. pp. 115-88.
16. Khoo K.M., Ting Y.P. Biosorption of gold by immobilized fungal biomass. *Biochem.J.* **8**: 51-9, 2001.
17. Leenen E.J.T.M., Dos Santos V.A.P., Grolle C.F., Tramper J., Wijffels R.H. Characteristics and selection criteria for support materials for cell immobilization in wastewater treatment. *Water Res.* **30**: 2985-96, 1996.
18. Liu Y., Yu T. Polymers and enzyme biosensors. *J.Macromol.Sci.-Rev.Macromol.Chem.Phys.* **C37**(3): 459-500, 1997.

19. Magdassi S., Vinetsky Y. In: Benita S. (Ed.), **Microencapsulation-methods and industrial applications**. M. Dekker, New York, 1996. p. 21.
20. Miura K., Kimura N., Suzuki H., Miyashita Y., Nishio Y. Thermal and viscoelastic properties of alginate/poly(vinyl alcohol) blends cross-linked with calcium tetraborate. **Carbohydr.Polym.** **39**: 139-44, 1999.
21. Moe S.T., Draget K.I., Break G.S., Smidsrod O. Alginates. In: Stephen A.M. **Food polysaccharides and their applications**. Marcel Dekker, New York, 1995. pp. 245-86.
22. Paiva A.L., Malcata F.X. Integration of reaction and separation with lipases: an overview. **J.Molec.Catal. B: Enzym.** **3**: 99-109, 1997.
23. Pavanetto F., Conti B., Genta I., Giunchedi P. Solvent evaporation, solvent extraction and spray drying for polylactide microsphere preparation. **Int.J.Pharm.** **84**: 151-9, 1992.
24. Saito R., Okamura S., Ishizu K. Synthesis of poly(vinyl alcohol) core-polystyrene Shell type microspheres. **Polym.** **36**(23): 4515-20, 1995.
25. Santaniello E., Ferraboschi P., Grisenti P. Lipase-catalyzed transesterification in organic solvents: applications to the preparation of enantiomerically pure compounds. **Enzym.Microb.Technol.** **15**: 367-82, 1993.
26. Shindo S., Kamimura M. Immobilization of yeast with hollow PVA gel beads. **J.Ferment.Bioeng.** **70**(4): 232-34, 1990.
27. Uhlich T., Ulbricht M., Tomaschewski G. Immobilization of enzymes in photochemically cross-linked poly(vinyl alcohol). **Enz.Microb.Technol.** **19**: 124-31, 1999.
28. Valivety R.H., Halling P.J., Peilow A .D., Macrae A .R. Lipases from different sources vary widely in dependence of catalytic activity on water activity. **Biochim.Biophys.Acta** **1122**: 143-6, 1992.
29. Villeneuve P., Muderhwa J.M., Graille J., Haas M.J. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. **J.Molec.Catalys. B: Enzym.** **9**: 113-48, 2000.
30. Wen S., Xiaonan Y., Stevenson W.T.K. Microcapsules through polymer complexation. **Biomaterials** **12**: 374-84, 1991.
31. Wu K.A., Wisecarver K.D. Cell immobilization using PVA crosslinked with boric acid. **Biotechnol.Bioeng.** **39**: 447-49, 1992.
32. Yonese M., Baba K., Kishimoto H. Viscoelastic properties of poly(vinyl alcohol)/alginate snake-cage hydrogels and interpenetrating hydrogels. **Polym.J.** **24**: 395-404, 1992.
33. Yahya A .R.M., Anderson W.A., Moo-Young M. Ester synthesis in lipase-catalyzed reactions. **Enzym.Microb.Technol.** **23**: 438-50, 1998.