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# Characterization of Sol-Gel Bioencapsulates for Ester Hydrolysis and Synthesis

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## Abstract

*Candida rugosa* lipase was entrapped in silica sol-gel particles prepared by hydrolysis of methyltrimethoxysilane and assayed by *p*-nitrophenyl palmitate hydrolysis, as a function of pH and temperature, giving pH optima of 7.8 (free enzyme) and 5.0–8.0 (immobilized enzyme). The optimum temperature for the immobilized enzyme (50–55°C) was 19°C higher than for the free enzyme. Thermal, operational, and storage stability were determined with *n*-butanol and butyric acid, giving at 45°C a half-life 2.7 times greater for the immobilized enzyme; storage time was 21 d at room temperature. For ester synthesis, the optimum temperature was 47°C, and high esterification conversions were obtained under repeated batch cycles (half-life of 138 h).

**Index Entries:** Thermal stability; sol-gel; immobilization; lipase; encapsulation.

## Introduction

Lipases have proved to be versatile and efficient biocatalysts, which can be used in an ample variety of reactions such as esterification, interesterification, and hydrolysis *(1,2)*. Although most lipases are more robust than many other enzymes, their industrial use as catalysts in synthetic organic chemistry has not reached a significant level *(3,4)*. This may be owing to their limited long-term stability, difficulties in separating the products from the lipase, and problems arising on reusing the biocatalyst. To solve these drawbacks, a number of different strategies have been developed *(3,5)*.

Many different methods have been described in this area *(5,6)*, and some researchers have suggested that hydrophobic materials are the most suitable supports for immobilization of lipase *(7,8)*, preferably by

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encapsulation within a polymer matrix, or silica, obtained by sol-gel techniques *(3,9,10)*.

The sol-gel process involves the transition of a system from a liquid "sol" (mostly colloidal) into a solid "gel" phase. By applying the sol-gel process, it is possible to fabricate ceramic or glass materials in a wide variety of forms: ultrafine or spherically shaped powders, thin film coatings, ceramic fibres, microporous inorganic membranes, monolithic ceramics and glasses, or extremely porous aerogel materials *(2,3,9,10)*.

In recent years, entrapment of enzymes in silica glasses has generated much interest, and the state of the art has been reviewed recently *(3,10)*. This technique is based on the sol-gel approach to generate silica matrices by acid- or base-catalyzed hydrolysis of hydrolyzable silane compounds such as tetraethyl orthosilicate (TEOS), methyltrimethoxysilane (MTMS), and tetramethoxysilane (TMOS) *(2,3,9,10)*.

This approach has several advantages because mechanical entrapment of enzymes using sol-gel materials allows stabilization of the proteins tertiary structure caused by the tight gel network *(3)*. Moreover, good results were obtained in a number of studies in which enzyme entrapment concerned the use of inorganic matrices such as silica gel *(3,11–14)*.

Despite the success of the sol-gel process, the fact that some proteins defy attempts to encapsulate them, coupled with the wide variation in reported activity for those that are entrapped, only highlights the complex nature of the biomolecules. It must be appreciated that biologic molecules are not "generic" but form numerous classes of diverse compounds, which not only differ in their function but also in their structure and environment in vivo. Because lipases are interphase-active enzymes with hydrophobic domains, it is also important to consider their structure and mode of catalytic action *(15)*. In certain types of lipases, it is the movement of short α-helical loops that uncovers the active site (lid mechanic) *(15,16)*. Another important parameter for lipases is the presence of an aqueous-lipid interface in the reaction medium, because its absence may cause modification of the hydration state of the enzyme, resulting in partial or total inhibition of enzyme activity *(10,17)*.

Pursuing our interest in the immobilization and subsequent use of lipases *(3,10)*, we investigated the feasibility of encapsulating microbial lipases in hydrophobic matrices obtained by the sol-gel process. The enzyme used was nonspecific lipase from *Candida rugosa*, and silicate sol-gel particles were prepared by acid- or base-catalyzed hydrolysis of silane compounds such as TEOS, MTMS, and poly(dimethylsiloxane) (PDMS) in the presence and absence of additives, such as polyethylene glycol (PEG) and polvinyl alcohol *(18,19)*. Based on these results, it appears that the entrapment and subsequent inorganic polymerization of TEOS or MTMS do affect the structure of the guest protein in a significant manner, or impose a diffusion phenomenon in both hydrolysis and esterification reactions.

Although systematic studies have been performed addressing questions related to the structure, morphology, and pore size of these hydrophobic sol-gel materials *(18,19)*, here we provide data on properties relevant for the practical use of *C. rugosa* lipase immobilized in silica sol-gel particles, prepared by hydrolysis of MTMS in aqueous and nonaqueous media. Comparative studies of free and immobilized lipase were carried out as a function of pH, temperature, and thermal stability. Enzymatic hydrolysis with the immobilized enzyme in the framework of the Michaelis-Menten mechanism is also reported. Thermoporometry analysis was used to obtain a more complete characterization of the support and biocatalyst porous structures.

## Materials and Methods

### *Lipase and Chemicals*

Commercial *C. rugosa* lipase (Type VII) was purchased from Sigma (St. Louis, MO). This lipase is substantially free of α-amylase and protease and contains lactose as an extender. Nominal specific lipase activity was 104.94 U/mg of protein. PEG (molecular mass: 1450 Daltons) (Merck, Darmstadt, Germany) was used as stabilizing agent. The silane precursor MTMS was supplied by Across Organic (N J) and used without further purification. Gum arabic, 2-propanol, heptane (all from Synth, Brazil), *p*-nitrophenyl palmitate (*p*-NPP), Triton X-100 (both from Sigma, St. Louis, MO), butanol (Merck), and butyric acid (Riedel-de Häen, Germany) were reagent grade. Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

### *Encapsulation of Lipase in Sol-Gel Matrix Using MTMS*

A modification of a published method *(10)* was used. *Candida rugosa* lipase was suspended in water (13.55 mg/mL), shaken for 15 min, and centrifuged to remove insoluble components. Ten milliliters of the supernatant of the lipase solution was added to a mixture containing aqueous sodium fluoride (1 *M*, 1 mL added) and PEG (4% [w/w] in water, 2 mL added) and water (1.64 mL). The solution was shaken and MTMS (8.57 mL) was added. The reaction mixture was vigorously shaken for 5 s on a vortex mixer and then gently shaken by hand. After 30 s, when the mixture formed a clear homogeneous solution and warmed up, it was placed in an ice bath until gelation occurred (1 h). The reaction vessel was left to stand closed for 24 h, then it was opened, and the gel was dried under vacuum at room temperature for 3 d. The dried gels were crushed to a powder with a particle size in the range of 180–250 μm.

### Differential Scanning Calorimeter Measurements

Pore diameter was determined for sol-gel matrix samples (support and immobilized lipase) by the thermoporometry method according to a modification described by Iza et al. *(20)*. The shift in freezing and melting temperatures of water held in mesoporous material was determined by differential scanning calorimeter (DSC) measurements. The DSC apparatus was a Perkin-Elmer DSC7 equipped with a liquid nitrogen–cooling accessory and calibration with indium. A sample of about 10–20 mg was put in a sealable aluminum pan, and one drop of the solvent was added to maintain the sample in an excess of solvent. Care was taken to avoid the undercooling effect by using the following procedure: The sample was first cooled below the freezing temperature of the pure liquid to –30°C and then heated up and kept at –20°C for 10 min. Thereafter, the pan was cooled down to –30°C at a rate of 1°C/min, which is slow enough to maintain thermodynamic equilibrium *(20)*.

### Scanning Electron Microscopy

Structural integrity and conformational changes, such as surface cavities in the support, set in by the lipase encapsulating procedure were observed by scanning electron microscopy (SEM) on a Leica LEO 440i microscope.

### Lipase Assay in Aqueous Medium

Hydrolytic activity was measured with emulsified *p*-NPP according to Kordel et al. *(21)*. One volume of a 16.5 m*M* solution of *p*-NPP in 2-propanol was mixed just before use with 9 vol. of 100 m*M* phosphate buffer, pH 7.0, containing 0.4% (w/v) Triton X-100 and 0.1% (w/v) gum arabic. Then, 2.7 mL of this mixture was preequilibrated at 37°C in a 1-mL cuvet of an ultraviolet-visible spectrophotometer (Varian UV-Carry; Varian). The reaction was started by the addition of 0.3 mL of enzyme solution at an appropriate dilution in 100 m*M* phosphate buffer, pH 7.0. The variation in the absorbance at 410 nm of the assay against a blank without enzyme was monitored for 2–5 min. Reaction rate was calculated from the slope of the absorbance curve vs time by using a molar extinction coefficient of $13 \times 10^6$ $cm^2/mol$ for *p*-nitrophenol. This value was determined from the absorbance of standard solutions of *p*-NPP in the reaction mixture. One enzyme unit was the amount of enzyme liberating 1 μmol of *p*-nitrophenol/min in the aforementioned conditions.

### Lipase Assay in Organic Medium

Reaction systems consisted of heptane (20 mL), *n*-butanol (114 m*M*), butyric acid (114 m*M*), and immobilized lipase in hydrophobic sol-gel matrices (0.5 g, dry wt). The mixture was incubated at 37°C for 8 h with continuous agitation at 150 rpm. Reactions were monitored by measuring

reactants and product concentrations by gas chromatography using a 6-ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA), and hexanol was used as an internal standard. Water concentrations in the liquid and solid phases were measured by the Karl Fischer method (DL 18, Mettler). Fatty acid concentrations were titrated with 0.02 *M* potassium hydroxide solutions with phenolphthalein as an indicator. Esterification activity was expressed as micromoles of butyl butyrate formed per minute per gram of dry support.

### *Catalytic Properties of Free and Immobilized Lipase in Aqueous Medium*

Free and immobilized hydrolytic activities were estimated with reaction mixtures containing 100 m*M* sodium phosphate buffer at different pH values in the range of 5.0–9.5 at 37°C. The effect of temperature on lipase activity was determined at temperatures from 30 to 60°C for the free and immobilized enzyme. For determination of thermal stability, both free and immobilized lipase preparations were incubated in sodium phosphate buffer (pH 7.0 and 7.5, respectively) at different temperatures (40–60°C) for 1 h. Samples were removed and assayed for residual activity as previously described (see Lipase Assay in Aqueous Medium); an unheated control was considered to be 100% active. The hydrolytic activity was measured as a function of long-term storage time at 4°C in aqueous medium for 2 mo.

### *Operational Stability of Immobilized Lipase*

The operational stability of the immobilized preparation was assayed for the hydrolysis of *p*-NPP and the synthesis of butyl butyrate in successive 24-h batches. At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with phosphate buffer or hexane to remove any substrate or product retained in the matrix. Then, the immobilized lipase was introduced into a fresh medium. The concentration of the reagents and product, and the residual enzyme activity, were determined at the end of each cycle. The biocatalyst half-life ($t_{1/2}$) was determined by applying the exponential decay model *(22)*.

## Results and Discussion

### *Surface Area, Pore Volume, and Pore Diameter*

In a previous work *(19)*, the mean pore diameter for sol-gel matrices obtained with MTMS as precursor was found to be 59.08 and 784.70 Å for the pure support and encapsulated lipase, respectively. In a representative study, Amano pure silica–containing gels derived from TMOS/MTMS (1:1) as well as the gel produced from TMOS alone were investigated. In this case, larger pore volumes were obtained *(3)*, compared with pore silica–containing gels derived from MTMS *(19)*. Mean pore diameter results indicated that

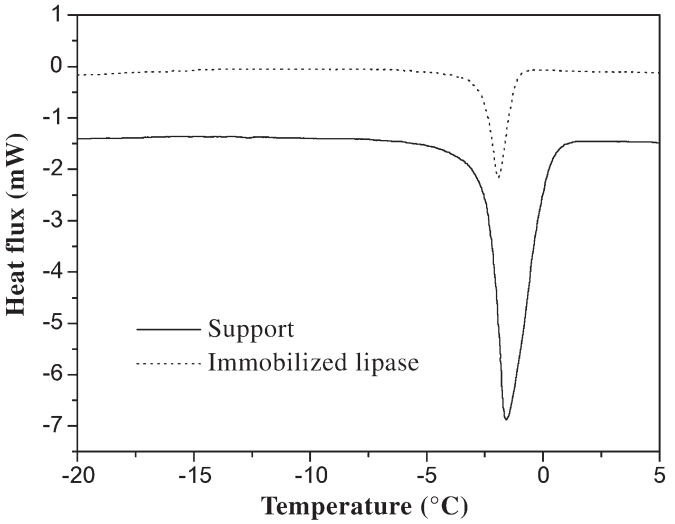


**Fig. 1.** Thermoporometry curves for silica gel and immobilized lipase.

the addition of an organic macromolecule additive (PEG) on the immobilized lipase increased the mean pore diameter of the immobilized enzyme matrix and that this was associated mainly with the inhibition of gel contraction during synthesis of the derivative. It should be pointed out, however, that the very small pore volume obtained for the MTMS is conducive to incorrect determinations of mean pore diameter. Consequently, results for the mean pore diameter for MTMS pure silica and immobilized lipase were taken cautiously *(19)*. Nevertheless, Brunauer, Emmet and Teller method (BET) is an accepted methodology to determine porous structure parameters with sol-gel materials, and sometimes thermoporometry can be an alternative method allowing better characterization of porous fragile and soft materials and hydrogels *(20)*. To reduce uncertainty in the determination of mean pore diameter with the MTMS materials, we decided to use thermoporometry methodology *(20)*. The solidification thermograms recorded for sol-gel saturated with water are given in Fig. 1, which represents the curves obtained for the immobilized lipase and pure silica. Solidification peaks at about 0°C were attributed to freezing of the excess bulk water. Temperature peaks corresponding to solidification of water inside the pore were detected between 0 and –5°C. From the solidification thermogram, the pore radius distribution curve was determined. Pore diameters obtained by DSC thermogram curves (Fig. 1) and Eq. 1, which represents the variation of pore diameter (Dp) with triple point temperature in a porous material saturated with water ($\Delta T = T - T_0$), were found to be 3.04 and 4.12 Å for pure silica and immobilized lipase, respectively.

$$Dp\ (\text{Å}) = 0.2 \times \left[ \left( \frac{64.67}{\Delta T} \right) + 0.57 \right] \quad (1)$$

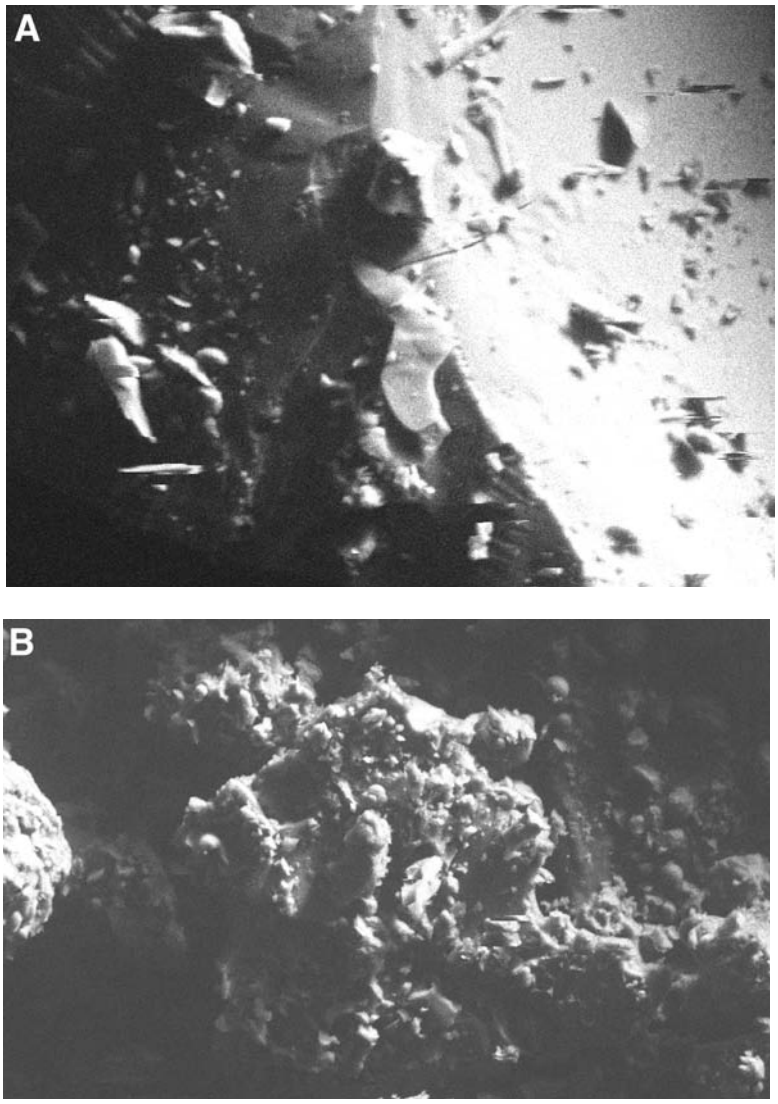


**Fig. 2**. SEM showing **(A)** MTMS gel support in absence of enzyme and **(B)** immobilized enzyme preparation obtained by entrapping *C. rugosa* lipase in MTMS gel.

## *Morphology*

SEM for the MTMS gel support (Fig. 2A) and for the *C. rugosa* lipase entrapped in the MTMS gel (Fig. 2B) showed that the surface of the pure silica had extremely low porosity or practically no porous structure, whereas the immobilized lipase preparation using MTMS as precursor utilizing PEG showed an irregular surface similar to that obtained by Reetz and colleagues *(3,10).* The porous and lipophilic character of the lipase-containing gels (Fig. 2B) results in a high activity for ester synthesis. The SEM studies provide information regarding only the general morphology

of the particles, not the actual conformation of the immobilized lipase. Further investigations to better characterize the immobilized lipase molecule center in the gel need to be carried out using special techniques, such as Si solid-state nuclear magnetic resonance (NMR) spectroscopy, C solid-state NMR spectroscopy, and X-ray powder diffraction *(7,3,10)*.

### *Characterization of Immobilized Lipase for Utilization in Aqueous Medium*

Our previous studies on the catalytic activity of the hydrolysis of an immobilized lipase preparation of olive oil *(18,19)* showed that the hydrolysis reaction was diffusion controlled for the gel prepared with MTMS as precursor, owing to the small pore size of the samples and consequent substrate diffusion limitations. In addition, aggregation of the enzyme molecules might occur at very high enzyme concentrations, resulting in a lower degree of enzyme dispersion in the gel matrix. When enzyme is immobilized within a porous support, in addition to possible external mass-transfer effects, there could also exist resistance to internal diffusion of substrate, because this must diffuse through the pores in order to reach the biocatalyst. The internal mass-transfer effects can be reduced, however, by decreasing the particle dimensions of the porous support containing the biocatalyst. A decrease in particle diameter results in a reduction in the distance from the outer support surface that the substrate must cross and, consequently, in a decrease in the substrate concentration gradient *(23)*. For the present work, we decided to use a small particle size and a substrate with lower molecular weight, *p*-NPP, and better results were obtained than with olive oil as substrate. Despite the small pore size, penetration of the substrate was achieved because the reported pore size is an average value and certainly larger pores than the average size were available for substrate diffusion.

The pH and temperature profiles of hydrolytic activities are shown in Figs. 3 and 4, respectively. The pH optimum shifted from 7.8 for the free enzyme to about 5.0–8.0 for the immobilized lipase (Fig. 3). The optimum temperature for the immobilized lipase (55°C) was 19°C higher than for the soluble enzyme (37°C), as shown in Fig. 4. The patterns of heat stability indicate that the immobilization process tends to stabilize the enzyme, as described in the literature *(7,22)*.

Table 1 provides the half-life and denaturation rate of the enzyme preparations. The results of the thermal stability tests confirmed the usual trend of the immobilization process, which tends to stabilize the enzyme. At 45°C, the thermal stability, measured as the ratio of the enzyme preparation half-life to the free enzyme half-life, was increased 1.73-fold. The free *C. rugosa* lipase showed a half-life of 0.41 h at 55°C, and at this temperature it maintained only about 20% of its original activity after 1 h, whereas the half-life of the encapsulated lipase at the same temperature was 1.87 h.

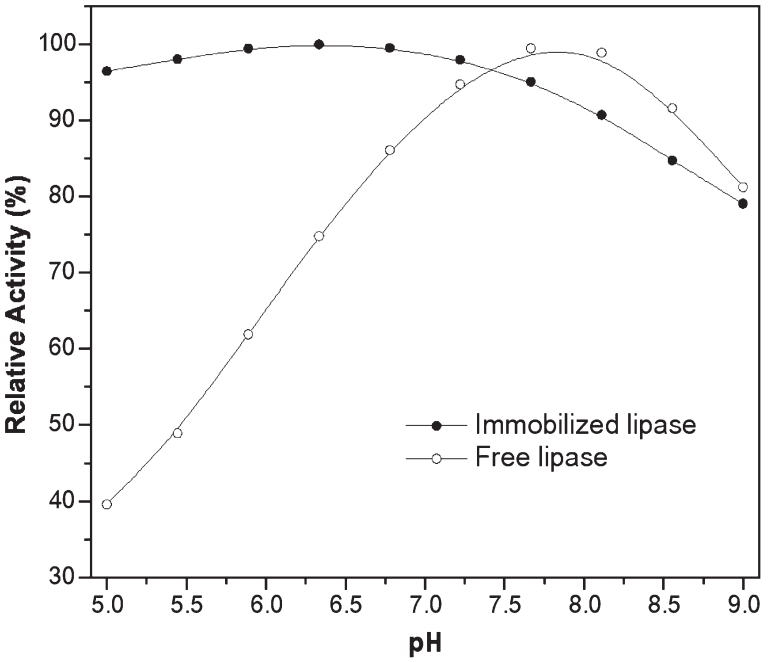


**Fig. 3.** Effect of reaction pH on hydrolytic activities of lipase preparations. Enzymes were assayed with *p*-NPP as substrate at 37°C.

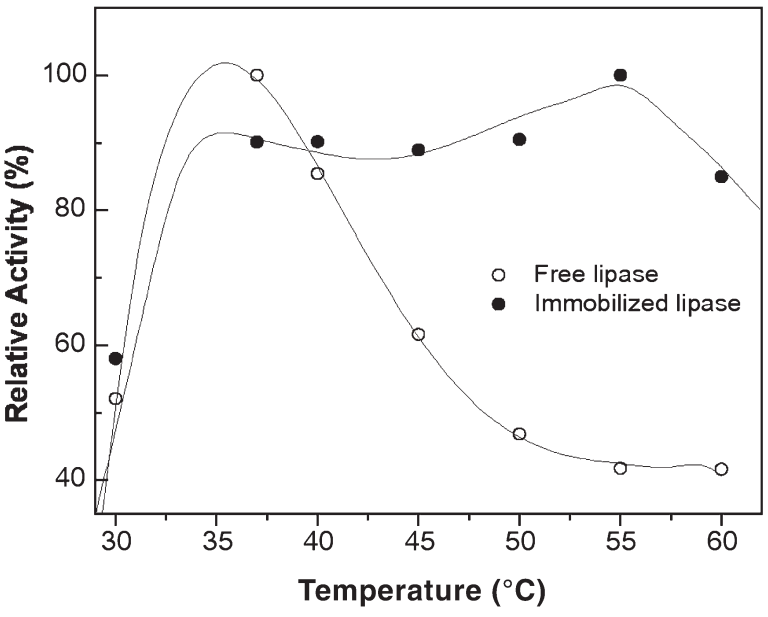


**Fig. 4**. Effect of reaction temperature on hydrolytic activities of lipase preparations. Enzymes were assayed with *p*-NPP as substrate at pH 7.0 and 7.5.

Table 1
Half-life ($t_{1/2}$) and Rate of Denaturation ($k_d$) for free and Immobilized Lipases

| | Rate of denaturation ($k_d$) ($h^{-1}$) | | Half-life ($t_{1/2}$) = $-\ln(1/2)/k_d$ ($h^{-1}$) | |
|---|---|---|---|---|
| Temperature (°C) | Free lipase | Immobilized lipase | Free lipase | Immobilized lipase |
| 45 | 0.52 | 0.30 | 1.33 | 2.29 |
| 50 | 0.98 | 0.37 | 0.71 | 1.87 |
| 55 | 1.69 | 0.37 | 0.41 | 1.87 |
| 60 | 2.45 | 0.37 | 0.28 | 1.87 |

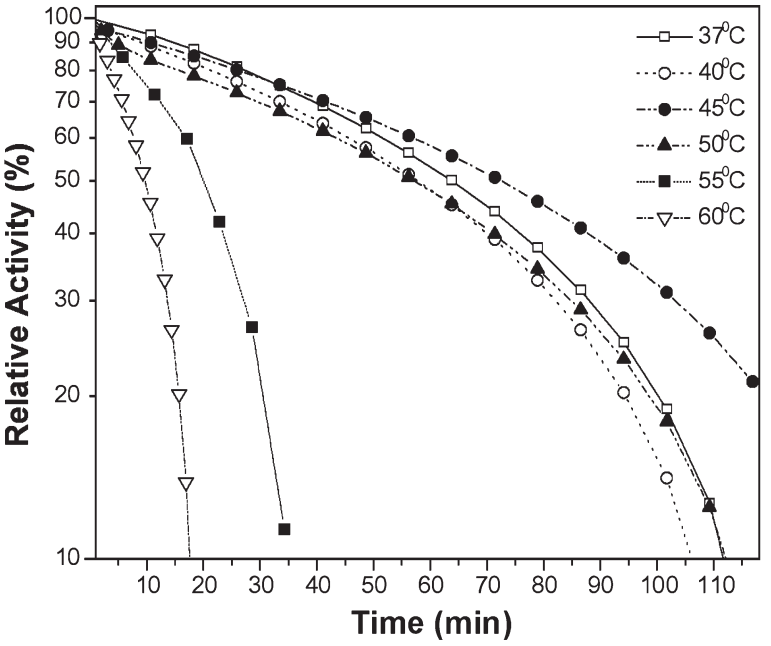


**Fig. 5.** Batch-operation stability tests at different temperatures, 37–60°C, for hydrolysis of *p*-NPP with immobilized lipase.

The immobilized lipase was repeatedly used in batch hydrolysis of *p*-NPP at different temperatures (Fig. 5). The retention of the biocatalyst activity after repeated use was assessed in terms of *p*-nitrophenol formation at the end of each cycle. *C. rugosa* lipase entrapped in silicate sol-gel particles exhibited higher stability in the range of 37–45°C, whereas at 50 and 55°C the immobilized lipase was practically inactivated after 12 recycles.

A higher thermal stability of the encapsulated derivative was also observed by thermogravimetric analysis as described previously *(19)*, in which the lower weight loss found for the encapsulated lipase was attributed to an increase in the thermal stability resulting from interactions between the silica matrix and organic components.

One of the most important aims of enzyme engineering is to enhance the conformational stability of enzymes. Protein stabilization has been achieved by several methods including the encapsulation of *C. rugosa* lipase by the sol-gel method. Immobilization enhanced the conformational stability of lipase as discussed in different articles *(5,7,8,9,22)*.

The influence of substrate concentration on hydrolytic activities was also analyzed for free and immobilized lipase in *p*-NPP solutions varying from 100 to 1000 μ*M* (Fig. 6 A,B) to determine the Michaelis-Menten constant ($K_m$) as the concentration of substrate at which half of the maximum reaction rate ($V_{max}$) is reached *(21)*. Plotting activity vs substrate concentration indicated that both lipase preparations obey the Michaelis-Menten equation, indicating that in the range studied, no inhibition by reaction products was detected. The values obtained for $K_m$ were 0.056 and 0.14 m*M* for the free and immobilized lipase, respectively. The maximum reaction rate ($V_{max}$) for *p*-NPP hydrolysis was 1469.5 μmol/(mg · min) with the free lipase and 1.31 μmol/(mg · min) for the immobilized lipase. For the case of lipase entrapped in the sol-gel matrix, apparent diffusion resistance was observed, as can be seen in Fig. 6, in spite of the small substrate chosen.

To complete the characterization of the encapsulated lipase, the hydrolytic activity was measured as a function of long-term storage time at room temperature, and after 21 d the activity decreased by 50%. Results from the literature show that immobilized lipase from *Rhizomucor miehei* in MTMS or MTMS/PDMS gel systems still retained up to 91% of its activity after storage in aqueous medium for 3 mo *(10)*. By contrast, commercially available *R. miehei* lipase adsorbed onto an ion-exchange resin (Lipozyme IM) retained only 6% of its initial activity under identical conditions *(10)*. Some of the sol-gel-entrapped lipases can be highly stable and may be stored at room temperature for months without loss of activity. For example, this was observed with *Pseudomonas cepacia* in MTMS or MTMS/PDMS gels that had been repeatedly tested in a batch esterification reaction of lauric acid with 1-octanol in isooctane *(10)*.

## *Characterization of Immobilized Lipase for Utilization in Organic Medium*

In organic medium, the immobilized lipase was tested for ester synthesis using a system consisting of *n*-butanol and butyric acid. The effect of temperature on the esterification activity of the immobilized lipase was monitored in the range of 37–60°C, and the final butyl butyrate concentrations after 24 h are given in Table 2. By increasing the reaction temperature, the rate of reaction increased up to 47°C and decreased for higher temperatures. Figure 7 shows high esterification activities under repeated 24-h batch cycles for the immobilized lipase, revealing a half-life of 138 h at 47°C. The increased stability of entrapped lipases might result from improved enzyme

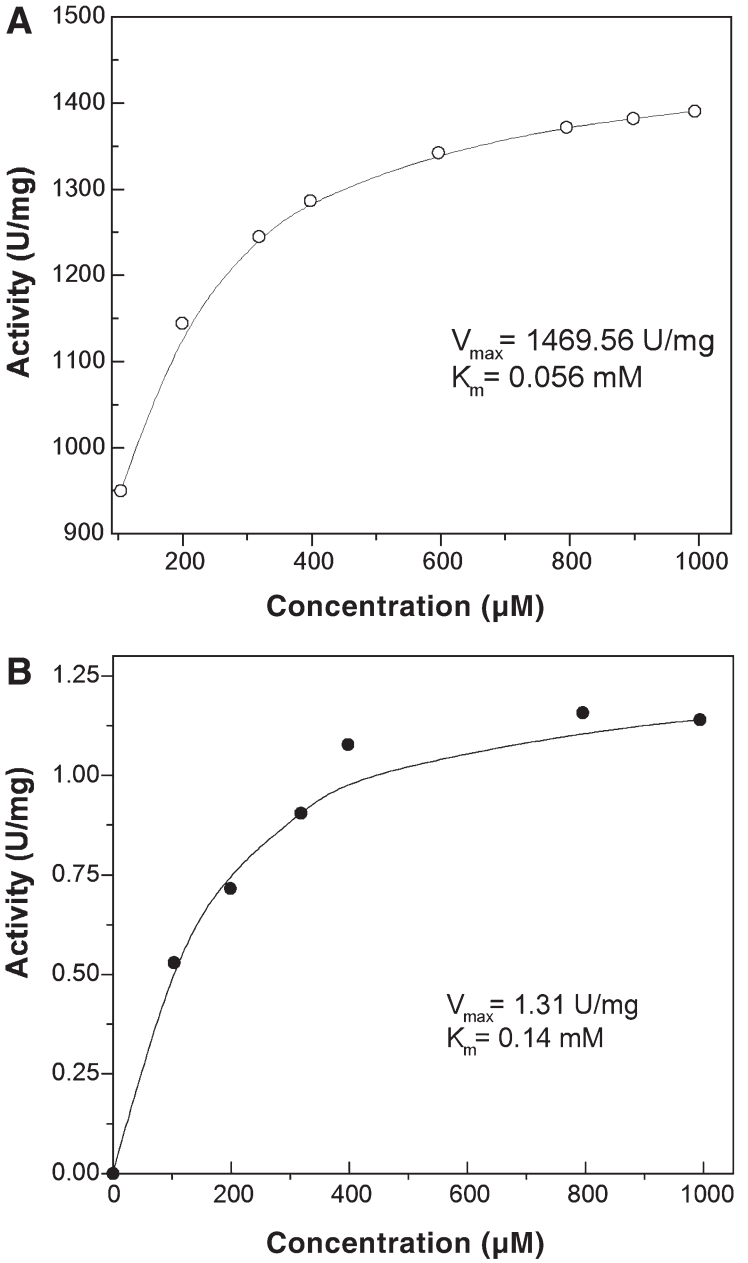


**Fig. 6.** Influence of substrate concentration on hydrolytic activities for **(A)** free lipase and **(B)** immobilized lipase. Reactions were carried out with *p*-NPP at different concentrations at 37°C.

retention compared with adsorption and from stabilizing interactions between lipase and gel matrix. It is not yet clear whether the lipase is simply physically entrapped or whether additional multipoint bonding by covalent, ionic, or hydrophobic interactions occurs *(10,14,18,19).*

Table 2
Effect of Reaction Temperature on Synthesis of Butyl Butyrate Using Lipase Entrapped in Sol-Gel Particles[a]

| Temperature (°C) | Butyric acid (g/L) | *n*-Butanol (g/L) | Butyl butyrate (g/L) |
|---|---|---|---|
| 37 | 4.09 | 2.15 | 11.96 |
| 42 | 2.15 | 2.5 | 11.65 |
| 47 | 0.72 | 1.17 | 15.44 |
| 52 | 2.02 | 2.28 | 10.71 |
| 60 | 3.08 | 2.84 | 8.90 |

[a]Concentrations after 24 h.

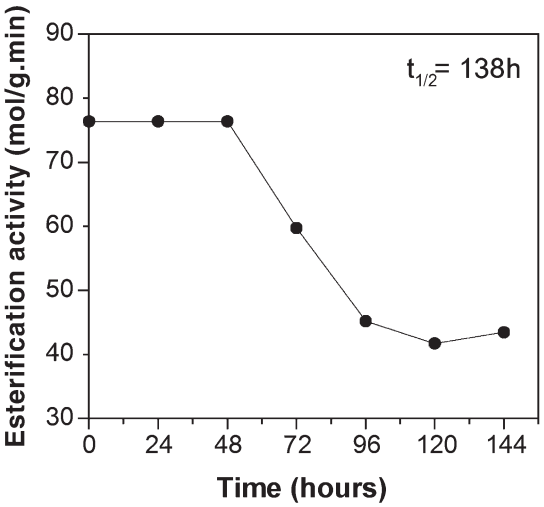


**Fig. 7.** Immobilized lipase batch-operation stability test for esterification reaction at 47°C.

Table 3
Properties of Both Free and Encapsulated *C. rugosa* Lipase in Silica Matrix Obtained by Sol-Gel

| Parameter | Free *C. rugosa* lipase value | Encapsulated *C. rugosa* lipase value |
|---|---|---|
| Optimum pH | 7.8 | 5.0–8.0 |
| Optimum temperature (°C) | 37 | 50–55 |
| Thermal inactivation constant ($k_d$, $h^{-1}$) at 50°C | — | 0.37 |
| $K_m$ (m*M*) | 1469.6 | 0.14 |
| $V_{max}$ (μmol/[mg·min]) | 0.056 | 1.31 |
| Stability tests | | |
| Storage, half-life (d) | — | 21 |
| Hydrolysis reaction, half-life (min) | — | 65.08 |
| Esterification reaction, half-life (h), at 47°C | — | 138 |

Encapsulation of *C. rugosa* lipase by the sol-gel method considerably enhanced its operational stability. We think that the stabilization effect results from the encapsulation in sol-gel protecting the enzyme activity under reaction conditions *(2,10)*. These results confirm the robustness of the encapsulated biocatalyst described in this work, as summarized in Table 3.

## Conclusion

Microbial lipase offers great potential for both hydrolytic and synthetic procedures. The possibilities and benefits of the application of *C. rugosa* lipase in silicate sol-gel particles have not yet been exploited in industrial biotechnology; however, the considerable significance of special mono- and diacylglycerols and carbohydrate fatty acid esters might suggest the application of the immobilized lipase in organic media. In our work, *C. rugosa* lipase was entrapped in silica sol-gel particles prepared by hydrolysis of MTMS. The experimental results show that free lipase had a pH optimum of 7.8 at 37°C, whereas that for immobilized lipase was reduced to 5.0–8.0. The thermal stability of the immobilized lipase increased 1.73-fold in comparison to the free enzyme, at 45°C. Studies on the operational stability of the immobilized derivative revealed good potential for recycling under aqueous and nonaqueous conditions. Our work also demonstrated the usefulness of thermoporometry in probing the structure of sol-gel matrices. In contrast to the classic techniques (such as BET, which underestimates pore size), thermoporometry analysis allowed determination of porous dimensions in the presence of a solvent that swells the material. More work is still necessary in this fast-moving field.

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