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Characterization and Utilization of *Candida rugosa* Lipase Immobilized on Controlled Pore Silica

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Abstract

Candida rugosa lipase was immobilized by covalent binding on controlled pore silica (CPS) using glutaraldehyde as cross-linking agent under aqueous and nonaqueous conditions. The immobilized *C. rugosa* was more active when the coupling procedure was performed in the presence of a nonpolar solvent, hexane. Similar optima pH (7.5–8.0) was found for both free and immobilized lipase. The optimum temperature for the immobilized lipase was about 10°C higher than that for the free lipase. The thermal stability of the CPS lipase was also greater than the original lipase preparation. Studies on the operational stability of CPS lipase revealed good potential for recycling under aqueous (olive-oil hydrolysis) and nonaqueous (butyl butyrate synthesis) conditions.

Index Entries: Lipase; immobilization; controlled pore silica; cross-linking; characterization; hydrolysis; esterification.

Introduction

The high catalytic activity and versatility for carrying out a variety of chemical transformations under mild reaction conditions in a stereoselective manner make the use of enzymes very attractive as industrial catalysts. Biotransformations using lipases, in particular, have enormous potential for several industrial applications owing to their remarkable properties under aqueous and nonaqueous media *(1,2)*.

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Most of the lipase-catalyzed reactions provide interesting alternatives to existing process in the oleochemical and fine chemical industries *(3,4)*. At the moment, lipase account for about 30% of all enzymes cited in the literature and this is expected to grow over the coming years as a wide range of lipase catalysts become available *(5)*. This renders a comparable biotechnology potential with proteases and carbohydrolases—enzymes used in large industry level—stimulating research on the optimization of lipase production, immobilization, and industrial applications.

Among these research lines, our group has dedicated efforts to establish procedures for immobilization of pancreatic and microbial lipases onto several support types because of the advantages of these derivatives, which include the possibility of enzyme reuse, easy product separation, and enzyme-improved stability *(6–8)*. An extensive literature is available on this subject and it covers publications dealing with different techniques of immobilizing lipases, characterization of the activated complex, and application under aqueous and nonaqueous media *(4,9)*. However, the search for the most effective procedure for immobilizing lipases is a complex task because the degree of stabilization depends on the enzyme structure, the immobilization method, and the type of support *(9,10)*. A great variety of natural or synthetic organic and inorganic materials with different characteristics (size, shape, and density) for the immobilization of lipases have been investigated, thus allowing the recovery of immobilized lipases under the form of particles, fibers, or even membranes *(4,9)*. Traditionally, the support material has been regarded as inert and without influence on the kinetic behavior of the biocatalyst. In fact, comparative kinetic studies indicated that differences related to the nature of the supports used for enzyme immobilization *(10–12)*. The data and experience reported in the literature can narrow the choice; however, an empirical approach is still necessary, because the requirements may change from case to case. For example, the advantages of using Celite as a support is clearly manifested as far as the activity and stability of these derivatives are concerned *(13)*. Processing constraints (e.g., low dispersion, low mechanical strength, and high pressure drop) may impose some limitations on the use of this kind of material. According to Ison et al. *(14)*, a packed bed of lipase immobilized on Celite operating in a column with flow of a solvent-free mixture was impractical owing to the high pressure drop. In order to select an immobilization technique for lipase, priority was given to fulfill the basic requirements to prepare a derivative that can be also applied in reactors working on a continuous fashion. Therefore, the suitability of support particle was the major concern. In this context, the work carried out by Zanin and De Moraes *(15)* provided the basis for the final decision. In this work, lipase was covalently attached on controlled pore silica (CPS). The lipase derivatives on CPS were characterized and applied to the synthesis of butyl butyrate.

Materials and Methods

Materials

Commercial *C. rugosa* lipase (Type VII) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis, MO). The lipase was a crude preparation with a nominal specific activity of 835 U/mg and 10% protein based on the Lowry method protein assay *(16)*. CPS was supplied by Corning Glass Works-USA (Corning, NY) and had the following characteristics: average particle porosity (ε), 0.566; particle matrix density (ρs), 2.178 g/cm^3; particle density (dry) (ρp), 0.948g/ cm^3; particle size, 37.5 nm containing pores of 375°A *(15,17)*. The silane γ-aminopropyltriethoxysilane (γ-APTS) and glutaraldehyde (25% solution) came from Sigma. Olive oil (low acidity) was purchased in a local market. Substrates for esterification reactions (n-butanol and butyric acid came from Merck, Darmstadt, Germany) and were dehydrated, with 0.32-cm molecular sieves (aluminum sodium silicate, type 13X BHD Chemicals, Toronto, Canada). Solvents were standard laboratory grade and other reagents were purchased either from Aldrich Chemical Co. (Milwaukee, WI) or Sigma.

Immobilization of Lipase on CPS

Lipase was immobilized by being covalently bound on CPS previously treated with γ-aminopropyltriethoxysilane (γ-APTS), followed by reaction of the pretreated beads with glutaraldehyde solution, according to the procedure described by Zanin and De Moraes *(15)*. Buffer (100 m*M* sodium phosphate buffer, pH 7.0) or hexane were used as a dispersion medium. For each gram of dry CPS, 0.3 g of lipase was used. The enzyme was dissolved in 10 mL of 100 m*M* of sodium phosphate buffer, pH 7.0, and mixed with the support under low stirring during 2 h at room temperature. After this, 10 mL of 100 m*M* sodium phosphate buffer, pH 7.0, or 10 mL hexane was added to the mixture enzyme-support and coupling took place overnight at 4°C. The derivative was filtered (Whatman filter paper No. 41) and thoroughly rinsed with 100 m*M* of sodium phosphate buffer or hexane. The quantitative evaluation of the immobilization procedure was carried out by carbon, hydrogen, and nitrogen contents of the enzyme, support, and immobilized lipase obtained by elemental analyzer (Perkin Elmer CHN 2400, Norwalk, CT). Analyses of the lipolytic activities carried out on initial and spent lipase solutions and immobilized preparations were also used to determine the coupling yield (η%) according to the following expression:

$$\eta\% = \text{Overall activity of the immobilized enzyme} / \times 100 / \text{Overall activity of the initial enzyme solution} \quad (1)$$

Lipolytic Activities

The activities of free and immobilized lipase were assayed by the olive-oil emulsion method *(18)*. The substrate was prepared by mixing 50 mL of the olive oil with 50 mL of emulsification reagent. The reaction mixture

consisting of 5 mL of the emulsion, 2 mL of 100 m*M* sodium phosphate buffer, pH 7.0, and either free (1 mL of lipase, 5 mg/mL) or immobilized (100–250 mg) lipase was incubated for 10 or 30 min at 37°C, respectively. The reaction was stopped by addition of 10 mL of acetone-ethanol solution (1:1). The liberated fatty acid was titrated with 25 m*M* potassium hydroxide solution in the presence of phenolphthalein as indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid/min under the assay conditions. Estimation of free and immobilized lipolytic actives at different pH values were carried out with reaction mixtures containing 100 m*M* of the phosphate buffer at pH in the range of 6.0–9.0 at 37°C. The effect of temperature on both lipase activities was determined from 40–60°C under the assay conditions.

Protein Assay

Protein was determined according to Lowry et al. *(17)* using bovine serum albumin (BSA) as a 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 in the filtrate and in the washing solutions.

Butyl Butyrate Synthesis

Ester synthesis was carried out in 20 mL of dry n-heptane containing 200 m*M* n-butanol, 250 m*M* butyric acid, and 25 mg/mL of immobilized lipase, except where noted. The mixture was incubated at 37°C with reciprocating agitation (150 rpm). The inhibitory effect of butyric-acid concentration on the esterification rate was studied by varying the initial acid concentration between 100 m*M* and 1000 m*M*, while keeping the initial butanol concentration at 200 m*M*. All the esterification reactions were run in parallel with blank experiments. These were conducted under similar conditions by using the same mass of support without enzyme, in order to assess the extent of substrate and/ or solvent evaporation.

Stability Measurements of the Immobilized Preparations

For determination of thermal stability, either free or CPS lipase preparations were incubated in 2 mL buffer or heptane at different temperatures at 40°, 50°, and 60°C for 1 h. Samples were withdrawn and assayed for residual activity as previously described, taking an unheated control as 100% active. The operational stability of the immobilized enzyme was assayed by using 0.5 g of immobilized lipase in successive batches carried out under the same conditions as described under butyl butyrate synthesis. Twenty-four hours after starting each batch, the immobilized lipase was removed from the reaction medium and rinsed with hexane, in order to extract any substrate or product eventually retained in the matrix. One hour later (length of time required for the solvent to evaporate) the immobilized derivative was introduced in a fresh medium.

Table 1
Covalent Immobilization of *Candida rugosa*
Using CPS and Different Dispersion Media

Dispersion media	Activity offered for immobilization (U)	Unbound activity (U)	Immobilized activity (U)		Immobilization yield (%) ([B/A] × 100)
			Theoretical (A)	Actual (B)	
Buffer	348	71.8	276.52	22.52	8.14
Hexane	348	69.64	279.08	51.24	18.36

Esterification Controls

The esterification reactions were monitored by measuring butanol and butyl butyrate concentrations by gas chromatography (GC) using a 6-ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA), and hexanol as internal standard. Water concentrations in the liquid and solid phases were measured by Karl Fischer potentiometric titration using a DL 18 Moisture Mettler (Zurich, Switzerland). The partition coefficients (support/ external organic solvent) of butanol and butyric acid were estimated according to Eq. 2 *(7)*.

$$P = [(C_0 - C)/C] \times [V_0/(V - V_0)] \quad (2)$$

where: P = partition coefficient, C_0 = initial concentration of compound, C = equilibrium concentration of compound, V_0 = total volume of system (organic phase + support), V = volume of organic phase. Partition experiments were conducted under the same conditions as their reaction counterparts. In order to estimate the support volume (V – Vo), a calibration curve of volume silica vs mass of the silica was established (volume of matrix [cm^3] = 0.829 × mass of silica (g) – 0.0835; correlation coefficient = 0.995).

Results

Immobilization of Lipase on CPS

Table 1 summarizes the results for the covalent immobilization of lipase using CPS. In this method, glutaraldehyde was used to activate the aminogroups on CPS and then to cross-link the enzyme to the support. Two types of dispersion media were used for immobilizing lipase and best results with respect to the recovery of total activity after immobilization were obtained using hexane. The immobilization yield of enzyme-support complex was also assessed by C, H, N mass balance determined by elemental analyzer with typical results as shown in Table 2. Our results clearly indicated that the immobilized lipase prepared by using hexane is superior than the corresponding preparation in buffer, not only with regard to the immobilized yield (protein retention of 60% and immobilization efficiency

Table 2
Mass Balance of Lipase Immobilized on CPS

Material	C (%)	H (%)	N (%)
Silica	0.27	0.08	0.06
Free lipase	25.04	4.88	2.64
CPS-lipase (buffer)	0.76	0.26	0.09
CPS-lipase (hexane)	3.91	0.78	0.42

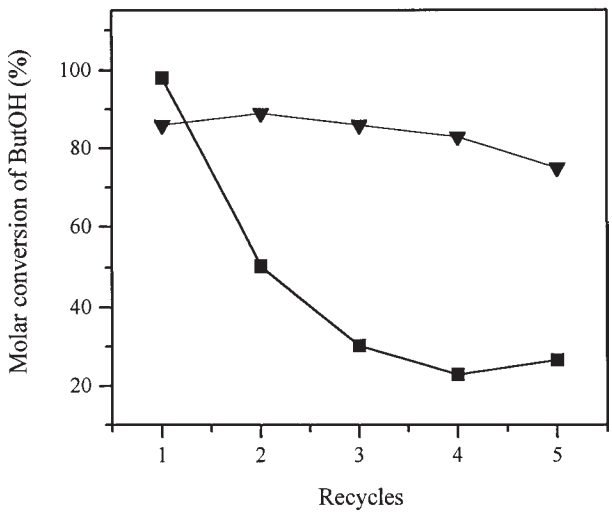

Fig. 1. Batch-operation stability test of the CPS-immobilized lipase preparations. Initial medium composition 200 m*M* butanol and 250 m*M* butyric acid in heptane. (■) CPS lipase on buffer; (▲) CPS lipase on hexane.

of 18%) but also in relation to its stability under operational conditions. As shown in Fig. 1, the lipase immobilized on CPS from buffer was initially active but gradually lost activity, whereas the immobilized derivative from hexane gave a stable enzyme preparation, and high conversions could be maintained for five sequential batch reactions. Given its satisfactory performance under repeated batch use, the later immobilized preparation was characterized and applied to the synthesis of butyl butyrate.

General Characteristics of CPS Immobilized Derivative from Hexane

A comparative study between free and immobilized lipase is provided in terms of pH, temperature, thermal stability, and operational stability. The pH and temperature profiles of lipolytic activities are shown in Figs. 2 and 3, respectively. Slightly lower value for optimum pH was found for the immobilized form in comparison with that displayed by the free lipase (Fig. 2). The optimal reaction temperature shifted from 40°C for the

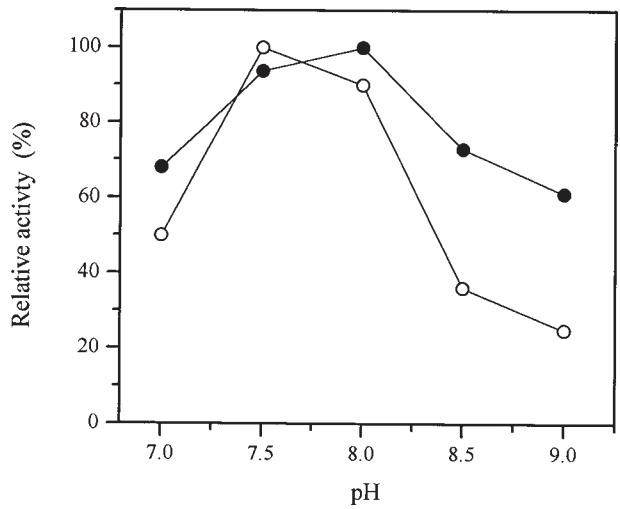

Fig. 2. Effect of reaction pH on the lipolytic activities of lipase preparations. The enzyme was assayed with olive-oil emulsion as substrate at 37°C; (●) free lipase, (○) CPS lipase.

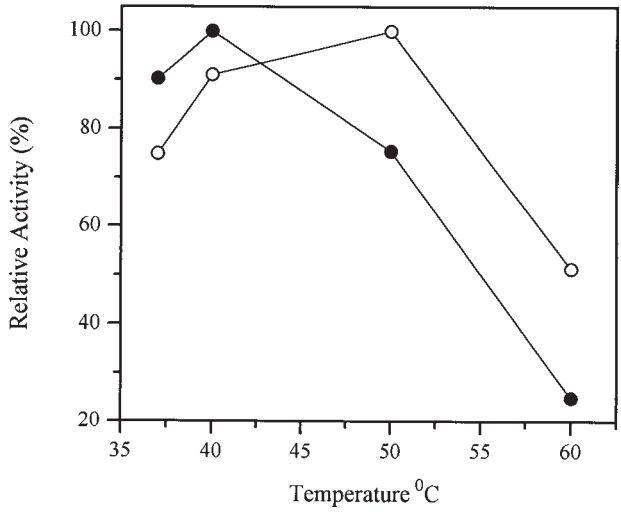

Fig. 3. Effect of reaction temperature on the lipolytic activities of the lipase preparations. The enzymes were assayed with oil emulsion as substrate at pH 7.5; (●) free lipase, (○) CPS lipase.

free lipase to 50°C for the CPS lipase. These results indicate that lipase is more stable when immobilized in the matrix. This hypothesis was further substantiated by the observation that greater thermal stability was also found with CPS lipase. The residual activities for both enzymatic preparations after incubation in the temperature range 40–60°C at pH 7.0 in 100 m*M*

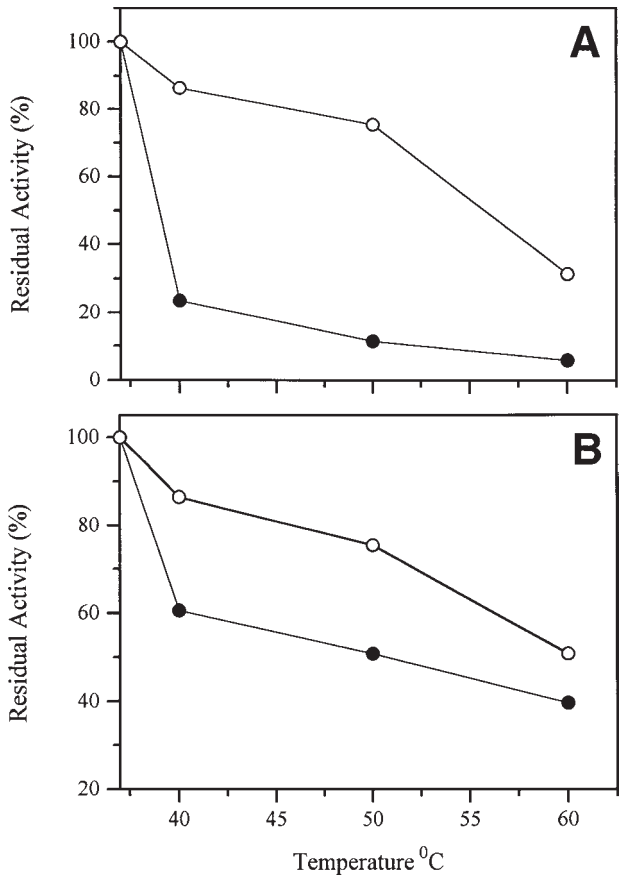

Fig. 4. Temperature inactivation of free (●) and CPS (○) lipase at different temperatures. Experiments were carried out in 100 m*M* phosphate buffer, pH 7.5, and heptane. The starting activities (free lipase, 800 U/mg; CPS lipase, 21.0 U/mg) were taken as 100%.

phosphate buffer and heptane for a period of 1 h are shown in Fig. 4. Under both conditions, the CPS lipase exhibited higher stabilities against heat than the soluble one. In the presence of buffer, while the soluble enzyme was practically inactivated during 1 h incubation, in buffer at 60°C the CPS lipase preserved about 40% of its original activity. The patterns of heat stability for the lipase preparations, in heptane, were also quite different and the residual activities differed by at least 25% in any of the tested temperatures on behalf of the CPS lipase. The operational stability of the CPS lipase was further tested. This was done by repeated assays (olive-oil hydrolysis) and after 20 batches (30 min/37°C), 70% of the initial CPS lipase activity was preservable (Fig. 5). Furthermore, significant activity loss was no longer noticeable after the 15th cycle. The general characteristics of immobilized lipase on CPS using hexane as a dispersion medium are summarized in Table 3.

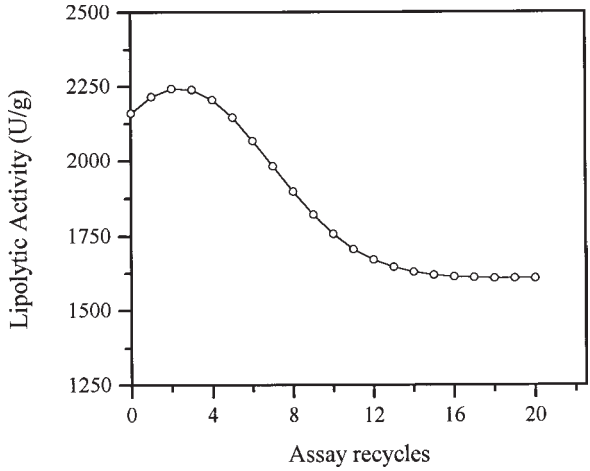

Fig. 5. Effect of repeated assays on the activity of CPS immobilized lipase from hexane. Incubation system, 5 mL of olive-oil emulsion; 3 mL of 0.1 *M* phosphate buffer, pH 7.5, and 0.5 g of CPS lipase; temperature 37°C, for 30 min.

Table 3
Characterization of CPS Immobilized Lipase
Using Hexane as the Dispersion Medium

Parameters	Established conditions
Carrier type	Controlled pore silica
Method of immobilization	Covalently bound
Yield of bound activity (%)	18.4
Lipolytic activity of bound enzyme (U/mg dry weight)	51.1
Water content (%)	5.0
Optimum pH	7.5
Optimum temperature (°C)	50.0
Stability on recycling (ester hydrolysis measurements)	Active over 20 recycling assays
Thermal deactivation constant at 40°C (in heptane, h^{-1})	0.13

Application in the Synthesis of Butyl Butyrate

For the chemical synthesis of esters, acid in excess is usually involved. This methodology can be also applied in the lipase-catalyzed esterification reaction, as a pattern reaction. However, short-chain organic acids may display some inhibitory effect on the enzyme activity. To check the effect of butyric acid on the enzyme performance, molar ratios between butyric acid (BA) and butanol (ButOH) in the range of 0.6–6.0 were used. As observed in Fig. 6, no inhibition of the enzyme activity was detected when butyric acid was used in excess. Actually, the esterification progress was even

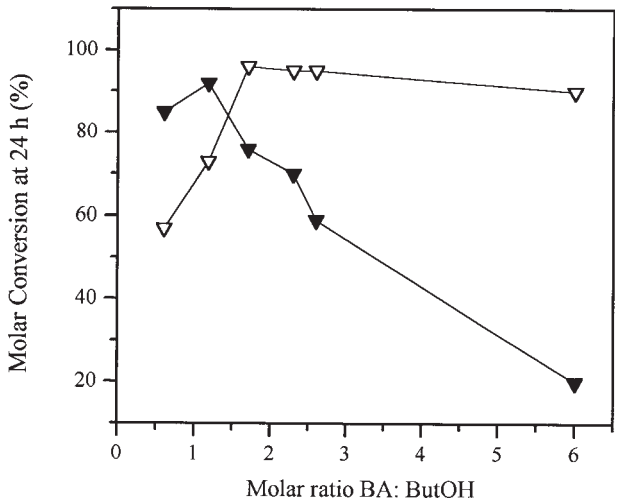

Fig. 6. Effect of the molar ratio of butyric acid to butanol in the molar conversion of butanol (▽) and butyric acid (▼) after 24 h of incubation with 0.5 g of CPS lipase at 37°C.

Table 4
Partition Coefficients of n-Butanol and Butyric Acid on CPS Lipase and Lipozyme

	Partition coefficient[a]	
Compound	Lipozyme[b]	Lipase-CPS
n-Butanol	2.43	1.97
Butyric acid	1.53	1.67

[a]Estimated according to the Eq. 2, in the concentration of 200 m*M* in heptane at 37°C under shaking.
[b]Data from our previous work *(8)*.

limited by the availability of butyric acid in the reactor vessel. At the lowest ratio tested (molar ratio of BA to ButOH of 0.6), a theoretical maximum value of 57% butanol molar conversion exists, so the end point was not reached. This conversion increases rapidly with additional amounts of butyric acid and then becomes essentially constant for molar ratios greater than 1.5. Such a relationship is quite similar to that attained with a commercial immobilized lipase preparation (Lipozyme) using the same reaction system *(8)*. The reason for this is probably the similarity between the partition coefficient values attained for both lipase preparations (CPS lipase and Lipozyme) as shown in Table 4. Both lipase preparations have higher partition coefficient values for butanol than for butyric acid, favoring in this way the migration of butanol to the enzyme solid phase. This suggests that butyl butyrate synthesis by either Lipozyme or CPS lipase might be limited by the butyric-acid concentration *(8)*. Here it is appropriate to mention that the enzymatic activity correlated closely with partition of sub-

Table 5
Influence of Molar ratio of Butyric Acid to Butanol
on the Esterification Performance Under Repeated use of CPS Lipase

Molar ratio BA:ButOH	ButOH molar conversion at 24 h[a] (%)			
	Recycle 1	Recycle 3	Recycle 4	Recycle 4
1:1	86	89	86	83
1:2.5	95	93	24	10

[a]0.5 g of CPS lipase was reacted for 24-h periods repeatedly in heptane containing 250 m*M* of butanol and butyric acid as follows: 250 m*M* and 625 m*M*.

strate/product so that partition coefficient values of reactants can be used as a parameter for substrate formulation.

Although butyric acid in excess seemed not to inhibit the activity of CPS lipase this should be avoided if reutilization of the immobilized preparation is envisaged. For example, when high butyric acid was used (molar ratio over 2.0) instability of the CPS lipase was noted with consequent decreased molar conversion during repeated runs. Stable repeated batches were achieved for nearly equimolar ratio (Table 5). Other researchers have also noted the influence of the reactants molar ratio for maintaining the stability of immobilized derivative during repeated batch runs *(19,20)*.

Discussion

Silica-based carriers and its derivatives with well-defined pore size are popular matrices for immobilization of enzymes. In this work, controlled porous silica was selected as a carrier for immobilizing *C. rugosa* lipase. The choice of this support was based on our previous background in using such kind of matrix for successful immobilization of several enzymes such as amyloglucosidase and inulinase *(15,17)*. In accordance with this methodology, the activation was achieved by silylation of the hydroxyl groups using aminoalkylethoxi-silane, followed by transformation of the aminoalkyl groups attached to the silica surfaces into Schiff's bases by treatment with glutaraldehyde. The direct coupling of lipase on the support structure as first proposed (using an aqueous-buffered dispersion medium) led to less stable biocatalyst preparation. Similar results were attained by several workers and possible reasons for such low performance were related to

1. Change in the conformation of the lipase upon immobilization;
2. A situation in which only a small amount of lipase is immobilized;
3. A decrease in the ability of substrates to reach the active site of the lipase (mass transfer limitations); or
4. The existence of steric hindrance imposed by the carrier matrix, which constrains the flexibility of the lipase molecule *(21,22)*.

To overcome this, several strategies have been investigated, including utilization of different type of silanes, addition of additive (sorbitol, polietilenoglicol or albumin), and use of different pore diameter size *(13,21,22)*. In the present work, a different approach was attempted in which hexane was used as a dispersion medium for enhancing the linkage between enzyme and support. Using such a method, lipase coupling yield of about 18% was achieved and even more important; higher operational stability was obtained.

The increased stability of immobilized lipase on CPS might result from the improved enzyme retention in nonpolar solvent. It is not yet clear whether the support expands in hexane, making a better distribution of the enzyme onto the surface area, or if polarity of the solvent may play an important role by not stripping off the bound water from the enzyme's surface. Although both mechanisms might have contributed to ensure high retention of lipase activity on the support material, it is most probably the use of a solvent that has lower polarity (higher log P value) that has allowed to maintain the conformational properties of the enzyme. This would not be surprising because study of the compatibility of a solvent with enzyme activity has shown that water-miscible hydrophilic solvents such as dimethylformamide (DMF) (log P = –1.01), dimethyl sulfoxide (DMSO) (log P = –1.35), lower alcohols (ethanol, log P = –0.31), and acetone (log P = –0.23) are usually incompatible, whereas water-immiscible hydrophobic solvents such as alkanes (hexane, log P = 3.50) retain an enzyme's high catalytic activity *(23)*. Similar results have been previously reported by several workers, including our group, using Celite as support material *(18,24,25)*.

Our hexane-based methodology for enzyme coupling to silica also enhanced the thermostability of the original *C. rugosa* lipase. The CPS lipase showed good stabilities at temperature over 40°C, even in an aqueous environment where free lipase is almost inactivated.

The lipase immobilized has been successfully applied to the synthesis of butyl butyrate provided that suitable reactant concentrations are used.

Acknowledgments

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