0273-2289/04/113/0307–0319/$25.00

Studies on Immobilized Lipase in Hydrophobic Sol-Gel

Cleide M. F. Soares,[1] Onelia A. dos Santos,[1] Heizir F. de Castro,[2] Flavio F. de Moraes,[1] and Gisella M. Zanin*,[1]

[1]State University of Maringa, Department of Chemical Engineering, Av. Colombo 5790, Bloco D-90, 87020-900, Maringa-PR, Brazil, E-mail: gisellazanin@maringa.com.br; and [2]Faculdade de Engenharia Quimica de Lorena, Department of Chemical Engineering, PO Box 116, 12606-970, Lorena-SP, Brazil.

Abstract

The hydrolysis of tetraethoxysilane using the sol-gel process was used to produce silica matrices, and these were tested for the immobilization of lipase from *Candida rugosa* by three methods: physical adsorption, covalent binding, and gel entrapment in the presence and absence of polyethylene glycol (PEG-1450). The silica matrices and their derivatives were characterized regarding particle size distribution, specific surface area, pore size distribution (Brunauer, Emmett, and Teller [B.E.T.] method), yield of grafting (thermogravimetric analyzer [TGA]), and chemical composition (Fourier transform infrared). Immobilization yields based on recovered lipase activity varied from 3.0 to 32.0%, and the highest efficiency was attained when lipase was encapsulated in the presence of PEG.

Index Entries: Silica matrices; immobilization; lipase; additive; sol-gel.

Introduction

The sol-gel process is the name given to a number of processes in which a solution, or sol, undergoes a sol-gel transition. In this broadest sense, the term *sol-gel* refers to the preparation of inorganic oxides by wet chemical methods, irrespective of final form product—monolith, crystalline, or amorphous *(1)*. Using sol-gel materials for mechanical entrapment of enzymes permitted stabilization of the proteins, tertiary structure owing to the tight gel network *(2)*. Moreover, the easy insertion of substituent groups into

*Author to whom all correspondence and reprint requests should be addressed.

silicate matrix may provide the entrapped enzymes with a beneficial microenvironment *(3)*. Therefore, it is not surprising that a great effort has been dedicated to the development of encapsulating processes for increasing the enzyme activity, especially those like lipases (EC 3.1.1.3) with potential application in a number of industrial processes. Lipases are used as additives to detergents, in the manufacture of food ingredients, in pitch control in the pulp industry, and as biocatalyst of stereoselective transformations *(4,5)*. The works of several research groups *(2–5)* are good examples of how this new technology has been successfully applied to these enzymes.

In the sol-gel methodology, it is essential to prepare a homogeneous solution containing the catalytic precursor in order to uniformly coagulate the solution. Since the rate of polymerization of different precursors varies considerably, it is necessary to test them with respect to their reactive levels in polar solvents to form the matrix, or to increase their condensation rate, with the addition of an acid or base. Systems already tested for lipases from either microbial or animal sources include the utilization of different precursors (tetramethoxysila, methyltrimethoxyla, ethyltrimethoxysila, polydimethysiloxa, and others), stabilizing additives (alcohol polyvinyl, albumin, gelatin, and others), and solvents such as methanol and ethanol. The drying conditions vary as follows: xerogels are dried by evaporation of the liquid, and aerogels are usually obtained by removing solvents in hypercritical conditions *(6)*.

Chemical derivatization of silica particles, which enables covalent linkage of various compounds (either biologic or not), leads to considerable changes in their physical and chemical properties. Indeed, reacting the silica hydroxyl groups with selected silane compounds can produce specific silica carriers for immobilization reactions. Since the reactions take place at the support surfaces, knowledge of the texture of the carriers is of crucial importance. It has been recognized that the particle surface area and, especially, the pore size distribution greatly control the final behavior of the supports. The smaller the pore diameter, the higher the surface area, but small-diameter pores may limit biologic compound immobilization as well as diffusion of substrates and reaction products. Therefore, an extensive knowledge of the support properties is essential for better understanding the carrier's performance *(7,8)*.

Here we report on the sol-gel immobilization of commercial *Candida rugosa* lipase (CRL) and analyze the effect of different immobilizing techniques on its activity, by modifying a procedure previously established for a chemical catalyst *(9)*. Hydrophobic silica gels were prepared according to Dutoit et al. *(9)*, in a procedure consisting of dissolving tetraethoxysilane (TEOS) in ethanol followed by condensation with ammonium solution. The matrix obtained was used to immobilize the lipase from *C. rugosa* by three methods: physical adsorption (ADS), covalent binding (CB and CB1), and gel entrapment in the absence (EN1) or presence (EN2) of polyethylene glycol (PEG-1450). The materials obtained were characterized with respect to their morphologic properties—particle size, surface area, and pore size

distribution—and for the biocatalyst samples obtained after lipase immobilization, the immobilization yield was also determined.

Materials and Methods

Lipase, Chemicals, and Reagents

Commercial CRL (Type VII) was purchased from Sigma (St. Louis, MO). The lipase was a crude preparation with a nominal activity of 104.94 U/mg. The silane γ-aminopropyltriethoxysilane (γ-APTS) and glutaraldehyde (25% solution) were from Sigma. PEG 1450 (Sigma) was used as stabilizing agent. The silano precursor TEOS was from Aldrich (Milwaukee, WI). Ethanol (minimum of 99%), ammonia (minimum of 28%), HCl (minimum of 36%), and gum Arabic were from Synth (São Paulo, Brazil). Olive oil (low acidity) was purchased at a local market. Solvents were standard laboratory grade and other reagents were purchased from either Aldrich or Sigma.

Preparation of Sol-Gel

Method 1: Without Addition of Enzyme

The methodology previously established by Dutoit et al. *(9)* was used with some modifications as follows: Thirty milliliters of TEOS was dissolved in 36 mL of absolute ethanol under inert nitrogen atmosphere. To this, 0.22 mL of HCl dissolved in 5 mL of ultrapure water was slowly added, and the mixture was agitated (200 rpm) for 90 min at 35°C. Then, 1 mL of ammonium hydroxide dissolved in 6 mL of ethanol was added (hydrolysis solution), and the mixture was maintained under static conditions for 24 h to complete the condensation. The material was collected and dried by evaporation, which leads to the formation of xerogels *(6)*. The support obtained was used for immobilizing commercial CRL by ADS and covalent binding (CB).

Immobilization of Lipase by ADS

Lipase was immobilized by ADS on the support following the methodology of Pereira et al. *(10)* with slight modifications. Pure silica (1 g dry wt) was soaked previously in hexane under agitation (100 rpm) for 1 h. Excess hexane was then removed with a 10 mL pipet, and 0.3 g of powdered lipase dissolved in 10 mL of distilled water was added. The fixation of lipase onto the support was performed under agitation for 3 h at room temperature and followed by an additional period of 18 h under static conditions at 4°C. The derivative was filtered (Whatman filter paper no. 41) and thoroughly rinsed with hexane.

Immobilization of Lipase by CB

Silica gel was silanized with γ-APTS followed by reaction with glutaraldehyde solution, and then lipase was immobilized by CB according to the procedure described by Soares et al. *(11)*. The enzyme (0.3 g) was

dissolved in 10 mL of distilled water and mixed with the support (1 g dry wt) under slow agitation for 2 h at room temperature. To observe the influence of an additive, PEG was added together with the enzyme solution (5 mg/g of support, 200 μL of aqueous solution containing 50 mg of PEG/mL). Then, 10 mL of hexane was added and the mixtures enzyme-support (CB1) and enzyme-support-additive (CB2) were incubated overnight at 4°C. The immobilized lipase derivatives were filtered (Whatman filter paper no. 41) and thoroughly rinsed with hexane.

Method 2: With Addition of Lipase During Gel Formation

For the preparation of lipase-encapsulated derivatives, a procedure was adopted similar to that described for Method 1, except, in this case, the lipase solution (2.70 g of enzyme diluted in 15 mL of ultrapure water) was added simultaneously with the hydrolysis solution (NH_4OH). The encapsulation of CRL in the hydrophobic silica gels was performed in the absence and presence of PEG-1450, resulting in the EN1 and EN2 derivatives, respectively.

Activity-Coupling Yield

Hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. *(11).* One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid/min under the assay conditions (37°C, pH 7.0, 150 rpm). Analyses of hydrolytic activities carried out on the lipase loading solution and immobilized preparations were used to determine the activity-coupling yield (η%), which measures the recovered enzymatic activity according to Eq. 1:

$$\eta(\%) = \frac{Us}{Uo} \times 100 \tag{1}$$

in which *Us* is the enzyme activity recovered on the support, and *Uo* is the total enzyme units offered for immobilization.

Assessment of Grafted Material

The amount of grafted material resulting from the lipase immobilization experiments was determined by weight loss in a TGA apparatus (TGA-50 Shimadzu Thermogravimetric Analyzer) over the range of 25–1000°C, with a heating rate of 20°C/min, using air as the purge gas. To eliminate error sources that could arise from the presence of residual reactants, the weight loss was only considered for temperatures above 130°C. The percentage of the grafted material was calculated using Eq. 2:

$$\text{Grafted material}\,(\%) = 100\left(w_i - w_f\right)/w_f \tag{2}$$

in which w_i and w_f are the initial and final weight of samples, respectively, the first taken at 130°C and the latter at 1000°C.

Table 1
Hydrolytic Activities and Activity-Coupling Yield
of Original Silica Gel Matrix and Immobilized Derivatives

Experiment	Hydrolytic activities (μmol/mg·min)	Activity-coupling yield (η%)[a]
ADS	41.5	12.04
CB1	27.3	7.93
CB2	24.8	7.20
EN1	66.4	13.92
EN2	128.9	31.98

[a]Calculated using Eq. 1.

Determination of Surface Area

The surface area measurements were performed by adsorption, using nitrogen as the adsorbate. The samples were previously degassed to below 50 mmHg at room temperature and the analyses were performed at 77 K, using liquid nitrogen. The equilibrium interval was 5 s. The surface area was calculated using the Brunauer, Emmett, and Teller (B.E.T.) method. Pore volume and area distributions based on BJH calculation (*8*) were evaluated by the B.E.T. apparatus software (NOVA 1200-Quantachrome).

Fourier Transform Infrared Spectroscopy

The samples of free lipase, pure silica (PS), silanized and activated silica, and immobilized derivatives were submitted to the Fourier Transform Infrared Spectroscopy (FTIR) analysis (Spectrophotometer FTIR BOMEM MB-100). The spectra were obtained in the wavelength range of 400–4000 cm^{-1} for evaluation of the immobilization procedures.

Results and Discussion

The support obtained by the sol-gel technique was used to immobilize commercial CRL following three procedures. In the first, the lipase was immobilized on PS by ADS; in the second, the enzyme was covalently bonded on the support previously silanized and activated with glutaraldehyde (SPS) in the absence (CB1) and presence of an additive (CB2); and, in the third, the enzyme was encapsulated in the absence (EN1) and presence of an additive (EN2).

Table 1 shows the activity-coupling yield (η%) results for the immobilization of lipase on hydrophobic silica gels. As expected, the activity-coupling yield for the lipase immobilized by ADS was very poor (η = 12.04%), probably owing to the inefficiency of this methodology for the silica support used. Similarly, experiments performed by covalent binding (CB1 and CB2) also gave low activity-coupling yield (η < 8.0%).

Table 2
Characterization of Original Silica Gel Matrix and Immobilized Derivatives

Experiment	Surface area (m^2/g)[a]	Mean pore diameter (Å)[a]	Pore volume (cm^3/g)[a]	w_i (mg)[b]	w_f (mg)[b]	Grafted material (%)[c]
PS	607.17	18.23	0.37	557	479	16.29
SPS	468.72	36.51	0.46	543	488	11.27
ADS	556.30	14.30	0.46	410	374	9.62
CB1	560.10	14.46	0.49	417	375	10.07
CB2	531.10	14.39	0.43	451	409	9.31
EN1	382.32	49.99	0.48	518	430	16.99
EN2	300.22	56.59	0.43	403	329	18.36

[a] Evaluated from B. E. T
[b] w_i and w_f are the initial and final weight of samples, respectively.
[c] Calculated using Eq. 2;

It is possible that the silanization and activation of the PS might induce conformational changes in the enzyme chains on its adhesion on the support surface leading to the low activity-coupling yields observed. These changes in structure may cause steric hindrance, which makes certain areas of the enzyme molecule inaccessible to the substrate (olive oil). In this case, the addition of an additive (PEG-1450) did not produce a beneficial effect. However, the positive effect of the PEG was noticed for the derivatives obtained by the encapsulation technique (EN2). The highest coupling yield (31.98%) was observed for the immobilized derivative obtained by lipase encapsulation in the presence of PEG-1450, confirming the effectiveness of this kind of additive *(12,13)*.

Concerning the physical characteristics (*see* Table 2), EN1 and EN2 derivatives showed the largest mean pore diameter, 49.99 and 56.58 Å, respectively. These values are greater than the mean pore diameter obtained by the traditional immobilization techniques: ADS, CB1, and CB2 (14.30, 14.46, and 14.39 Å, respectively), which gave very close results.

Figure 1 shows the pore size distribution of primary supports and the immobilized derivatives in silica matrix on an incremental (derivative) basis, dV/d Log (D), to highlight the differences among the immobilized derivatives in silica gels. The computation of pore size distribution, from gas adsorption, was based on the BJH method using the desorption branch. From these plots it was observed that all solid samples (PS, SPS, ADS, CB1, CB2, EN1, and EN2) exhibit a unimodal distribution of pores, and the smaller mean pore diameters were observed with the samples PS, ADS, CB1, and CB2 (Table 2).

Pore volume, surface area, and mean pore diameter were influenced by the methodology of producing the silica derivatives, and the greatest

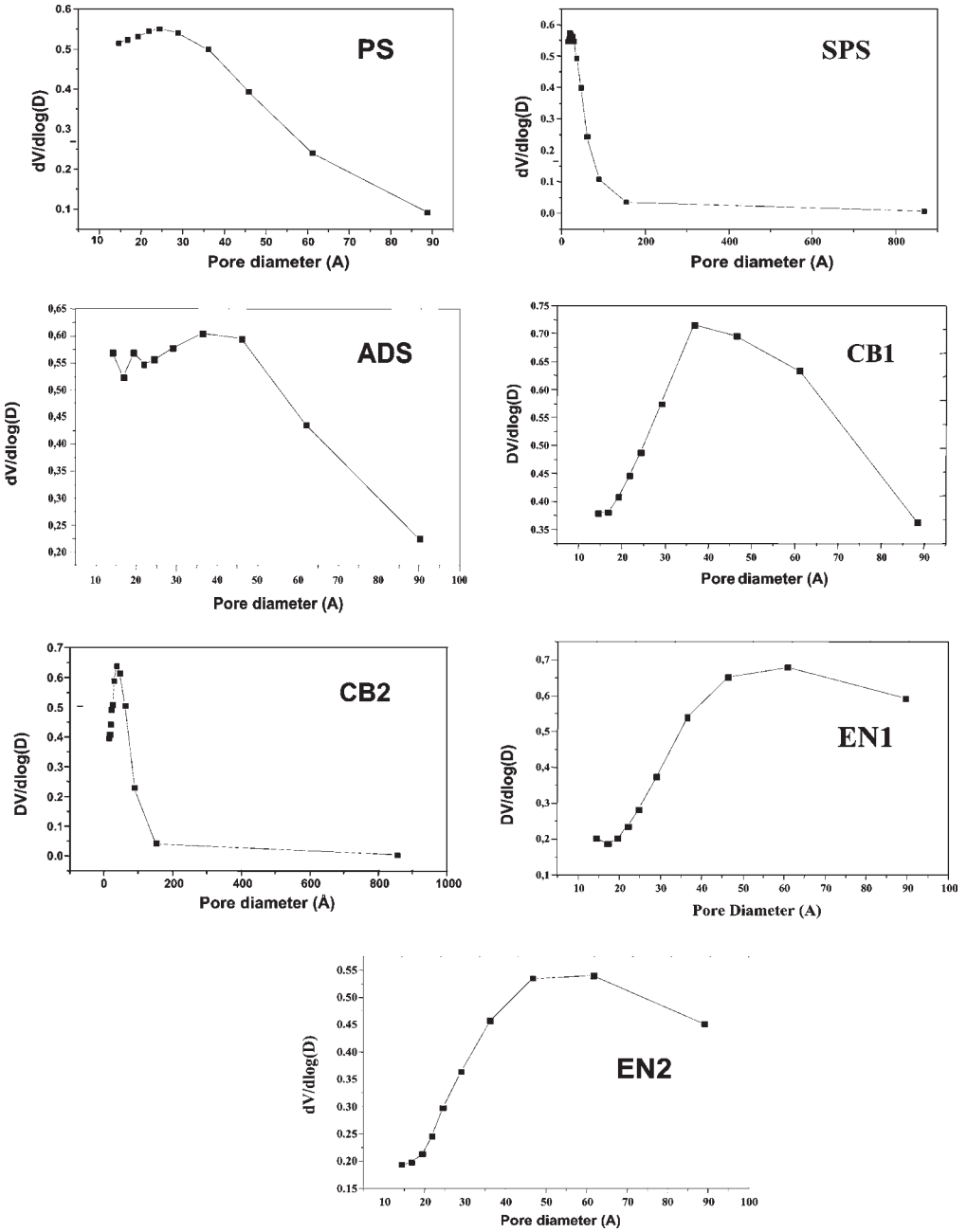

Fig. 1. Pore size distributions (dV/d Log [D]) of pure silica gel (PS), silanized and activated silica (SPS), and immobilized derivatives in silica gels (ADS, silica with lipase physically adsorbed; CB1, silica with lipase covalently bonded; CB2, silica with lipase covalently bonded in the presence of PEG; EN1, silica with entrapped lipase; EN2, silica with lipase entrapped in presence of PEG).

deviations of the mean behavior were observed with the biocatalysts produced by the encapsulation technique. Lipase encapsulated in the presence of the additive PEG showed the largest mean pore size (56.59 Å), and an increase of about 6.6 Å in relation to the mean pore size of the lipase encapsulated in the absence of PEG (Table 2 and Fig. 1). Results described by Keeling-Tucker et al. *(14)* also showed an increase in the pore size with the addition of organic macromolecules, and this was associated mainly with the encapsulation of those macromolecules that inhibit gel contraction during the synthesis of the support.

Several works have explained the occurrence of a step change in adsorption isotherms by the structural orientation of the adsorbed species and also by the manner in which the adsorption occurred *(15)*. However, none related the activity-coupling yield observed with different techniques of lipase immobilization in hydrophobic sol-gels to the adsorption isotherms of the supports.

Similar adsorption isotherms were obtained for samples PS and SPS, and for the lipase immobilized derivatives of silica gels (ADS, CB1, CB2, EN1, and EN2). Typical examples are shown in Fig. 2. We observed hysteresis loops in the adsorption-desorption isotherms, denoting materials with well-defined ordered structures, and the presence of mesopores, with hysteresis loops between isotherms types H1 and H2 *(8,16)*. Primarily on the mesopores of silica, a multilayer of adsorbate is formed, increasing the relative pressure, and depending on the mean pore diameter, at $P/P_0 \approx 0.4$, capillary condensation takes place on the multilayer, resulting in a further increase in pore volume *(1)*.

The parameters of the pore structure, such as surface area, pore volume, and mean pore diameter, can generally be used for a formal description of the porous systems, irrespective of their chemical composition and their origin, and for a more detailed study of the pore formation mechanism, the geometric aspects of pore structure are important. This picture, however, oversimplifies the situation because it provides a pore uniformity that is far from reality. Thorough attempts have been made to achieve the mathematical description of porous matter. Researchers discussed the cause of porosity in various materials and concluded that there are two main types of material based on pore structure that can be classified as corpuscular and spongy systems. In the case of the silica matrices obtained with TEOS and other precursors, the porous structure seems to be of the corpuscular type, in which the pores consist of the interstices between discrete particles of the solid material. In such a system, the pore structure depends on the pores mutual arrangements, and the dimensions of the pores are controlled by the size of the interparticle volumes *(1)*.

The stepped intrusion curves clearly denote different pore size ranges. In the case of experiments EN1 and EN2, this is possibly the reason for the mean pore diameter and activity-coupling yield being the highest. The specific surface areas obtained for all experiments are listed in Table 2.

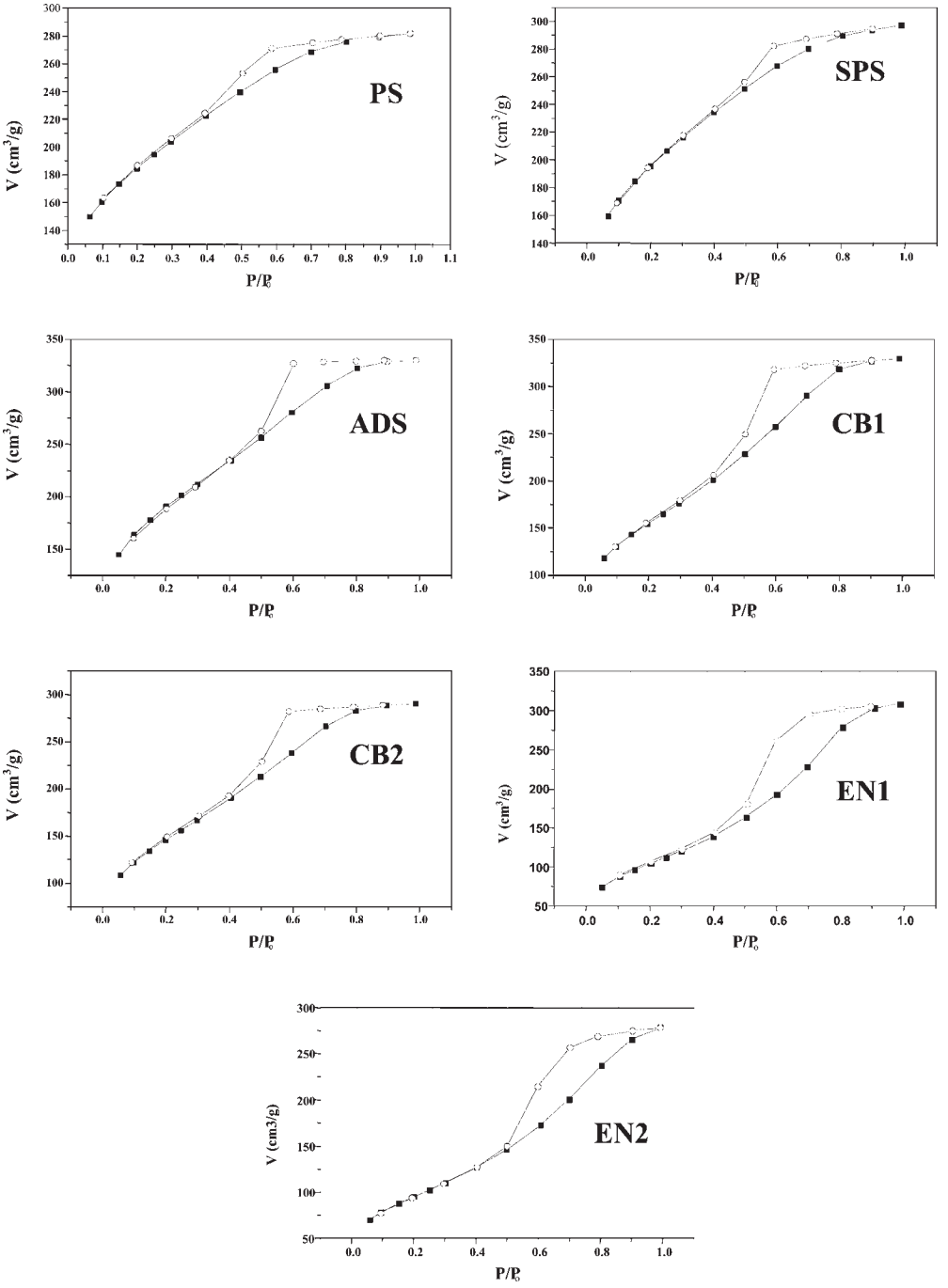

Fig. 2. Gas adsorption/desorption isotherms obtained for pure silica gel (PS), silanized and activated silica (SPS), and immobilized derivatives in silica gels (ADS, silica with lipase physically adsorbed; CB1, silica with lipase covalently bonded; CB2, silica with lipase covalently bonded in presence of PEG; EN1, silica with entrapped lipase; EN2, silica with lipase entrapped in presence of PEG). (○),Adsorption; (■), desorption.

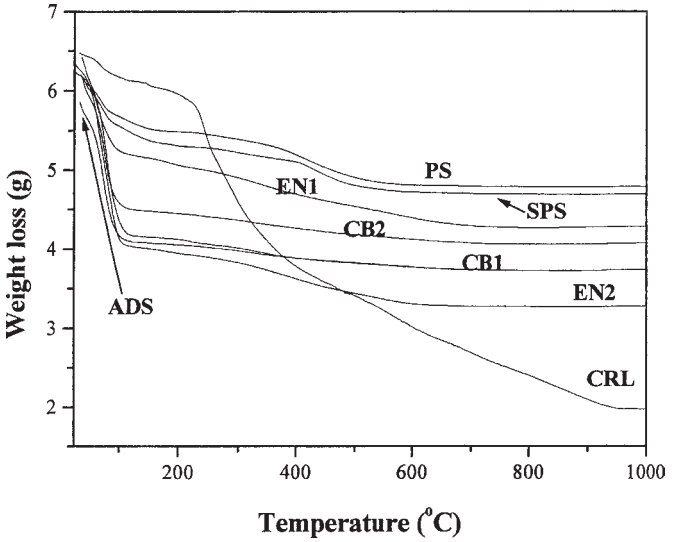

Fig. 3. Loss of grafted mass by heating of immobilized lipase samples. TGA profiles are shown for the free lipase (CRL), original silica (PS), silanized derivative (SPS), and immobilized derivatives (ADS, silica with lipase physically adsorbed; CB1, silica with lipase covalently bonded; CB2, silica with lipase covalently bonded in presence of PEG; EN1, silica with entrapped lipase; EN2, silica with lipase entrapped in presence of PEG).

Although the knowledge of the sample surface area is important, as already pointed out, the pore size distribution is even more critical, since it greatly affects the activity-coupling yield of the biologic immobilization because of the diffusion-controlled phenomena.

From the results presented in Tables 1 and 2, it can be observed that in general the greater the mean pore diameter, the higher the percentage of grafted material (calculated using Eq. 2 and taking into consideration the weight loss as a function of temperature, as shown in Fig. 3). The percentage of grafted material can also be correlated with the activity-coupling yield, as displayed in Fig. 4. In this set of experiments, the highest values for the activity-coupling yield and for the percentage of grafted material were observed for the derivatives obtained by encapsulation in the presence of an additive (EN2).

To verify the efficiency of the methodology in relation to the incorporation of lipase in silica gel matrices, infrared spectroscopy was used. Figure 5 shows the spectra of the free lipase (CRL), pure silica gel (PS), silanized and activated silica (SPS), and immobilized derivatives in silica gels (ADS, CB1, CB2, EN1, and EN2).

The immobilized lipase derivatives have shown the same characteristic bands—950 (Si-O-Si stretching), 810 (Si-O-Si- stretching), and 600 (Si-O-Si bending) cm^{-1} bands *(7,17)*, as the supports (PS and SPS). The

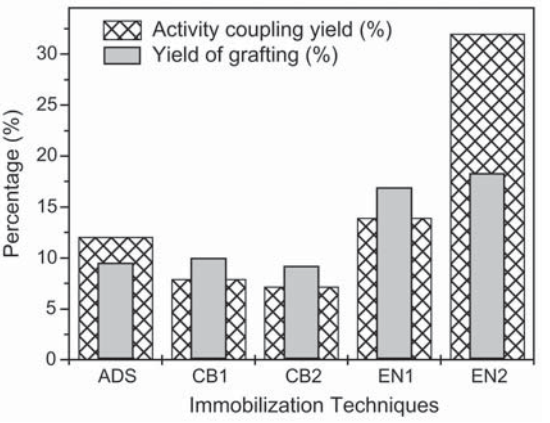

Fig. 4. Comparison of activity-coupling yield (η%) and yield of grafting for samples of CRL immobilized on silica hydrophobic matrices by different methodologies.

negative effect in the recovered activity yield of the silane used to activate the support for covalent binding can be observed in the considerable differences in the percentage of –OH groups (3400 cm^{-1} bands) exhibited in the spectra of the PS and SPS samples (Fig. 5A,B).

As described in the literature *(7,17)*, the lipase enzyme has two characteristic bands at 1650 and 1600 cm^{-1} (primary and secondary amino groups), and these are clearly shown in Fig. 5A,B. These bands are also displayed in the spectra for the immobilized derivatives (ADS, CB1, CB2, EN1, and EN2), revealing, owing to the greater peak height, a higher efficiency for the encapsulation technique in the presence of PEG (EN2), when compared with the other tested methods. Further information on the catalytic activity was obtained by testing the derivatives according to our method for synthetic applications—i.e., the esterification of *n*-butanol with butyric acid *(10–12)*. This reaction was selected because it gave measurable results with great accuracy in a short span of time and with a minimum amount of lipase *(11,12)*. In addition, this reaction system has been used by our group as a standard reaction system for testing lipases immobilized in several supports *(10,11,18)*.

Conclusion

Various lipase immobilization methods were tested with different silica matrices, and the immobilized enzyme samples were examined by morphologic, physicochemical, and biochemical characterization methods. The results allowed correlation of the activity-coupling yield of different immobilization methods in relation to the incorporation of lipase in the silica gels and showed that the most active biocatalyst resulted from the encapsulation of commercial CRL in the presence of PEG.

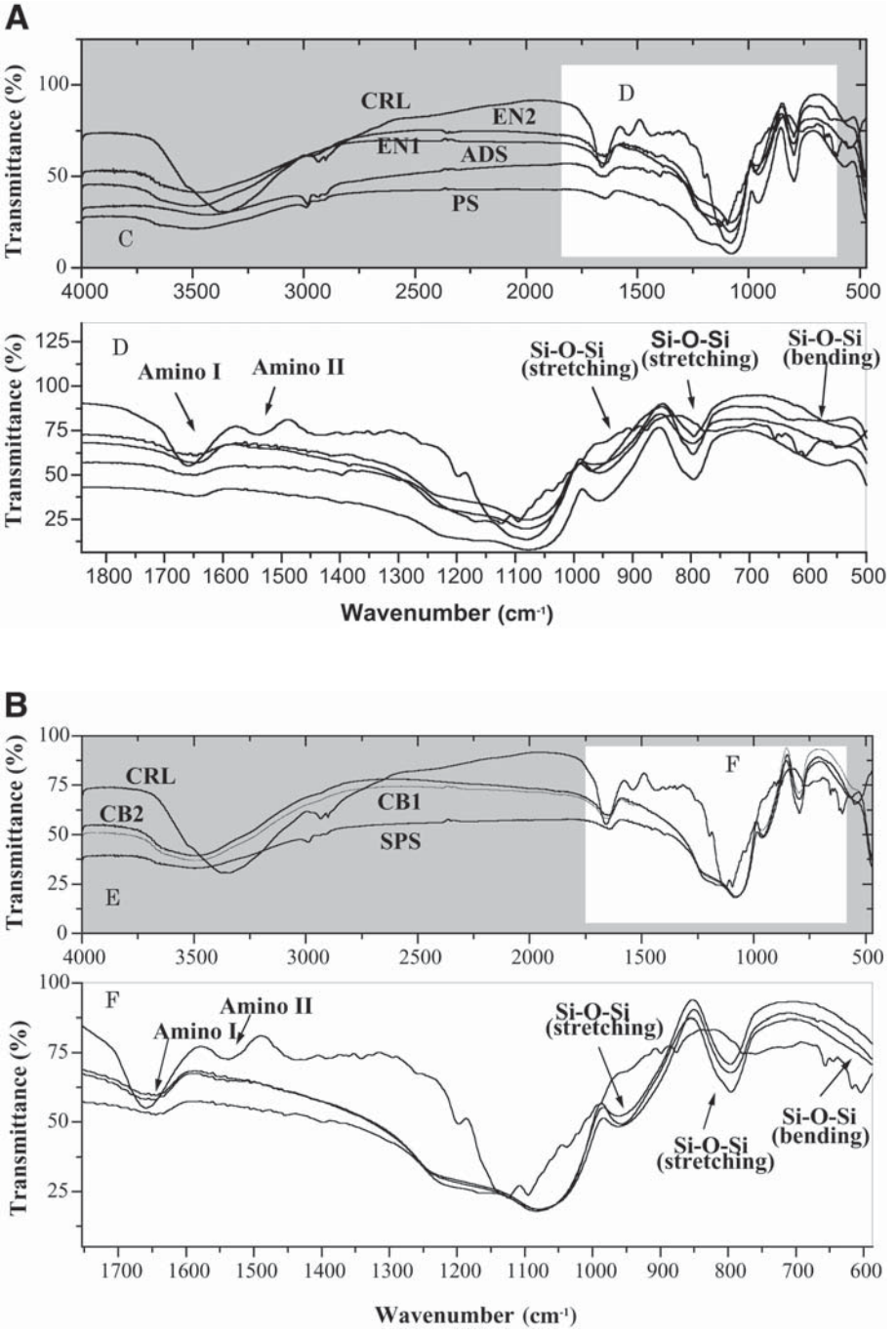

Fig. 5. **(A)** FTIR spectra for free lipase (CRL), original silica (PS), and immobilized derivatives (ADS, silica with lipase physically adsorbed; EN1, silica with entrapped lipase; EN2, silica with entrapped lipase in presence of PEG). **(B)** FTIR spectra for free lipase (CRL), silanized derivative (SPS), and immobilized derivatives (CB1, silica with lipase covalently bonded; CB2, silica with lipase covalently bonded in presence of PEG).

Acknowledgments

We acknowledge financial assistance from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

References

1. Unger, K. K. (1979), *Porous Silica: Its Properties and Use as Support in Column Liquid Chromatography*, Elsevier, New York, NY.
2. Kauffmann, C. and Mandelbaum, R. T. (1998), *J. Biotechnol.* **62,** 169–176.
3. Reetz, M. T., Zonta, A., and Simpelkamp, J. (1996), *Biotechnol. Bioeng.* **49,** 527–534.
4. Pierre, A. and Buisson, P. (2001), *J. Mol. Catal. B: Enzymatic* **11,** 639–647.
5. Buisson, P., Hernandez, C., Pierre, M., and Pierre, A. C. (2001), *J. Non-Crystalline Solids* **285,** 295–302.
6. Siouffi, A. M. (2003), *J. Chromatogr.* **1000,** 801–818.
7. Bosley, J. A. and Peilow, A. D. (1997), *J. Amer. Oil Chem. Soc.* **74(2),** 107–111.
8. Ramos, M. A., Gil, M. H., Schact, E., Matthys, G., Mondelaers, W., and Figueiredo, M. M. (1998), *Powder Technol.* **99,** 79–85.
9. Dutoit, D.C. M., Schneider, M., Fabrizioli, P., and Baiker, A. (1996), *Chem. Mater.* **8,** 734–743.
10. Pereira, E. B. (1999) MS thesis, Chemical Engineering Department, State University of Maringa, Maringa-PR, Brazil.
11. Soares, C. M. F., Castro, H. F., Moraes, F. F., and Zanin, G. M. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 745–758.
12. Soares, C. M. F., Castro, H. F., Santana, M. H., and Zanin, G. M. (2001), *Appl. Biochem. Biotechnol.* **91–93,** 715–719.
13. Soares, C. M. F., Castro, H. F., Santana, M. H., and Zanin, G. M. (2002), *Appl. Biochem. Biotechnol.* **98–100,** 703–718.
14. Keeling-Tucker, T., Rakic, M., Spong, C., and Brennan, J. D. (2000), *Chem. Mater.* **12,** 3695–4704.
15. Al-Duri, B. and Yong, Y. P. (2000), *Biochem.l Eng. J.* **4,** 207–215.
16. Ravikovitch, P. I. and Neimark, A. V. (2000), *Langmuir* **18,** 9830–9837.
17. Assis, O. B. G. (2003), *Braz. J. Chem. Eng.* **20(3),** 339–342.
18. De Castro, H. F., Oliveira, P. C., Soares, C. M. F., and Zanin, G. M. (1999), *J. Amer. Oil Chem. Soc.* **76(1),** 125–131.