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Influence of gelation time on the morphological and physico-chemical properties of the sol–gel entrapped lipase

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Abstract

Different gelation times (4, 18, 24 and 48 h) were used for the preparation of silica sol–gel supports and encapsulated *Candida rugosa* lipase using tetraethoxysilane (TEOS) as precursor. The hydrophobic matrices and immobilized lipases produced were characterized with regard to pore volume and size by nitrogen adsorption (BJH method), weight loss upon heating (TGA), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), chemical composition (FTIR) and percentage of hydrolysis (POH%) of olive oil. These structural parameters were found to change with the gelation time, but no direct relation was found between the percentage of oil hydrolysis (POH%) and the gelation time. The best combination of high thermal stability and high POH% (99.5%) occurred for encapsulated lipase produced with 24 h gelation time.

Keywords: Gelation time; Sol–gel; *Candida rugosa*; Entrapped lipase; Hydrolysis

1. Introduction

The sol–gel process, in which inorganic oxides can be prepared from liquid precursors at low temperatures, allows biological molecules to be immobilized in amorphous inorganic matrices. The successful encapsulation of biomolecules such as enzymes and other proteins within the pores of sol–gel derived glasses has been well documented [1–3]. The sol–gel immobilized enzymes retain biological activity, and in some cases, proteins experience an increased stability upon encapsulation. Moreover, the encapsulation approach produces a more robust biologically active material. However, the nature of the solid network generated by aggregation of colloids or polymerization of alkoxides depends on the diffusion of the elemental species and the growing clusters. It also depends on the probability of aggregation during the contact between species in the procedure [4–6].

In a previous study [7], biocatalysts with encapsulated lipase in silica gels produced by acid- or base-catalyzed hydrolysis of silane compounds such as tetraethoxysilane (TEOS) or methyltrimethoxysilane (MTMS), in the presence of the additive polyethylene glycol (PEG) were developed. These gels were characterized with regard to mean pore diameter, specific surface area, pore size distribution (B.E.T. method), weight loss upon heating (TGA) and chemical composition (FTIR). The behavior of the sol–gel encapsulated lipase systems depends on the physical and structural properties of the support, and the biochemical properties of the lipase. The conformation of the encapsulated lipase in a gel can largely be improved by grafting appropriate functionality, such as alkyl-substituted silanes and additives, on the gel network, which surrounds the enzyme. The encapsulation of *Candida rugosa* lipase (CRL) in sol–gel prepared by the hydrolysis of alkyl-substituted silanes like TEOS, in the presence of PEG showed considerable hydrolytic and esterification activity, depending on the water content of the immobilized enzyme [7,8]. On the other hand, the encapsulation of CRL in sol–gel prepared by the hydrolysis of MTMS, in the presence of PEG only showed high activity for esterification [8]. This was related to the interactions caused by the resulting hydrophobic–hydrophilic nature of the support. The most efficient systems appeared to be worthy of further study, owing to their potential application as biocatalysts. In addition, further characterization of the gel structural properties was warranted, given the difficulty in correlating these properties with the enzymatic activity. The esterification or hydrolytic activity

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and the functional stability of *C. rugosa* lipase immobilized by the sol–gel process, using precursor MTMS or TEOS, in the presence of PEG, were analyzed in the light of the gel texture and structure which was characterized by ^{29}Si and ^{13}C NMR [9].

After these studies, it was required to determine the ideal gelation time to enable an adequate aggregation of the solid network generated during the contact between species of the procedure. The stability of the sol–gel structure due to the gelation time and the characteristics of the hydrophobic matrices and immobilized preparations were characterized with regard to the mean pore volume, specific surface area, pore size distribution (B.E.T. method), weight loss upon heating (TGA), chemical composition (FTIR). The catalytic activities of the immobilized preparations were assayed by the hydrolysis of olive oil.

2. Experimental procedures

2.1. Enzyme and chemicals

Commercial *C. rugosa* lipase (CRL, Type VII, product No L1754) was purchased from Sigma Chemical Co. (St Louis, MO, United States). 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^{-1} protein. Polyethylene glycol (PEG, MW 1450, Merck) was used as stabilizing agent. The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and, gum Arabic were from Synth (São Paulo, Brazil). Olive oil was purchased at a local market. 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.

2.2. Encapsulation of lipase from C. rugosa (CRL) in sol–gel matrices and gelation time

The methodology previously established by the Patent PI0306829-3 [10] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under nitrogen inert atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water was slowly added and the mixture was agitated (200 rpm) for 90 min at 35 °C. Then, 10 mL of lipase solution (18.29 mg mL^{-1}), PEG solution (5 mg mL^{-1}, 8 mL added) and 1 mL of ammonium hydroxide dissolved in 6 mL of ethanol were added (hydrolysis solution) and the mixture was kept under static conditions for gelation times of 4, 18, 24 and 48 h, to proceed with the chemical condensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 24 h.

2.3. Determination of the percentage of hydrolysis

The methodology previously described by Pinheiro et al. [11] was used. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of gum Arabic solution (7%, w/v). The reaction mixture containing 50 mL of the oil emulsion, 10 mL of 100 mM sodium phosphate buffer (pH 7.0) and either free (1 mL, 5 mg mL^{-1}) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for 60 min at 37 °C. After this period, the percentage of hydrolysis was determined by titration of the released fatty acids and defined as the percentage weight of free fatty acids in the sample, in comparison with the maximum theoretical amount of free fatty acids that could be produced if all the oil in the sample was hydrolyzed:

$$\mathrm{POH}\,(\%) = \frac{\mathrm{Na} \times 0.02 \times 10^{-3} \times \mathrm{MM}}{W_\mathrm{t} \times f_0 \times f_1} \times 100 \quad (1)$$

where f_0 is the fraction of oil in the sample at the start of reaction ($g_{\mathrm{lipids}}/g_{\mathrm{sample}}$), f_1 the ratio of the mass of fatty acids to the mass of oil, after total oil hydrolysis ($g_{\mathrm{fatty\ acids}}/g_{\mathrm{lipids}}$), MM the average molecular weight of fatty acids in the olive oil (825.30 g mol^{-1}), N_a the volume of sodium hydroxide solution required during fatty acids titration (mL), and W_t is the weight of the sample (g).

2.4. Sample characterization

The characterization of the porosity of hydrophobic matrices and immobilized biocatalysts is a complex issue when the total porosity, the pore size and the pore size distribution should be further analyzed. Methods based on gas adsorption are the most convenient for the study of microporous and mesoporous materials, using volumetric measurements of the adsorbed gas quantities. Pure silica gel or encapsulated lipase samples were characterized based on the BJH calculations [12] which were evaluated by the B.E.T. (Brunauer–Emmett–Teller) apparatus software (NOVA 1200-Quantachrome).

The samples weight loss upon heating was determined in a thermo gravimetric analysis-TGA apparatus (TGA-50 Shimadzu-Thermogravimetric Analyzer) over the range 25–1000 °C, with a heating rate of 20 °C min^{-1}, using air as the purge gas. Differential scanning calorimetry data were taken in a PerkinElmer DSC-50 differential scanning calorimeter. Free lipase, pure silica or immobilized lipase samples weighting 6 mg were put in a sealable aluminum pan, which was heated from 25 to 500 °C at a rate of 10 °C/min. Free lipase, pure silica and immobilized lipase samples were also submitted to the Fourier transform infrared—FTIR analysis (Spectrophotometer FTIR BOMEM MB-100). The spectra were obtained in the wavelength range from 400 to 4000 cm^{-1} for evaluation of the immobilization procedures, in accordance with Soares et al. [7].

Scanning electron microscopy (SEM) was used to characterize the surface of pure silica matrices and immobilized lipase samples.

3. Results and discussion

3.1. Characterization of the porosity of hydrophobic matrices and immobilized biocatalyst

Data for pore volume distributions obtained for the different gelation times and the percentage of micropores and mesopores

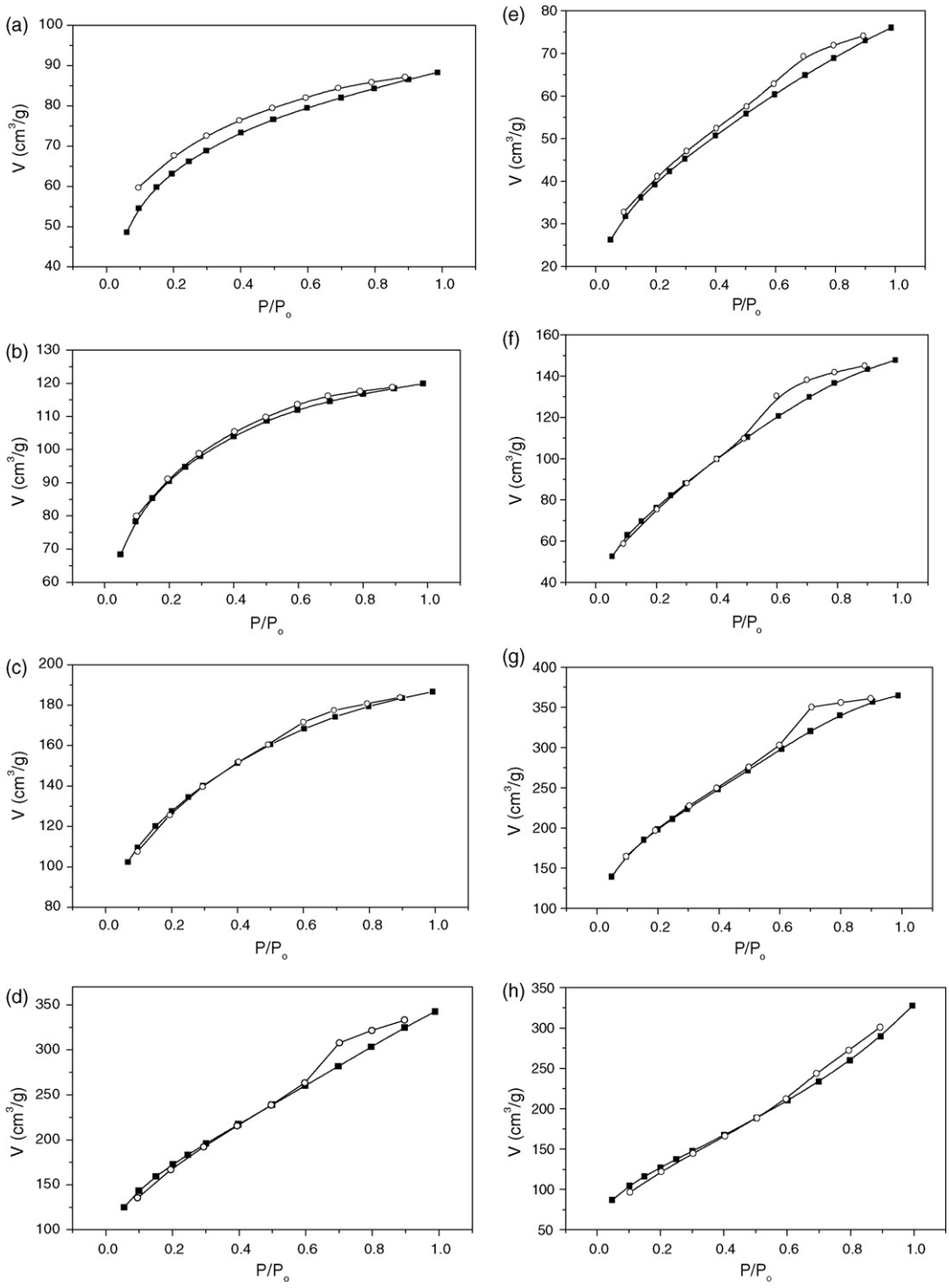

Fig. 1. Nitrogen adsorption–desorption isotherms for hydrophobic matrices and immobilized preparations as described in Table 1.

is shown, respectively, in Fig. 1 and Table 1, which illustrate the effect of the length of the wet gel aging period on the porous structure of the pure silica and encapsulated lipase samples.

As can be seen in Fig. 1a–h the total pore volume was significantly affected by different gelation times and by the lipase presence into the matrix. Pore volume goes through a maximum as the aging period varied from 4 to 48 h and the presence of the enzyme into the solid matrix clearly increases it for the samples of 18 and 24 h gelation time. The hydrophobic matrices and immobilized preparations pore volume distributions reported in Fig. 1a–h were processed by the BJH method, because it corresponds to more stable adsorption conditions and it correlates better with the enzyme immobilization data.

Table 1 shows that the percentage of micropores and mesopores in the solid matrices was slightly affected by different gelation times and by the presence of the encapsulated lipase. As the gelation time varied from 4 to 48 h, the mean percentage of micropores varied less than 1% for the pure silica and biocatalyst matrices, while the presence of lipase increased the mean micropores percentage in less than 2%. The same com-

Table 1
Textural properties of hydrophobic matrices and immobilized biocatalysts determined from nitrogen adsorption measurements

Sample	Gelation period (Fig. 1 correspondence)	%Micropores	Mean ± standard deviation	% Mesopores	Mean ± standard deviation
Pure Silica	4 (a)	70.73	71.18 ± 0.63	29.27	28.83 ± 0.63
	18 (b)	71.49		28.51	
	24 (c)	70.58		29.42	
	48 (d)	71.9		28.1	
Encapsulated lipase	4 (e)	72.35	72.33 ± 0.61	27.65	27.69 ± 0.63
	18 (f)	72.71		27.29	
	24 (g)	71.46		28.59	
	48 (h)	72.79		27.21	

parisons for the mesopores give circa 2 and 4%, respectively, but note that the mean percentage of mesopores has decreased while the mean percentage of micropores has increased in the presence of lipase. Unfortunately, in the case of hydrophobic matrices and immobilized biocatalysts, the amount of micropores or mesopores is small to be precisely characterized by the nitrogen adsorption isotherms. The monolithic structure of the bulk gels is usually not preserved during gelation time. Evaporation can be reduced or stopped during and after gelation in order to promote reinforcement of the inorganic network.

Several works have explained the occurrence of a step change in the adsorption isotherms due to the structural orientation of the adsorbed species and also, to the manner in which the adsorption occurred. However, none have related the percentage of hydrolysis observed to the adsorption isotherms of the supports produced with different gelation times. We observed hyteresis 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. On the mesopores of silica, a multilayer of adsorbate is formed, as the relative pressure is increased, and depending on the mean pore diameter, at $P/P_0 \approx 0.45$, capillary condensation takes place on the multilayer, resulting in a further increase of pore volume (Fig. 1h).

The nature of the solid network generated by aggregation of colloids or polymerization of alkoxides depends on the diffusion of the elemental species and this affects the growing of hydrophobic matrices or immobilized preparations (effects of dilution, viscosity and temperature). It also depends on the probability of the aggregation during the contact between species for the formation of the hydrophobic matrices and immobilized biocatalysts (reactivity). Consequently, the nitrogen adsorption measurements were influenced by the limiting mechanisms for the growth of the solid matrices network, being diffusion-limited for the pure silica matrices and reaction-limited for the immobilized biocatalyst.

The studied materials show two length levels of porosity: microporosity within the polymeric silica clusters and mesoporosity between clusters as is usually observed in the case of two-step acid–base-catalyzed silica gels. The aging enables the maintenance of a high porous volume in the dried gel, which is essentially associated with the large inter-cluster mesoporosity initially present in the wet gel.

3.2. TGA results

In order to obtain the formation of the solid network the aggregation of the colloidal particles must be promoted. In the case of the preparation of hydrophobic matrices and immobilized biocatalysts, the starting sols are only very slightly aggregated if at all. Thermogravimetric analysis (TGA) provides an important tool for gelation time studies of macromolecules. This technique allows determining the temperature range at which a heated sample undergoes a major conformational change by means

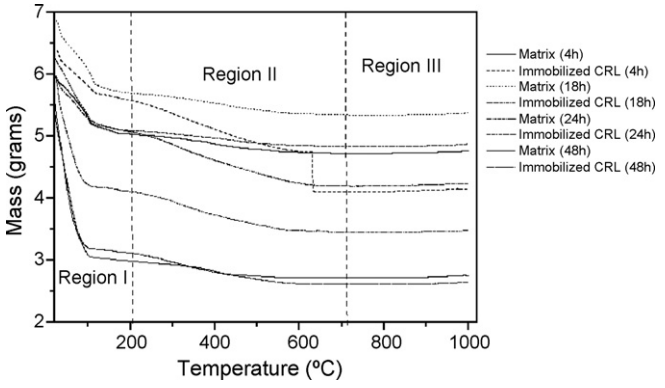

Fig. 2. Thermogravimetric curves for hydrophobic matrices and immobilized preparations.

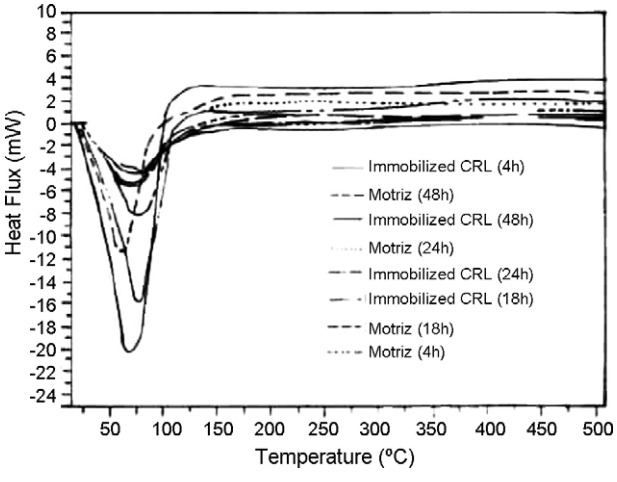

Fig. 3. DSC for the hydrophobic matrices and immobilized preparations.

of monitoring the thermal weight loss profile. The weight loss curves were divided into three regions as shown in Fig. 2, according to Brinker and Scherer [4], to simplify the thermogravimetric analysis. Comparison between hydrophobic matrices and immobilized biocatalysts with different gelation times (4, 18, 24 and 48 h) indicated that low values obtained for the weight loss associated with the lipase encapsulated biocatalysts is the result of an increased matrix thermal stability resulting from interactions among silane precursors and organic components in the case of 4 and 18 h gelation times. When the gelation time of both hydrophobic matrices and immobilized biocatalysts was 4 h the weight loss curve profile for the TEOS derivatives exhibited a sharp weight loss (Regions I, II and III, Fig. 2), and this difference can be attributed to the formation of the solid network with a lower mechanical strength. This was caused probability by a weak aggregation of the colloidal particles formed from TEOS, which aid the formation of matrix structures that incorporate molecular bound water.

3.3. Differential scanning calorimetry (DSC)

Fig. 3 illustrates that peaks appearing circa 100 °C are related to the evaporation of water and ethanol, and the denaturation of free enzyme, due to the activity loss temperature effect. The same kind of results were observed for the DSC curve profiles obtained for the immobilized preparations with 4 and 18 h gelation times.

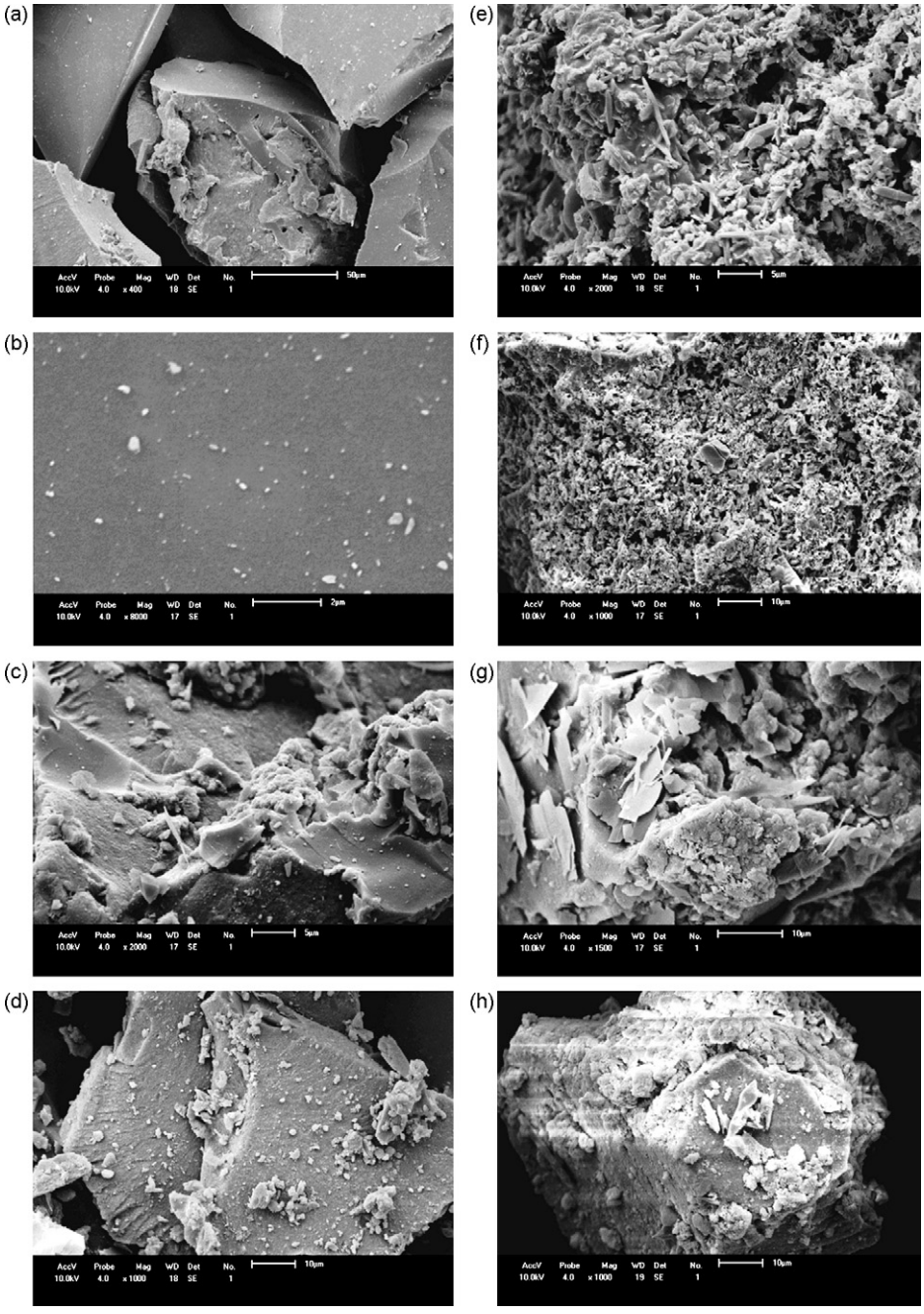

Fig. 4. Scanning electron microscopy for gelation times 4, 18, 24 and 48 h (SEM): (a–d) hydrophobic matrices and (e–h) immobilized preparations.

For 24 h gelation time an inverted behavior was observed. This was related to the decreased heat exchange associated with larger pore sizes, which are found at greater percentages for the smaller gelation times. The thermal decomposition of the structure with large pores demands less energy. The porous structure of the wet gels is filled by the liquid phase and the evaporation of the liquid contained in the pores results in capillary stresses on the solid network that cause shrinkage.

3.4. Scanning electron microscopy (SEM)

Scanning electron microscopy photos are shown for the hydrophobic matrices (Fig. 4a–d) and immobilized preparations (Fig. 4e–h). The more porous surface and lipophylic character of the lipase-containing gels (Fig. 4) results in a favorable activity for hydrolysis. The SEM studies only deliver information regarding the general morphology of the particles, and not on the actual conformation of the internal porous structure. It can be observed that the pure silica matrices showed very low superficial porosity for the different gelation times (Fig. 4a–d). Conversely the encapsulated lipases (Fig. 4e–h) showed a porous surface that appears to be more porous for the lower gelation times. Further investigations to better characterize hydrophobic matrices and immobilized biocatalysts in the gel need to be carried out using special techniques.

3.5. Fourier transformed infrared spectroscopy (FTIR)

Hydrophobic matrix and immobilized lipase samples were characterized by FTIR. For all samples, the FTIR spectrum (cm^{-1}) displayed characteristic bands referred to the silica; 810 (Si–O–Si silica), 110 (Si–O–Si silica) and 3400 cm^{-1} (O–H silica) (Fig. 5a–d).

Free enzyme showed a typical spectrum of proteins; with bands associated to their characteristic amide group (CONH). In this case, in the range of 1650–1540 cm^{-1}, there is an amide I band due to the double bond CO stretching, the CN stretching and NH bending [13]. Those bands are very faint in the spectra for the immobilized preparations, but reveal the presence of primary and secondary amino groups (lipase), particularly in the samples of 18 and 24 h gelation times. FTIR investigation of single-step acid and base-catalyzed silica by Brinker and Scherer [4] showed that acid-catalyzed conditions resulted in larger concentration of internal hydroxyl groups. Fig. 5a–d sequences of infrared spectra show a progressive reduction in the ~3400 cm^{-1} band assigned to internal and mutually hydrogen-bonded surface hydroxyls, for samples 18, 24, and 48 h, associated with Region II in the TGA curves (Fig. 5a–d). Similar behavior has been documented in numerous silicate and multicomponent silicate systems, confirming the relationship between shrinkage and condensation reactions of different precursor types in Region II as displayed in Fig. 2.

3.6. Olive oil hydrolysis

Olive oil hydrolysis relative POH% results obtained with the immobilized preparations were 100, 91.52, 99.50 and 69.82 after 60 min reaction time, for the gelation times of 4, 18, 24 and 48 h, respectively. Therefore, the highest relative percentage of hydrolysis of the olive oil was attained with the immobilized lipase produced with 4 h gelation time. However, the structure and texture characterization of this immobilized biocatalyst by thermogravimetric analysis (TGA) showed higher weight loss upon heating, as observed in Fig. 2, thus suggesting mechanical strength limitations for its use in reactors. The POH% value was not proportional to the immobilized biocatalyst gelation time and its variation was not monotonic, suggesting that different mechanisms are influencing it. The higher external surface porosity observed with the SEM tests for the lower gelation times is consistent with the fact that the immobilized biocatalyst produced with 4–24 h gelation times gave higher values for the POH%, suggesting that for the latter material external diffusion restrictions were less severe than for the 48 h immobilized biocatalyst. Taking into consideration the conjunction of high thermal stability as shown in the TGA tests and high performance for hydrolysis, the immobilized biocatalyst produced with 24 h gelation time is favored. This may be correlated to the diffusional limitations in the hydrolysis reaction caused by the cluster size distribution resulting from increasing gelation time, which influenced cluster growth in the hydrophobic matrices or immobilized biocatalyst.

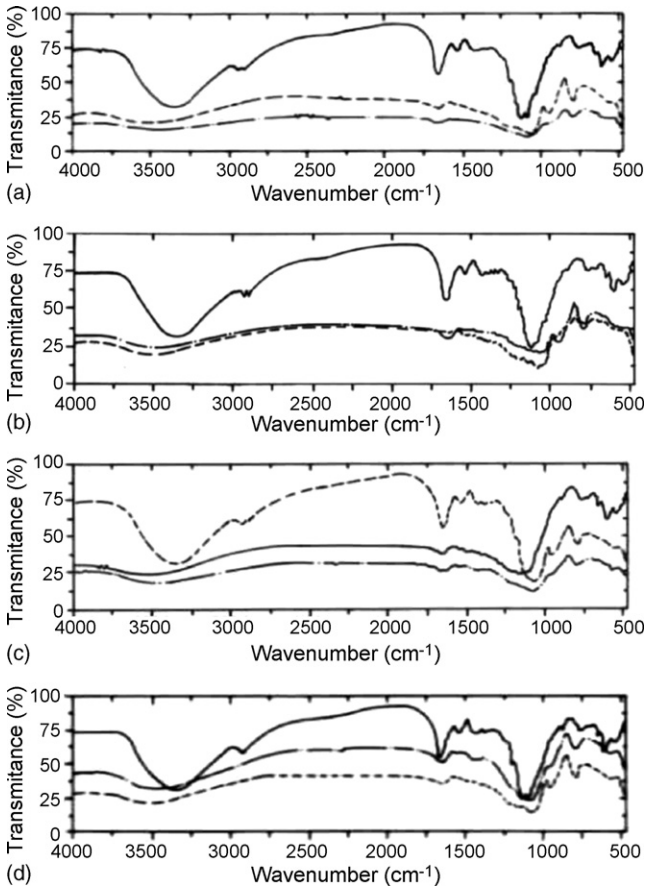

Fig. 5. FTIR for free lipase (solid line); hydrophobic matrices (dashed line) and immobilized preparations (solid-dashed line) using different gelation times: (a) 4 h; (b) 18 h; (c) 24 h and (d) 48 h.

4. Conclusions

Different gelation times (4,18, 24 and 48 h) used for the preparation of silica sol–gel encapsulated *C. rugosa* lipase, using tetraethoxysilane (TEOS) as precursor, did not show significant differences in the percentage of micropores and mesopores in the immobilized biocatalyst solid matrices, but pore volume with pure silica, initially increased with aging and then passed through a maximum. The presence of lipase increased pore volume for aging periods of 18 and 24 h. Taking into consideration the thermal stability of the immobilized biocatalyst and its performance for olive oil hydrolysis, 24 h of gelation time produced the encapsulated lipase with the most adequate set of parameters.

Acknowledgments

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