Combined Sol-gel Entrapment and Adsorption Method to Obtain Solid-Phase Lipase Biocatalyts

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Sol-gel entrapment combined with adsorption was used to obtain biocatalysts for kinetic resolution of secondary alcohols. Lipase AK "Amano" 20 (from Pseudomonas fluorescens) was immobilized using methoxy- or ethoxysilanes with alkyl or phenyl nonhydrolyzable groups as precursors and Celite 545 as deposition support. Increasing the hydrophobicity of methyltriethoxy-/tetraethoxysilane matrix was advantageous for the activity of the immobilized preparate up to 2:1 molar ratio, resulting in 87% recovered total activity for 2-octanol substrate. Enantioselectivities of preparates based on methoxysilanes were higher than for ethoxysilane precursors, the enantiomeric ratio E of 2-heptanol substrate reaching 37 when octyltrimethoxysilane and tetramethoxysilane have been used in 1:1 molar ratio.

Keywords: immobilized lipase, sol-gel entrapment, adsorption, enantioselective acylation, secondary alcohols

Enzymes represent the most promising biomolecules with potentially large-scale applications in synthetic organic chemistry, but high price of the biocatalyst and non-satisfactory operational stability limited their industrial applications mostly to high-value compounds, as chiral intermediates or products [1-3]. Immobilization has been proved as one of the best techniques to maintain biological activity in potentially adverse conditions for a longer period. The main advantage of immobilization is the facility to isolate the biocatalyst from the reaction mixture and reuse it several reaction cycles in order to increase its productivity. Various immobilization methods have been developed, and several issues have to be addressed to select the optimal one for a specified application [4, 5]:

- the immobilization should not involve the amino acid residues from the catalytic center of the enzyme;
- the immobilization reaction conditions should not be inhibitory for the enzyme;
- enzyme leakage from the carrier during operation must be minimal;
- the immobilization procedure must be cheap and reproducible.

The immobilization method might differ for every enzyme and application, but it is difficult to meet all requirements. Each technique is unique and therefore the possibilities to improve enzyme characteristics like activity, selectivity, and stability, are limited. It looks obvious that a rational combination of immobilization methods could maintain the advantages of every technique and eliminate the drawbacks, but complementarity is necessary in order to insure that the "preimmobilized" enzyme could be subjected to the second method. Such a possibility is to combine enzyme immobilization based on adsorption with a subsequent method, like entrapment. Adsorption is an easily available and cheap technique having as main drawback the leakage of the immobilized enzyme from the carrier [6]. To prevent leakage, the adsorbed enzyme can be further entrapped in a gel matrix. Penicillin V acylase was immobilized by such a way, first adsorbed on kieselguhr and then entrapped in polyacrylamide gel [7].

The sol-gel entrapment is an immobilization method that involves the entrapment of enzyme within a porous polymer matrix that allows reagents and products to flow through. Sol-gel processes are well studied in materials chemistry. The main advantage of using silica gels for enzyme immobilization is that they can be easily tailored to a large range of porous textures, network structures, surface functionalities and processing conditions. Moreover, the *p*H, gelation time, or hydrophobicity of the matrix can be adapted to a particular enzyme or application [8].

Sol-gel processes can be classified based on the precursor's inorganic or alcoxide nature. The alcoxide route is more important for enzyme encapsulation, as the hydrolysis and condensation reaction conditions are mild and the enzyme is not inactivated during the process. Sol-gels are obtained from alcoxysilanes by hydrolysis and condensation polymerization reactions, leading to the formation of a porous gel structure (fig. 1).

Additionally, the silica precursor can hold various nonhydrolizable organic functionalities directly linked to the silicium, that will be incorporated in the immobilization matrix allowing tailoring its hydrophobicity and assuring an appropriate microenvironment for the enzyme catalytic action. It was demonstrated that for successful sol-gel immobilization of lipases hydrophobic silica matrices prepared from tetraalcoxysilane and alkyl-substituted silane precursors $RSi(OCH_3)_3$, (R = alkyl) are required. The encapsulation of the activated lipase is possibly accompanied by the interaction of the lipophylic domain of the biocatalyst with the hydrophobic groups of the silica matrix. A displacement of the lid covering the active center of the lipase takes place, resulting in activation of the enzyme [9]. Another advantage of sol-gel encapsulation of enzymes is a possible stabilization of the enzyme tertiary structure by the relative rigidity of the gel structure [10].

Combination of adsorption and sol-gel entrapment could result in an immobilization procedure able to take advantage of both individual methods. As experimental technique, it can be realized by mixing the protein-

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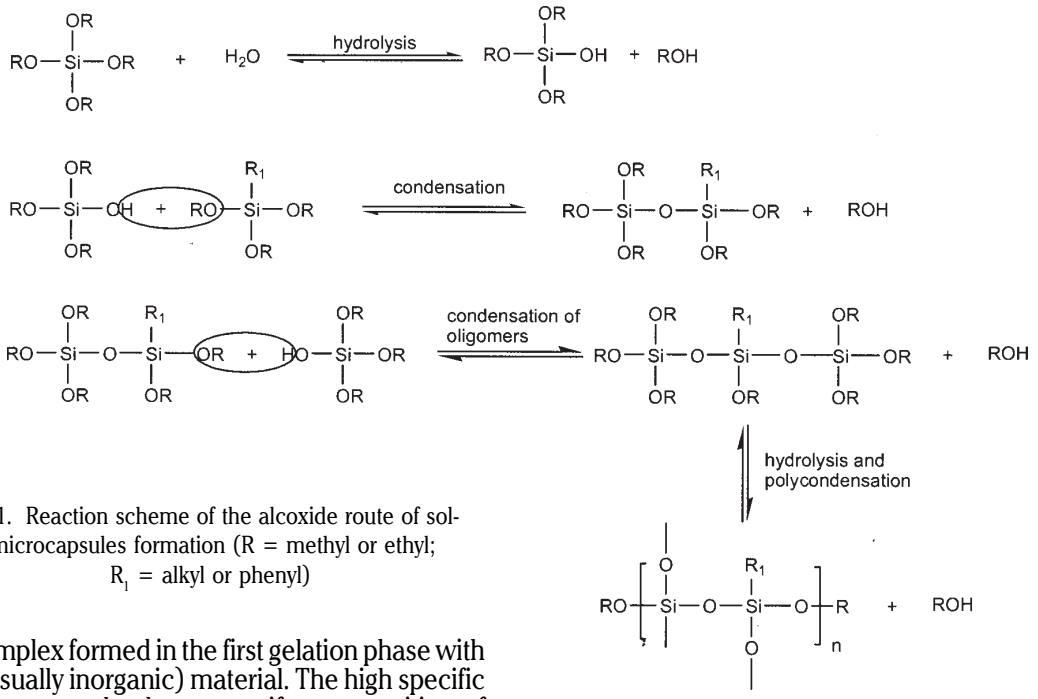

Fig 1. Reaction scheme of the alcoxide route of sol-gel microcapsules formation (R = methyl or ethyl; R_1 = alkyl or phenyl)

macromer complex formed in the first gelation phase with the support (usually inorganic) material. The high specific surface of the support leads to an uniform repartition of enzyme, avoiding aggregation and resulting in increased stability and better mechanical properties of the obtained preparate. The enzyme could also be stabilized against thermal inactivation resulted from protein unfolding. Previous studies proved that using high-porosity supports the gelation occurred in the internal part of pores and activity was low. Supports with lower porosity (as Celite 545) led to formation of gel only at the external surface of the support, resulting in higher activity [11, 12].

We have no knowledge about studies concerning the influence of combined sol-gel entrapment and adsorption immobilization on the enzyme enantioselectivity. Immobilization is considered a method to improve enzyme stability and not to influence enantioselectivity. However, our previous studies [10, 13] showed that lipase enantioselectivity could also be influenced by the immobilization procedure. In kinetic resolutions based on different reaction rates of the two enantiomers, steric and conformational effects are particularly important, and they can be influenced by the post-immobilization micro-environment around the catalytically active zone.

The present work was focused on study of activity and enantioselectivity of lipase immobilized by combined sol-gel entrapment and adsorption in acylation reactions of secondary alcohols. Vinyl acetate as acylation reagent and different reaction media have been used, influence of immobilization on thermal stability of the immobilized enzyme being tested, too.

Experimental part

Lipase AK "Amano" 20 (from *Pseudomonas fluorescens*) was a generous gift of Amano Enzyme Inc. (Japan). The silane precursors methyl- (MeTEOS), ethyl- (EtTEOS) and phenyltriethoxysilane (PhTEOS) were from Aldrich. Tetraethoxysilane (TEOS), propyl- (PrTMOS), octyl- (OcTMOS), and phenyltrimethoxysilane (PhTMOS), sodium fluoride, vinyl acetate, polyethylene glycol (PEG, M=20.000), were purchased from Fluka. Methyl-trimethoxysilane (MeTMOS), tetramethoxysilane (TMOS), Celite 545, n-hexane (98%), and racemic secondary alcohols: 2-butanol, 2-pentanol, 2-hexanol, 2-heptanol and 2-octanol were from Merck. Acetone (99%), and isopropyl alcohol (99.7%) have been bought from Chimopar, Romania. n-Hexadecane (99%, Merck) and n-decane (95%, Riedel de Haën) were used as internal standards for the chromatographic analysis.

Sol-gel entrapment was realized as previously described [10]. Lipase AK (100 mg/mL) was suspended in TRIS/HCl 0.1 M, pH 8.0 buffer, stirred at room temperature for 30 min, centrifuged, and the supernatant (protein content 24.7 mg/mL) was used for immobilization. In a 4 mL glass vial, Lipase AK filtrate (780µL), 4% w/v PEG (200µL), 1M NaF (100µL), and isopropyl alcohol (200µL) were mixed. By continuous stirring, a mixture of silane precursors (total 6 mmoles) was added. The resulting mixture was stirred at room temperature until the gelation started. The gel was kept for 24 h in the refrigerator to complete polymerization. The bulk gel was washed with isopropyl alcohol (10 mL), TRIS/HCl 0.1M, *p*H 8.0 buffer (10 mL), isopropyl alcohol (10mL) and hexane (10 mL) and dried at room temperature 48 h. Finally, it was crushed in a mortar and kept in the refrigerator.

Sol-gel entrapment with deposition on Celite was performed employing the method proposed by Kawakami and Yoshida [11], modified by supplementing with NaF catalyst. For the sol prepolymer, 6 mmole silane precursors (in different ratios) were mixed with 1 mL ethanol, 0.5 ml distilled water and 11 µL HCl 0.04 M. This mixture was magnetically stirred for 1h and subsequently 150 mg lipase suspended in 2.2 mL TRIS/HCl buffer (*p*H 8.0, 0.1M) and 100 µL NaF 1M were introduced. 1 g Celite 545 was added to this mixture when the gelation started. The resulted material was kept for 24 h at room temperature to complete maturation, then washed with isopropyl alcohol, TRIS/HCl buffer (0.1M, pH 8.0), isopropyl alcohol again and hexane. Finally it was dried 48 h at room temperature.

Lipase adsorption on Celite was realized by adding 1 g Celite to a solution of lipase (150 mg in 4 mL TRIS/HCl 0.1 M, *p*H 8.0 buffer). The suspension was stirred 30 min, then 40 mL cold acetone (-15°C) were added, in droplets. The resulted suspension was filtered, washed with cold acetone and dried at room temperature.

The reaction mixture for the acylation studies consisted from secondary alcohol (1 mmole), vinyl acetate (3 mmole), hexane (2 mL) and free (5 mg) or immobilized (50 mg) Amano AK lipase. 4 mL glass vials containing the reaction mixture were stirred at 300 strokes/min (MIR S-

100 Sanyo orbital shaker), at 40°C (POL EKO-CLN 115 STD incubator), for 24 h.

Samples taken at different intervals were analyzed for conversion and enantiomeric excess by a Dani 86.10 gas chromatograph equipped with flame ionization detector, using a 30 m x 0.32 mm CYDEX-B chiral column (SGE, Australia). The analysis conditions were set as follows: oven temperature: 50 to 120°C with 10°C/min heating rate, injector temperature 240°C, detector temperature 280°C, carrier gas (helium) flow 0.6 mL/min. Only the enantiomers of the formed ester products have been separated on this column. Transesterification efficiencies were calculated based on the alcohol conversion at 24 h and expressed as the average amount of the obtained 2-acetoxy-ester (in micromoles) per hour by 1 mg of free or immobilized enzyme. The total activity recovery yields (in %) have been calculated as the molar ratio of the acylated alcohol in 24 h by 1 mg of sol-gel immobilized lipase and 1 mg of free enzyme, respectively. Based on and literature data [14], it was assumed that Amano AK lipase is (*R*)-specific in the acylation reaction of the tested secondary alcohols, therefore the main product was considered the (*R*)-ester. Enantiomeric excess (ee_p) of the resulted ester product was calculated based on the enantiomers peak area. Enantiomeric ratio (*E*) was calculated according to Sih et al., [15], using the formula:

$$E = \frac{ln[1 - C(1 + ee_P)]}{ln[1 - C(1 - ee_P)]}$$ C representing the conversion

SEM images have been recorded using an Inspect S + EDAX Genesis XM 2i Scanning Electron Microscope (FEI Company).

Results and discussion

Activity and selectivity of the immobilized Amano AK lipase preparations resulted by combined sol-gel entrapment and adsorption have been studied. The model reaction was acylation of secondary aliphatic alcohols by vinyl acetate in organic solvent (hexane). Native lipase and lipase immobilized by simple adsorption were used as reference. Tetramethoxy- or tetraethoxysilane were used as precursors, mixed with alkyl- or phenyl-substituted trimethoxy- and triethoxysilanes, respectively. Reaction temperature was set at 40°C. The parameters employed to characterize the immobilization efficiency were effective activity, total activity yield, enantiomeric excess and enantiomeric ratio, all measured at 24 h reaction time.

A study of influence of silane precursors molar ratio was accomplished using methyl-triethoxysilane (MeTEOS) as the second precursor and four secondary alcohols: 2-butanol, 2-pentanol, 2-hexanol and 2-octanol as substrates. The activities, activity yields, and enantioselectivities of the resulted preparates are shown in table 1. Simple adsorption gave higher activities but lower enantio-selectivities than sol-gel entrapment combined with adsorption. Entrapment in sol-gels made only from TMOS resulted in xerogels without transesterification activity, and at low methyl group concentration (1:1 molar ratio) also significant activity loss of the immobilized enzyme was observed (30-45% total activity was recovered). Increase of methyl group concentration in the precursor mixture was beneficial until 2:1 MeTEOS/TEOS molar ratio, but increase to 4:1 caused only a slight further increase or even a decrease of activity.

Substrate	Immobilization method/ MeTEOS:TEOS molar ratio	Activity[a] (μmol·h^{-1}·mg^{-1})	Activity yield[b] (%)	ee	E
2-butanol	Native lipase /-	2.458	-	7	1
	Adsorption/-	0.398	160	4	1
	SGEA /1:1	0,115	39	8	1
	SGEA /2:1	0,247	84	13	2
	SGEA /4:1	0,263	89	10	2
2-pentanol	Native lipase	2.380	-	47	7
	Adsorption	0.343	142	13	4
	SGEA /1:1	0,071	25	71	7
	SGEA /2:1	0,180	63	61	7
	SGEA /4:1	0,189	66	56	7
2-hexanol	Native lipase	2.446	-	73	17
	Adsorption	0,345	139	26	6
	SGEA /1:1	0,132	45	78	12
	SGEA /2:1	0,211	72	78	18
	SGEA /4:1	0,198	67	72	14
2-octanol	Native lipase	2.095	-	55	6
	Adsorption	0.388	182	31	7
	SGEA /1:1	0,085	34	62	5
	SGEA /2:1	0,217	87	58	7
	SGEA /4:1	0,174	69	59	6

Table 1
INFLUENCE OF NONHYDROLYZABLE GROUP CONCENTRATION ON ACTIVITY AND ENANTIOSELECTIVITY OF AMANO AK LIPASE IMMOBILIZED BY SOL-GEL ENTRAPMENT AND ADSORPTION (SGEA)

[a] transesterification activity measured at 24h reaction time

[b] total immobilized activity vs. total activity of the enzyme subjected to immobilization

Table 2
INFLUENCE OF SILANE PRECURSOR NATURE ON 2-HEXANOL ACYLATION BY VINYL ACETATE, USING AMANO AK LIPASE IMMOBILIZED BY SOL-GEL ENTRAPMENT COMBINED WITH ADSORPTION

Silane precursors	Activity[a] ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)	Activity yield[b] (%)	ee	E
Native lipase	2.446	-	73	17
Adsorption	0,345	139	26	6
MeTEOS:TEOS	0,132	45	78	12
EtTEOS:TEOS	0.213	88	70	11
PhTEOS:TEOS	0,232	101	69	15
MeTMOS:TMOS	0,057	22	99	233
PrTMOS:TMOS	0,106	45	84	15
OcTMOS:TMOS	0,182	86	75	13
PhTMOS:TMOS	0,113	37	83	14

a. transesterification activity measured at 24h reaction time

b. total immobilized activity vs. total activity of the enzyme subjected to immobilization

As concerns enantioselectivity, neither the native enzyme nor the immobilized preparates were able to discriminate the optical isomers of 2-butanol. The other secondary alcohols were acylated at slightly higher enantiomeric excess by the immobilized preparates than by the native lipase, 2-hexanol being the best substrate. Simple adsorption led to an important decrease of enantioselectivity. The highest enantiomeric excess and enantiomeric ratio values were observed for the preparate obtained at 1:1 precursor molar ratio, but associated with lower activities, as mentioned. Therefore, it can be concluded that the optimum precursor ratio for immobilization by entrapment and adsorption using MeTEOS and TEOS was 2:1, resulting in 63-87% recovered total activity and 58-78% enantiomeric excess values for acylation of the mentioned secondary alcohols.

The nonhydrolizable group nature showed considerable influence on the efficiency of the obtained biocatalyst. Preparates obtained with different precursors showed total activity recovery values in a wide range, between 22-101%, for the acylation of 2-hexanol (table 2). Molar ratio of precursors was 1:1, reactions were carried out at 40°C, in hexane, and 3:1 vinyl acetate/alcohol molar ratio. It is usual to have a partial inactivation of the enzyme during the immobilization processes. In sol-gel entrapments ethanol and methanol, introduced as reaction medium for sol formation or resulted from the polycondensation, could have inhibitory effect. As the nonhydrolyzable group was phenyl or methyl, important differences between the preparates based on ethoxy- or methoxysilanes have been noticed, suggesting a more significant inhibitory effect of methanol. Increase of the alkyl chain length was beneficial for the activity, demonstrating that hydrophobic effects in the immobilization matrix are important even if adsorption interactions are also present. Phenyl group was the most effective for preparates obtained from ethoxysilane-based precursors, and octyl for those based on methoxysilanes. Excepting the MeTMOS/TMOS preparate, the enantiomeric ratio (E) values were not significantly different compared to the native lipase, even though the enantiomeric excess values at 24h reaction time were higher for the immobilized lipases. The advantage of high enantiomeric ratio value (E=233), obtained for the above mentioned preparate was invalidated for a possible large-scale use of the biocatalyst by the associated low activity. We tried several different experimental protocols to overcome this problem, but notable improvements could not be achieved (data not shown).

It would be important to know how the immobilization process occurs, by entrapment of the enzyme and simultaneous deposition of the sol-gel layer, or by formation of a sol-gel layer around the previously adsorbed enzyme. We tried to facilitate the first mechanism, using an immobilization technique that involved addition of the adsorbent (Celite) only after beginning of gelation. Scanning electron microscopy (SEM) was used to investigate the morphology of the immobilized preparates. From figure 2 looks plausible that the sol-gel entrapped enzyme was deposited on the adsorbent surface. The whole preparate is amorphous and we can suppose that the adsorption was realized mainly at the surface, not in the internal structure of the adsorbent. The enzyme deposited in inner pores partially could loose its activity due to more difficult access of the reagents to the active center.

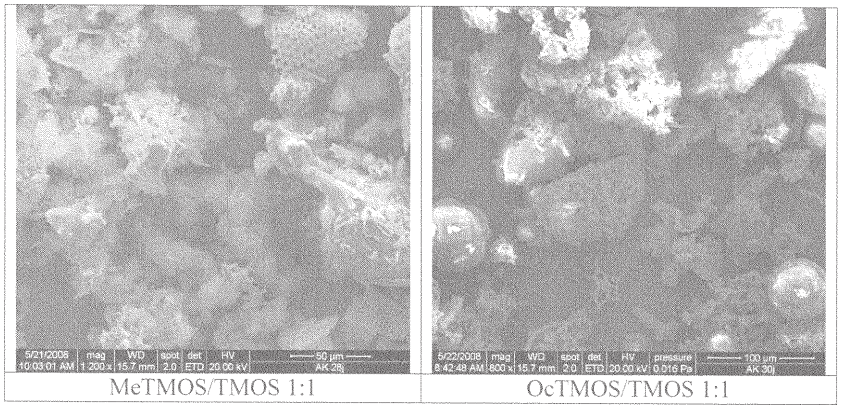

Fig. 2. Scanning electron micrograph images of immobilized Amano AK lipase preparates obtained by sol-gel entrapment and adsorption, employing tetramethoxysilane and (**a.**) methyl-trimethoxy- and (**b.**) octyl-trimethoxysilane, respectively

Table 3

INFLUENCE OF SECONDARY ALCOHOL CHAIN LENGTH ON CATALYTIC EFFICIENCY OF AMANO AK LIPASE IMMOBILIZED BY THE COMBINED SOL-GEL ENTRAPMENT AND ADSORPTION METHOD. SILANE PRECURSORS WERE OCTMOS AND TMOS, 1:1 MOLAR RATIO

Substrate	Activity[a] ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)		Activity yield[b] (%)	Enantiomeric excess (%)		E	
	Native	Immob.		Native	Immob.	Native	Immob.
2-butanol	2.458	0.103	48	7	11	1	1
2-pentanol	2.380	0,155	75	47	64	7	7
2-hexanol	2.446	0,182	86	73	75	17	13
2-heptanol	2.630	0,146	64	74	91	9	37
2-octanol	2.095	0,230	127	55	60	6	9

a. transesterification activity measured at 24h reaction time

b. total immobilized activity vs. total activity of the enzyme subjected to immobilization

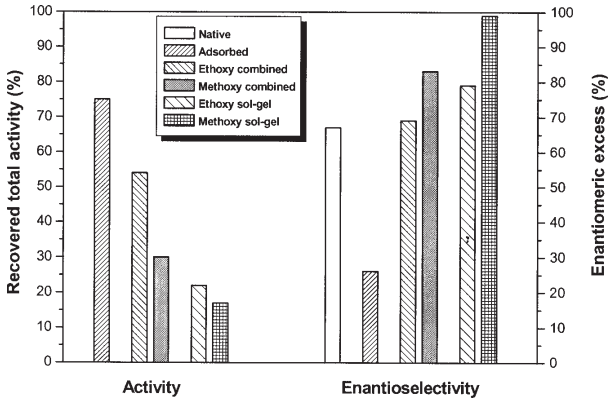

Fig. 3. Activity and enantioselectivity of Amano AK lipase immobilized by different methods: adsorption, sol-gel entrapment based on ethoxy- and methoxy-substituted silanes, and sol-gel entrapment combined with adsorption

A study of secondary alcohol chain length was carried out for the most efficient preparate from methoxysilane precursors, based on OcTMOS and TMOS. Acylation reactions were carried out 24h by vinyl acetate in hexane at 40°C. As results from table 3, the highest activity yield of immobilization was measured for the secondary alcohol with the longest alkyl chain (2-octanol), and the enantioselectivity reached the highest value for 2-heptanol. A more effective hydrophobic character, not only concerning the sol-gel matrix but also the substrate structure, was beneficial for the immobilized preparate activity. The recovered total activity increased above 100%, demonstrating the efficiency of the immobilization method. The enantiomeric excess was in all cases higher for the immobilized enzyme compared to the native lipase, even though the differences were not spectacular. As concerns enatiomeric ratio (E) values, only in the case of 2-heptanol the immobilized preparate demonstrated a notable enantioselectivity increase, associated with good activity.

A comparative evaluation of immobilization by adsorption, sol-gel entrapment and combined method was made for enantioselective acylation of 2-hexanol, using silane precursors containing phenyl nonhydrolizable group (PhTMOS/TMOS and PhTEOS/TEOS in 1:1 molar ratio, respectively). As results from figure 3, immobilization by adsorption gave the highest activity yield, but the enantioselectivity of the resulted preparate was low. Considering both sol-gel entrapment and combined methods, ethoxy-silane precursors have been more efficient as concerns the activity yield of the resulted immobilized enzymes, while the enantioselectivities of the preparates obtained from methoxy-silane precursors were higher. Comparing the results of sol-gel entrapment and combined method, it is obvious that deposition of the sol-gel entrapped enzyme on Celite led to higher recovery of the total activity. The explanation is relatively easy, as the repartition of the entrapped enzyme on a large surface can reduce the risk of enzyme aggregation during the immobilization process and avoid diffusional limitations. As concerns the enantioselectivity, it was higher for sol-gel entrapped preparations, but those obtained by the combined method also showed higher values as the native enzyme.

Conclusions

Solid-phase lipase biocatalysts have been already proved as important synthesis tools for organic chemists, especially in chiral resolutions of racemic mixtures. Sol-gel entrapment combined with adsorption was demonstrated to improve enzyme stability and activity, but did not change significantly the enantioselectivity of the preparates compared to the native enzyme. Both ethoxysilane and methoxysilane derivatives are valuable precursors of the sol-gel matrix, ethoxysilanes leading to preparates with higher activities as gelation time is longer and the thermic effect of the condensation reactions do not generate over-heating of the microenvironment that could inactivate the enzyme. The obtained preparations will be considered as biocatalysts to obtain derivatives of 3-hydroxy-butyric acid, too.

Acknowledgements: The authors would like to acknowledge Amano Enzymes Inc. for supplying the lipase and Ms. Paula Sfârloagă from the National Institute of Electrochemistry and Condensed Matter Timişoara for the SEM analysis. This work was supported by the National Authority for Scientific Research (ANCS) by grant PNII-IDEI nr. 368/2007.

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Manuscript received: 13.10.2008