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Preparation and properties of xerogels obtained by ionic liquid incorporation during the immobilization of lipase by the sol–gel method

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ABSTRACT

Lipase from *Pseudomonas fluorescens* (Amano AK) has been immobilized by the sol–gel method using tetramethoxysilane and trimethoxysilanes with alkyl or aryl groups as precursors and ionic liquids as immobilization additives. Room temperature ionic liquids (ILs) based on imidazolium cations with different hydrophobicities and inorganic or organic anions were investigated. The biocatalytic activity in the acylation reaction of secondary aliphatic alcohols by vinyl acetate increased at lower polarity of the IL, being 58% higher in case of octyl than for ethyl substituent of the alkylimidazolium cation. The optimum IL/silane molar ratio in the immobilization mixture was around 0.2. Hydrophobic groups from both the silane precursor and IL converge to the optimal microenvironment for the enzyme action. ILs as reaction media led to higher activities as common organic solvents in the kinetic resolution of secondary alcohols, but the highest enantioselectivities (enantiomeric ratio *E* values > 50) have been observed in acetone and tetrahydrofuran. Structural investigation of the preparates by SEM–EDX and FT-IR has confirmed the partial confinement of the IL in the xerogel structure.

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1. Introduction

Biocatalytic reactions in non-aqueous solvents have been attested in the last decades as realistic alternatives to replace conventional chemical procedures, especially when increased regio- or enantioselectivity was needed [1–3]. However, utilization of volatile organic solvents could be harmful for the environment, even if their recovery is usually performed at the end of the reaction. ILs have been attracted a lot of interest among non-aqueous reaction media, recommended by favorable properties as very low vapor pressure, high thermal stability, miscibility adjusted by the appropriate selection of cationic and/or anionic moiety [4]. Biocatalytic performance of enzymes in ILs was the subject of extensive investigations and it was obviously demonstrated that in several cases was not only maintained, but rather increased compared to conventional media [5,6]. Gubicza et al. showed that *Candida rugosa* lipase exhibited higher activity and enantioselectivity in the esterification of (*R*,*S*)-2-chloropropanoic acid in ionic liquids compared to that in hexane [7,8]. As enzymes are not soluble in most ILs when used as pure solvents, they have been employed either in immobilized or suspended native form. Based on process efficiency parameters, immobilized enzymes seem to be the right option, considering their improved operational and thermal stability [9]. Novozyme 435 has been used with excellent results for the synthesis of isoamyl acetate by direct esterification in ILs, maintaining its catalytic activity at the same level following 10 reuse cycles [10].

Although ionic liquids are documented primarily as valuable reaction media to replace volatile organic solvents, they could have other interesting applications like additives during the sol–gel immobilization process of enzymes. Sol–gel encapsulation has proved to be a versatile technique for the immobilization of a large variety of biomolecules [11]. Hybrid organic–inorganic sol–gel matrices are formed by the hydrolysis of silane precursors, tetra-alcoxysilanes mixed with alcoxysilanes carrying hydrophobic groups, followed by condensation reactions to yield silica. Silica particles grow gradually as condensation proceeds, leading to the formation of colloidal sols and further gels. Whether the formed gels are allowed to maturate and dry at room temperature, a porous silica network is formed, with the enzyme entrapped in the sol–gel matrix and the hydrophobic groups partly covering the surface [12]. Lee et al. showed that ILs can be used to protect *Candida rugosa* lipase against inactivation by the released alcohol and shrinking of gel during the maturation and drying step of the sol–gel immobi-

Abbreviations: $[Emim]^+$, 1-ethyl-3-methyl-imidazolium; $[Pmim]^+$, 1-propyl-3-methyl-imidazolium; $[Bmim]^+$, 1-butyl-3-methyl-imidazolium; $[Hmim]^+$, 1-hexyl-3-methyl-imidazolium; $[Omim]^+$, 1-octyl-3-methyl-imidazolium; $[Tf_2N]^-$, bis(trifluoromethyl-sulfonyl)imide; MeTMOS, methyl-trimethoxysilane; PrTMOS, propyl-trimethoxysilane; OcTMOS, octyl-trimethoxysilane; PhTMOS, phenyl-trimethoxysilane; TMOS, tetramethoxysilane; DMF, N,N-dimethylformamide; THF, tetrahydrofuran; MTBE, methyl *t*-butyl ether; IL(s), ionic liquid(s); SEM–EDX, scanning electron microscopy coupled with energy dispersive X-ray spectroscopy.

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lization process [13]. Karout and Pierre find that ILs as additives increased the gelation time, influenced the pore size distribution and some residual ILs remained attached to the sol–gel network [14]. Our preliminary results demonstrated that the catalytic efficiency of microbial lipases in the acylation reaction of secondary alcohols could be enhanced by the presence of hydrophobic alkyl groups in the hybrid sol–gel matrix and utilization of ILs as non-structural template compounds [15].

In the present work, ionic liquids containing dialkylimidazolium cations with different hydrophobicities and organic or inorganic anions have been investigated as structure-directing immobilization additives for sol–gel entrapped *Pseudomonas fluorescens* (Amano AK) lipase. The influence of immobilization conditions and reaction media on catalytic efficiency of the immobilized preparates was also studied in ILs, compared to common organic solvents.

2. Experimental

2.1. Materials

Lipase from *Pseudomonas fluorescens* (Amano Lipase AK) was purchased from Aldrich. Silane precursors propyl- (PrTMOS), octyl- (OcTMOS), and phenyl-trimethoxysilane (PhTMOS), as well as N,N-dimethylformamide (DMF) were purchased from Fluka. Methyl-trimethoxysilane (MeTMOS), tetramethoxysilane (TMOS), n-hexane, 2-propanol, acetonitrile, tetrahydrofuran (THF), toluene, methyl *t*-butyl ether (MTBE), sodium fluoride, vinyl acetate, 2-hexanol, and 2-heptanol were from Merck. All the specified reagents were of analytical grade and have been used as purchased. Dodecane (99%, Merck) and decane (>99%, Aldrich) were used as internal standards for the gas-chromatographic analysis.

Ionic liquids 1-ethyl-3-methyl-imidazolium tetrafluoroborate [Emim]BF_4, 1-ethyl-3-methyl-imidazolium acetate [Emim][$COOCH_3$], 1-ethyl-3-methyl-imidazolium trifluoroacetate [Emim][$COOCF_3$], 1-propyl-3-methyl-imidazolium tetrafluoroborate [Pmim][BF_4], 1-butyl-3-methyl-imidazolium tetrafluoroborate [Bmim][BF_4], 1-hexyl-3-methyl-imidazolium tetrafluoroborate [Hmim][BF_4], 1-butyl-3-methyl-imidazolium hexafluorophosphate [Bmim][PF_6], 1-butyl-3-methyl-imidazolium bis(trifluoromethyl-sulfonyl)imide [Bmim][Tf_2N], were purchased from Merck at the highest available quality. 1-Octyl-3-methyl-imidazolium tetrafluoroborate [Omim][BF_4] was from Fluka.

2.2. Immobilization Method 1, for preparates from silane precursors PrTMOS, OcTMOS and PhTMOS

The immobilization method previously described [16] and based on the Reetz procedure [17] has been used, modified by replacing polyethyleneglycol additive with ionic liquid, as indicated in the scheme presented in Fig. 1. Lipase AK (100 mg/mL) was suspended in Tris/HCl buffer solution 0.1 M, pH 8.0, stirred at room temperature for 30 min, centrifuged 5 min at 25 °C and 3000 rot/min (Boeco U-320R centrifuge, Boeckel, Germany), and 780 μL of the supernatant (containing 74 mg dissolved enzyme) have been used for immobilization. The immobilization was accomplished in a 4 mL glass vial, by mixing the lipase solution with 200 μL ionic liquid, 100 μL 1 M NaF aqueous solution and 200 μL isopropyl alcohol (magnetic stirring, 200 rot/min). Silane precursors (total 6 mmoles) were added, and mixing continued at room temperature until the gelation started. The formed gel was kept for 24 h at room temperature to complete polymerization. Subsequently, the bulk gel was washed to eliminate unreacted monomers and additives with isopropyl alcohol (10 mL), Tris/HCl buffer solution 0.1 M, pH 8.0 (10 mL), isopropyl alcohol again (10 mL) and hexane (10 mL). The sol–gel encapsulated enzyme was dried in a vacuum oven at 25 °C for 8 h, crushed in a mortar and kept in a closed vessel in the refrigerator.

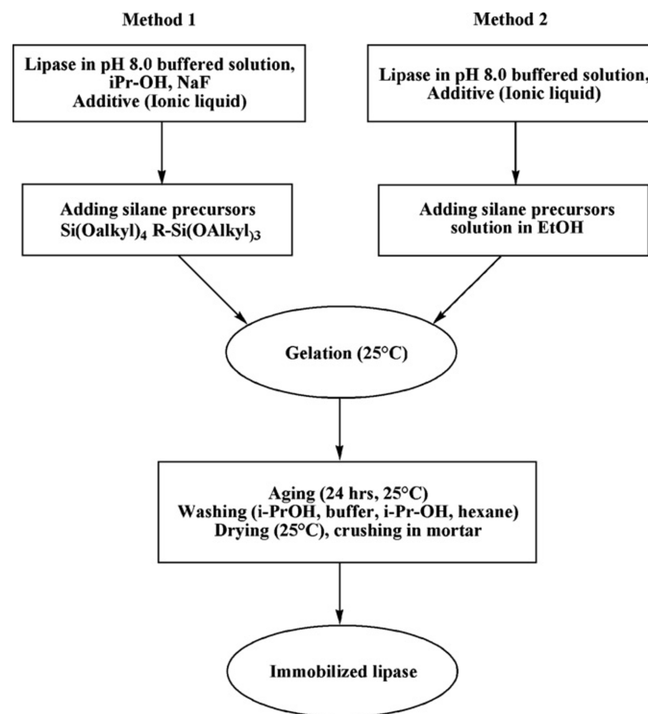

Fig. 1. Scheme of immobilization methods used.

2.3. Immobilization Method 2, for preparates from silane precursor MeTMOS

This method, based on the methodology proposed by Orçaire et al. [18] was employed for preparates obtained from MeTMOS and TMOS silane precursors, as those obtained according to *Method 1* exhibited very low activities, probably resulted from a too dense structure of the sol–gel. In a 4 mL vial 780 μL of lipase AK solution in Tris/HCl buffer 0.1 M, pH 8.0, obtained as indicated in *Method 1*, were mixed with 200 μL ionic liquid and magnetically stirred for 15 min. In a second 4 mL vial, the silane precursors (total 6 mmoles) and ethanol (0.5 mL) were mixed for 15 min (magnetic stirring, 200 rot/min, room temperature). Subsequently, the two solutions were mixed, and magnetically stirred until gelation occurred. The resulting gel was kept at room temperature for 24 h to complete polymerization. The bulk gel was washed and dried as described in *Method 1*.

2.4. Acylation of racemic secondary alcohols by vinyl acetate

Acylations have been made in 4 mL capacity glass vials, charged with a mixture of secondary alcohol (2-hexanol or 2-heptanol, 1 mmole), vinyl acetate (3 mmole), reaction medium (organic solvent or ionic liquid, 2 mL) and free (5 mg) or sol–gel immobilized (25 mg) AK lipase. The biocatalyst amounts were chosen to ensure an appropriate conversion during the selected reaction time (24 h). The solvents, alcohols and ILs used were separately equilibrated to 0.328 water activity at 25 °C over saturated $MgCl_2$ solution for 48 h, as indicated by Bell et al. [19]. The mixture was stirred using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40 °C (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). Samples taken at different time intervals were analyzed for conversion and

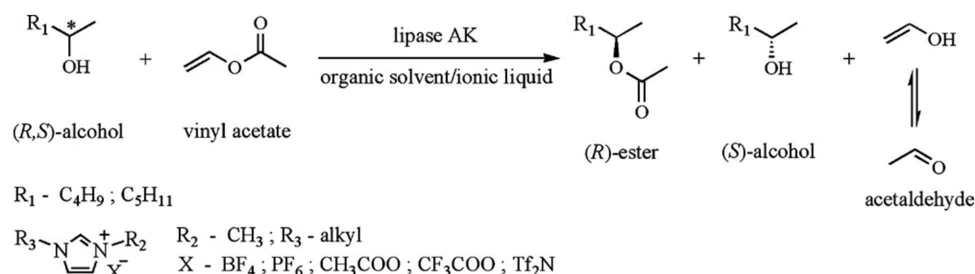

Fig. 2. Reaction scheme and structures of employed ionic liquids.

enantiomeric excess by a Varian 450-GC chromatograph equipped with flame ionization detector, using a 30 m × 0.25 mm Elite-Cyclosil B chiral column, 0.25 mm film thickness (PerkinElmer, USA). The analysis conditions were set as follows: oven temperature: 50–120 °C with 10 °C/min heating rate, injector temperature 240 °C, detector temperature 280 °C, carrier gas (hydrogen) flow 1.2 mL/min. The conversions were calculated based on the internal standard method, using the appropriate calibration curve for every alcohol.

Transesterification activities were calculated based on the alcohol conversion at 24 h and expressed as the average amount of formed 2-acetoxy-alcohol (in micromole) in 1 h interval by 1 mg of free or immobilized enzyme. The reaction without enzyme did not give any product in the same conditions. To characterize the overall efficiency of the immobilization process, total activity yield was calculated as % of the total enzymatic activity recovered following immobilization, related to the total activity of the lipase subjected to immobilization. As only the enantiomers of the ester products have been separated on this column, the enantiomeric excess of the resulted ester product (ee_p) was determined based on the enantiomers peak area. Accordingly, the enantiomeric ratio (E) values were calculated based on conversion and ee_p values, as proposed by Chen et al. [20].

All acylations have been run in duplicate and sampling was also made in duplicate. Because the differences between the four data points for the same assay were less than 2% for conversion and 1% for the enantiomeric excess, average values have been calculated and presented in the tables and figures.

2.5. Structural analysis

FT-IR spectra were recorded on a Prestige21 spectrophotometer (Shimadzu, Japan) on 400–4000 cm^{-1} range at 4 cm^{-1} spectral resolution, using the attenuated total reflectance (ATR) technique. Scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) was performed with an Inspect S + EDAX Genesis XM 2i system (FEI Company, The Netherlands). The analysis parameters were: pressure 1.5×10^{-2} Pa, resolution <10 nm at 3 kV.

3. Results and discussion

3.1. ILs as immobilization additives

Utilization of ILs as immobilization additives could have multiple influences on both the entrapment process and catalytic efficiency of immobilized enzyme. The polarity of IL and its miscibility with the silane precursors and water may influence the homogeneity of the mixture subjected to the sol–gel process, gelation characteristics, and structure of the formed hybrid sol–gel matrix. As regards catalytic efficiency, Dang et al. [21] demonstrated that pretreatment of lipase with ILs could result in enhanced activity and stability and it is obvious to presume such an effect in the course of lipase immobilization by the sol–gel method when ILs are used as additives.

To evaluate the catalytic efficiency of sol–gel immobilized lipase, we used as model reaction the kinetic resolution of two secondary alcohols, 2-hexanol and 2-heptanol, by acylation with vinyl acetate (Fig. 2). *Pseudomonas fluorescens* lipase is not highly enantioselective in this process, therefore it was also possible to investigate the effect of immobilization on the enantioselectivity. The time course of the mentioned reaction was not influenced by sol–gel immobilization. In case of the 2-heptanol substrate showed in Fig. 3, both conversion and enantiomeric excess curves were similar for native and immobilized lipase, in hexane reaction medium. The reaction progress curve over the studied time period (48 h) was not linear, as it is usual for the majority of biocatalytic reactions. Our aim was to characterize the catalytic performance of the immobilized enzyme, therefore we calculated the transesterification activity from a single-point experiment, based on the conversion at 24 h. Calculation of initial rates based on the linear period of the reaction to obtain kinetically valid activity values would have been difficult for preparates with low activities, as were those obtained with MeTMOS as precursor. Therefore, the term "activity" used in the present work and expressed as μmole h^{-1} mg $lipase^{-1}$, must be considered only for comparative evaluation of catalytic efficiency, not for the kinetic behavior of the biocatalyst. The transesterification activity (related to 1 mg biocatalyst) was obviously higher for the native enzyme, as the enzyme usually represents only 8–10% (w/w) of the sol–gel preparate amount. To characterize the global efficiency of the immobilization process, it is significant that the

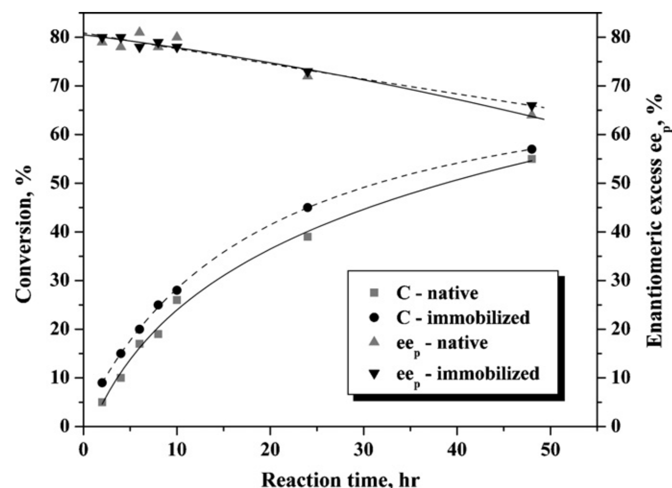

Fig. 3. Time course of 2-heptanol acylation reaction by vinyl acetate, in hexane at 40 °C, catalyzed by *Pseudomonas fluorescens* lipase, native and immobilized by the sol–gel method with OcTMOS and TMOS precursors (1:1 molar ratio) and [Omim][BF_4] as additive, at 0.322 IL/silane molar ratio. Conversion (C) curves, in the lower part, and enantiomeric excess (ee_p) curves in the upper part of the diagram were plotted only to illustrate the tendency of these parameters evolution, without kinetic signification.

Table 1
Influence of IL additive structure and polarity on the catalytic efficiency of sol–gel entrapped lipase in the acylation of racemic 2-heptanol by vinyl acetate in hexane medium. Immobilization was performed with OcTMOS and TMOS silane precursors at 1:1 molar ratio, IL/silane molar ratio was 0.129.

Ionic liquid	E_T^N	Conversion[e] (%)	Activity (μmole h^{-1} mg^{-1})	Total activity yield[f] (%)	ee$_p$ (%)	E
Reference (native lipase)	–	39	1.383	100	72	10
[Emim][BF$_4$]	0.71[a]	34	0.295	128	77	11
[Pmim][BF$_4$]	0.69[a]	43	0.370	209	73	11
[Bmim][BF$_4$]	0.68[a]	40	0.352	199	78	13
[Hmim][BF$_4$]	0.70[b]	42	0.360	177	75	12
[Omim][BF$_4$]	0.64[c]	53	0.467	229	68	12
[Emim][COOCH$_3$]	0.60[d]	41	0.344	167	74	11
[Emim][COOCF$_3$]	0.66[d]	32	0.276	116	79	12

[a] From Ref. [24].
[b] From Ref. [23].
[c] From Ref. [13].
[d] Estimations, based on the values for [Bmim]COOCH$_3$ and [Bmim]COOCF$_3$ from Ref. [23].
[e] Measured at 24-h reaction time.
[f] Total activity recovery yield following immobilization, as defined in Methods.

total activity recovered after immobilization was usually higher than the total activity of the enzyme subjected to immobilization. Particularly, for the reaction presented in Fig. 3, the transesterification activities at 24 h were 1.60 and 0.35 μmole h^{-1} mg lipase^{-1} for the native and immobilized lipase, respectively, resulting a total activity yield value of 156%. The enantiomeric excess was influenced by the conversion and decreased slowly during the reaction, but the enantiomeric ratio E remained in the same range (9–14) for both native and immobilized lipase.

Polarity is an important parameter influencing the interactions of ILs with enzymes. There are many possibilities to evaluate the polarity of ILs and organic solvents, defined as overall solvation capability. We used the empirical scale of relative solvent polarity E_T^N determined by the solvatochromic standard Reichardt's dye. This scale employs water (E_T^N 1.00) and tetramethylsilane (E_T^N 0.00) as reference solvents for high and low polarity, respectively [22,23].

Using 2-heptanol as model substrate, the catalytic efficiency of Amano AK lipase immobilized using IL as additive was among 20–34%, related to the native enzyme (Table 1). The total enzymatic activity increased next to immobilization for all ILs tested, as we found total activity up to 2.3-fold higher compared to the native enzyme.

Correlation of IL polarity with catalytic efficiency of the immobilized lipase is difficult, because E_T^N values reported by various groups for the same IL could differ considerably, probably influenced by the remaining water content or traces of other polar impurities. Nevertheless, it can be observed that increasing polarity of the IL resulted in lower activities. In the series of ILs with 1-alkyl-3-methyl-imidazolium cations and tetrafluoroborate anion, the activity of the preparate increased with the length of the alkyl group, being 58% higher in case of octyl than for ethyl. Lee et al. [13] reported results with opposite tendency compared to ours, as the specific activity of *Candida rugosa* lipase immobilized with [Emim][BF$_4$] additive was 8.7-fold higher than with [Omim][BF$_4$], in the acylation reaction of benzyl alcohol. Such differences could be explained by the different precursors and immobilization protocols employed. Conformational differences between the microbial lipases used in the two investigations could also affect the immobilized catalyst properties. However, our results are consistent with the well-known fact that a more hydrophobic microenvironment around the enzyme is beneficial for lipase activity. Among ILs with the same cation [Emim], the highest activity was also measured for the less polar [Emim][COOCH$_3$].

We also expected possible changes of enantioselectivity induced by the ionic liquid additive, but it was not the case. Although the measured ee$_p$ values of the product at 24-h reaction time showed some fluctuations (±5% from the mean value), the enantiomeric ratio E values were almost the same. Previously, we found more important changes of enantioselectivity in relation to the silane precursor's nature and relative concentration in the immobilization mixture [25]. It seems that the silane structure has a more significant function in this respect than the nature of IL additive.

The influence of increasing amount of IL additive was studied for the acylation reaction of 2-hexanol at 40 °C, in hexane reaction medium. [Omim][BF$_4$] has been used in molar ratios from 0.064 to 0.322, related to total silanes (Table 2). This IL is not miscible with water, but miscible with the silanes used as precursors. Increasing the relative amount of IL in the immobilization mixture allows maximizing its positive effect as structure-directing additive during the sol–gel process. The highest transesterification activities were obtained for an IL/silane molar ratio around 0.2. The enantioselectivity of the immobilized lipase was not strongly influenced by the IL amount, but it was slightly improved related to the native enzyme.

3.2. Catalytic properties of the sol–gel immobilized lipases in ionic liquids and organic solvents

The biocatalytic properties of the sol–gel immobilized lipases are strongly influenced by the nature and concentration of the nonhydrolyzable alkyl or aryl group of the silane precursor (RTMOS) used in combination with TMOS. Our previous results showed that a 2:1 RTMOS/TMOS molar ratio was still accurate to ensure both high activity and physical robustness of immobilized preparates [25]. This influence has been studied for three different ILs used as additives. The investigated model reaction was the acylation of 2-hexanol by vinyl acetate in hexane, at 40 °C.

Using silane precursors with different nonhydrolyzable groups and different ILs as additives resulted in immobilized lipases with activities ranging on a wide scale. Increasing the hydrophobic groups density in the sol–gel matrix was generally beneficial for

Table 2
Influence of [Omim][BF$_4$] additive amount on catalytic efficiency and enantioselectivity of 2-hexanol acylation catalyzed by sol–gel immobilized lipase. Silane precursors for immobilization were OcTMOS and TMOS (1:1 molar ratio), acylation has been run in hexane.

IL/silane molar ratio	Conversion (%)	Activity (μmole h^{-1} mg^{-1})	ee$_p$ (%)	E
Reference (native lipase)	29	1.217	78	11
0.064	50	0.411	75	16
0.129	59	0.494	63	13
0.193	67	0.591	49	15
0.257	63	0.548	57	14
0.322	47	0.419	75	14

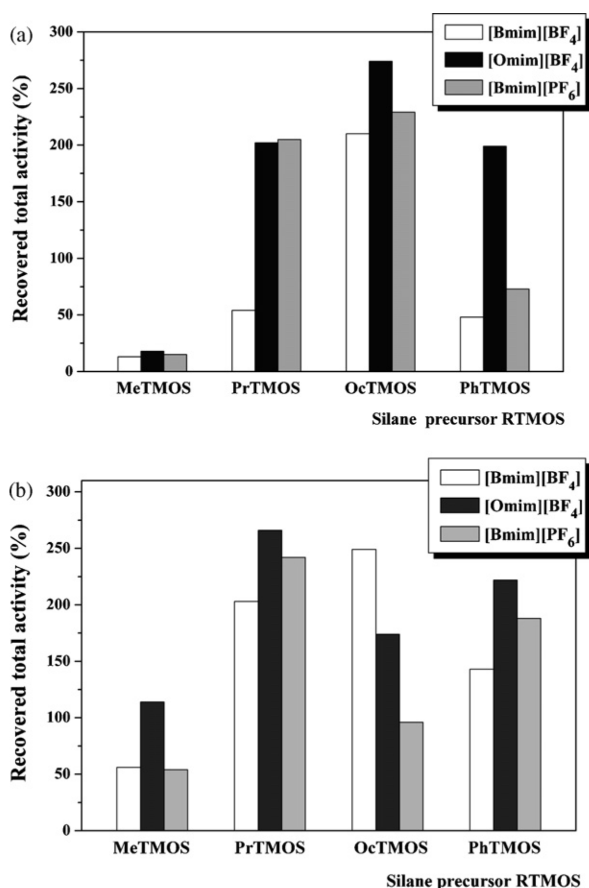

Fig. 4. Influence of the nonhydrolyzable group of silane precursor and ionic liquid structure on the relative total activity recovered after sol–gel immobilization of *Pseudomonas fluorescens* lipase, at (a) 1:1, and (b) 2:1 RTMOS/TMOS molar ratio. Acylation of 2-hexanol was made by vinyl acetate at 40 °C, in hexane.

lipase immobilization efficiency, expressed as total activity recovery yield (Fig. 4).

At 1:1 precursor molar ratio, the tendency was the same for every ionic liquid tested as additive, namely the activity increased with the alkyl group chain length from methyl to octyl (Fig. 4a). Using silane precursor with phenyl instead of alkyl nonhydrolyzable group, the activity of the best preparate was comparable, but the gelation process was usually slowed down and the reproducibility of immobilization results was uncertain. At 1:1 silane precursor molar ratio a more hydrophobic substituent in both silane precursor and cationic part of IL additive is needed to obtain a sol–gel immobilized lipase with increased activity, [Omim][BF_4] being the additive with the highest improvement of catalytic efficiency. However, using alkyltrimethoxysilanes with large alkyl groups at higher concentration could be detrimental for the immobilization process by decreasing the gelation rate and hindering the formation of the porous structure (Fig. 4b). Increasing the hydrophobic group density in the sol–gel matrix (2:1 RTMOS/TMOS molar ratio), the maximum immobilization efficiency shifted to the preparate obtained with the PrTMOS precursor. Considering the biocatalysts obtained with OcTMOS precursor and different IL additives, at this molar ratio the highest efficiency was assayed for [Bmim][BF_4], with a shorter alkyl chain. It results that the optimal content of hydrophobic groups in the silica matrix must be tuned carefully, considering all the components of this system as well as the structure of substrate.

The influence of reaction medium on catalytic efficiency of the lipase immobilized by the sol–gel method with IL additive was investigated in various ILs, as well as in common organic solvents. The immobilized preparates were obtained from OcTMOS and TMOS precursors with [Omim][BF_4] as IL additive. Selection of a suitable reaction medium is a difficult issue in biocatalysis because some organic solvents, commonly used in organic syntheses, can inactivate the enzymes. As regards lipases, the enzymes with the largest number of synthetic applications, it was demonstrated an overall trend toward higher activities in hydrophobic reaction media. The explanation of this phenomenon is related to nonpolar interactions leading to the displacement of a helical oligopeptide unit called "lid" which covers the active center, facilitating an easier access of the substrate [26]. Lipase immobilization by the sol–gel method with IL as additive did not change this tendency, although it was not possible to obtain a direct correlation between the activities and solvent polarities on the Reichardt normalized scale (Table 3). The highest conversion and activity at 24-h reaction time was achieved in hexane and the lowest in acetonitrile. The reduced activity of hydrolases in water-miscible organic solvents was explained as well by the capability of these compounds to strip the tightly bound water layer from the enzyme molecule, which is essential to maintain the catalytic activity [27]. In our study, the solvents were equilibrated before the reaction to a constant water activity, therefore we can presume that the mentioned essential water layer was not disturbed and consequently the enzyme activity remained quite high also in water-miscible solvents as acetone or THF. Hara et al. [28] associated the lower activity of *Pseudomonas cepacia* lipase in more hydrophilic solvents with the competing hydrolysis reaction of the ester and acylation agent by the water present in system. We used 3:1 molar excess of vinyl acetate to overcome negative effects of possible hydrolysis of the acylation agent. Although slight hydrolysis of the ester product cannot be excluded, it was not significant since comparable conversions in water-miscible and water-immiscible solvents have been achieved. The absence of activity in DMF was not caused by excessive polarity, it could be attributed to the well-known denaturating effect of some solvents as DMF or dimethylsulfoxide on synthetic activity of lipases and other hydrolases [24,29].

The enantioselectivity of kinetic resolution 2-hexanol was strongly influenced by the nature of reaction medium. Acetone and THF, water-miscible solvents with intermediate polarity, demonstrated high efficiency in this respect.

Table 3
Influence of organic solvent nature and polarity on catalytic efficiency of 2-hexanol acylation by sol–gel immobilized lipase. Silane precursors for immobilization were OcTMOS and TMOS (1:1 molar ratio), [Omim][BF_4] was employed as additive at 0.13 IL/silane molar ratio.

Reaction medium (organic solvent)	E_T^N	Conversion (%)	Activity (μmole h^{-1} mg^{-1})	ee_p (%)	E
Hexane	0.009[a]	59	0.494	63	13
Toluene	0.099[a]	49	0.397	72	13
MTBE	0.124[a]	56	0.458	77	34
THF	0.207[a]	43	0.339	92	50
Acetone	0.355[a]	47	0.395	91	53
DMF	0.405[a]	0	0	0	0
Acetonitrile	0.460[a]	33	0.273	83	16

[a] From Ref. [22].

Table 4
Influence of IL reaction medium structure and polarity on catalytic efficiency of 2-hexanol acylation by sol–gel immobilized lipase. Silane precursors for immobilization were OcTMOS and TMOS (1:1 molar ratio), [Omim][BF_4] was employed as additive at 0.13 IL/silane molar ratio.

Reaction medium (ionic liquid)	E_T^N	Conversion (%)	Activity (μmol h^{-1} mg^{-1})	ee$_p$ (%)	*E*
[Emim][BF_4]	0.71[a]	72	0.662	38	9
[Pmim][BF_4]	0.69[a]	59	0.484	56	8
[Bmim][BF_4]	0.68[a]	38	0.316	78	13
[Omim][BF_4]	0.64[b]	36	0.314	84	18
[Bmim][Tf_2N]	0.64[c]	48	0.392	62	8
[Bmim][PF_6]	0.67[c]	49	0.412	32	4
[Omim][PF_6]	0.63[c]	61	0.511	42	5

[a] From Ref. [21].
[b] From Ref. [13].
[c] From Ref. [23].

ILs might be an attractive solution as polar reaction media, particularly to achieve high conversion values for substrates that are not soluble in solvents that usually allow high lipase activity. As discussed, the synthetic activity of lipases is usually lower in polar solvents, but in ILs could be different due to the specific properties of these compounds. The nature of cation and anion are both important in this topic. Seven ILs have been tested as reaction media for the acylation of 2-hexanol by vinyl acetate. The polarity of these compounds ranked from 0.71 to 0.63, at considerably higher values compared to acetonitrile, the most polar organic solvent previously studied. In the series of ILs with [BF_4] anion, the transesterification activity of the sol–gel immobilized lipase increased with increasing polarity of the IL and decreased with the alkyl chain length of imidazolium cation (Table 4). Such an apparently strange behavior of lipases in ILs based on 1-alkyl-3-methyl-imidazolium cation and [BF_4] anion was also reported by De Diego et al. [30] for the acylation of 1-butanol by vinyl propionate catalyzed by commercially available immobilized lipases. At contrary, Lee et al. [31], using the same enzymes and ILs with [Tf_2N] anion, measured the highest initial rate for the acylation of benzyl alcohol by vinyl acetate for the more hydrophobic [Omim][Tf_2N].

For ILs with the same cation ([Bmim] or [Omim], Table 4), the activity increased with decreasing polarity. In this case, the anion's nucleophilicity seems to have a more important role than the reaction medium polarity. Probably, ILs with increased nucleophilicity shall coordinate more strongly to positively charged sites at the enzyme surface, leading to conformational changes and decrease of enzyme activity, as it was firstly suggested by Sheldon et al. [32]. The [BF_4] anion is more nucleophilic than [PF_6] and might induce lower activities, as confirmed by our experimental results.

The observed discrepancies concerning the influence of IL structure on lipase activity could be also attributed to the different microenvironments in the vicinity of enzyme active center, determined by the combined effect of immobilization support, reaction medium, and substrate structure. The purities of ILs used in the experiments, especially the water content, and their purification method are very important, as well. We used commercially available ILs at the highest possible purity and from the same supplier, but it is difficult to compare the conditions with those reported elsewhere. For the acylation reaction of 2-hexanol by vinyl acetate, the best IL reaction medium was [Omim]PF_6, yielding higher activities than the best organic solvent.

The influence of ILs as reaction media on the enantioselectivity of lipases is also controversial. Enhancement of enantioselectivity in the resolution of chiral alcohols was reported in some cases. Kim et al. [33] obtained an important increase of the enantiomeric ratio *E* in ILs, principally in [Bmim]PF_6, but their study was made for reactions with very high enantioselectivities in organic solvents, too. Substantially increased enantioselectivities in ILs compared to organic solvents have been reported by Schöfer et al. [34] for lipase from *Alcaligenes* sp., but they tested only one organic solvent (MTBE).

Our results show that enantioselectivity of *Pseudomonas fluorescens* lipase in the acylation reaction of 2-hexanol was lower in the best IL than in the best organic solvents, THF and acetone, but was comparable or higher compared to other organic solvents (Tables 3 and 4). Probably, by interactions between the electrically charged ions from the reaction medium and specific residues situated close to the active center may affect the conformational rigidity of the enzyme molecule, resulting in diminution of the kinetic discrimination between the two isomers. For this type of substrates and enzyme, utilization of ILs is not recommended when the main target is the synthesis of products with high optical purity, organic solvents still remaining the best choice. We could not set up a direct correlation between the IL structure and the enantioselectivity of lipase, but for ILs with [BF_4] anion the enantiomeric ratio values slightly increased at higher hydrophobicity of the alkyl substituent from the imidazolium cation.

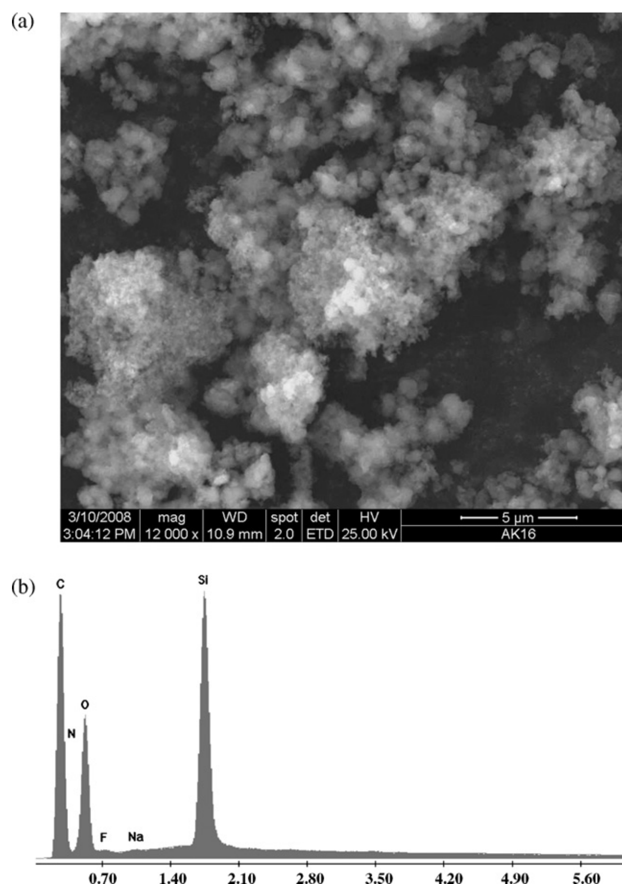

Fig. 5. SEM image (a) and EDX spectrum (b) of the sol–gel entrapped AK lipase obtained from OcTMOS/TMOS precursors at 1:1 molar ratio.

3.3. Structural characteristics of preparates immobilized with ionic liquid additive

Detailed structural investigation of the sol–gel immobilized lipase with ILs as additives was not the aim of this study. We only attempted to identify how the presence of ILs can modify the morphology of the sol–gel preparates and to have a possible confirmation of the partial incorporation of the IL in the silica matrix.

Encapsulating microbial lipases in hybrid inorganic–organic matrices with significant content of hydrophobic groups could induce important differences compared to the porous structure characteristic for inorganic sol–gel materials. SEM images can provide useful information regarding the surface morphology, and EDX spectra allows qualitative and semi-quantitative identification of the elements present in the sample.

The SEM image of the lipase immobilized using OcTMOS and TMOS as silane precursors without IL additive (Fig. 5a) shows an irregular porous structure, with spherical nanoparticles. In the EDX spectrum (Fig. 5b) the presence of F and Na elements can be observed, indicating that at least a part of the NaF catalyst was not eliminated during the gel washing step. The presence of F might be also a consequence of $[BF_4]^-$ or $[PF_6]^-$ anions decomposition in the presence of moisture, even though they not react with water [35,36]. The C/Si relative ratio (w/w) in the silane precursors employed for this immobilization was 2.9. The semi-quantitative analysis of the EDX spectrum gives a very close value (2.8) of this ratio, suggesting that in our case the hydrophobic groups were uniformly distributed in the sol–gel matrix, not concentrated at the surface. Adding an IL additive during the immobilization process resulted in important modifications of the general surface morphology of the biocatalyst. The SEM image shows a more amorphous structure (Fig. 6a), and in the elemental composition resulted from the EDX spectrum the boron element can be identified (Fig. 6b). It results that a part of the ionic liquid used as additive [Omim]BF_4 was confined in the sol–gel structure. The semi-quantitative elemental analysis gives a much lower C/Si ratio than previously (1.6), indicating a different distribution of hydrophobic groups in the surface region of the sol–gel matrix.

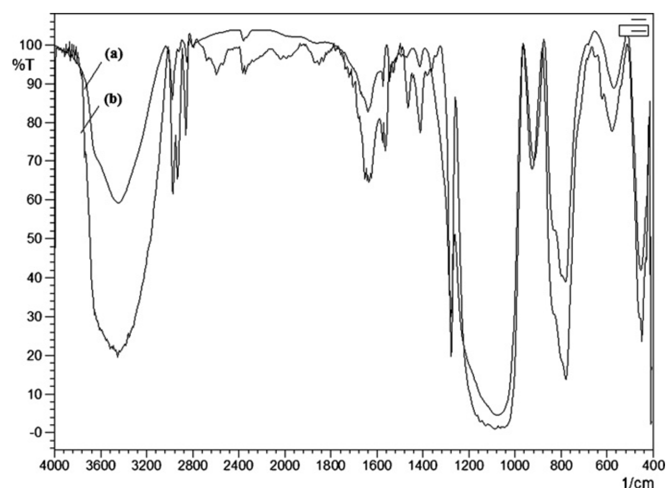

Fig. 7. FT-IR spectra of sol–gel entrapped preparates obtained from MeTMOS and TMOS precursors at 1:1 molar ratio (a) without, and (b) with [Omim]BF_4 as additive.

The inclusion of a part of the IL additive in the sol–gel material during the immobilization process was also confirmed by FT-IR spectroscopy (Fig. 7). We selected preparates obtained using MeTMOS as one of the silane precursors, to demonstrate the incorporation of the additive by the presence of the symmetrical and asymmetrical stretching bands of methylene groups at 2858 cm^{-1} and 2928 cm^{-1}, that cannot be found in the matrix obtained without [Omim][BF_4]. More specific are the symmetrical and asymmetrical stretching vibrational bands of imidazolium skeleton, which occur at 1463 cm^{-1} and 1562 cm^{-1}, respectively. The attribution of the mentioned IR vibrational bands was made according to literature data [37].

The presented results confirm that the IL remained partially confined in the sol–gel structure, though the gel was carefully washed with aqueous buffer, isopropanol, and hexane. The exact nature of interaction between the IL and the sol–gel matrix needs more sophisticated techniques. Shi and Deng [38] reported about physical confinement of ILs in nano-porous silica gel during the sol–gel process and nano-scale dispersion of the IL within the matrix. It looks feasible that a similar process takes place in the course of enzyme immobilization, but it could be strongly influenced by interactions between the enzyme and IL.

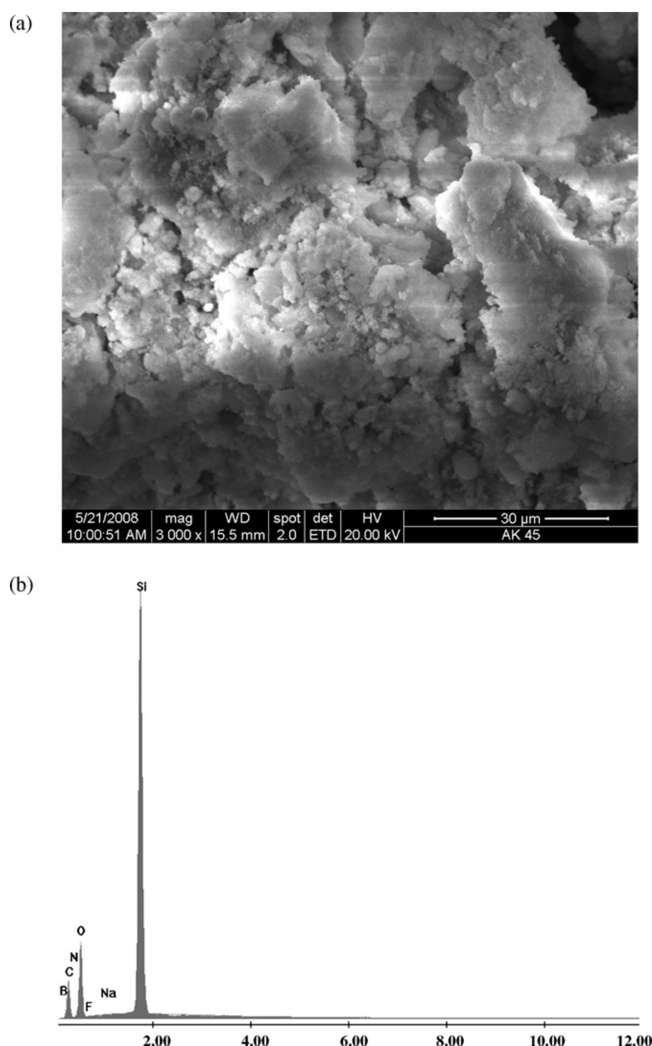

Fig. 6. SEM image (a) and EDX spectrum (b) of the sol–gel entrapped AK lipase obtained from OcTMOS/TMOS precursors and [Omim]BF_4 as additive.

4. Conclusions

Immobilization of lipase in hybrid sol–gel matrices using specific ILs as additives resulted in biocatalysts with high catalytic efficiency. The recovery yield of total activity following immobilization was usually higher than 100%, the best value being recorded for [Omim]BF_4 as additive. Kinetic resolution of secondary alcohols is an important aim in biocatalysis. Although native *Pseudomonas*

fluorescens lipase is not highly enantioselective for this process, we demonstrated that using the sol–gel entrapped biocatalyst and an appropriate selection of the reaction medium *E* values higher than 50 can be achieved for the acylation of 2-hexanol. Considering this reaction, ILs are better reaction media than organic solvents, but with lower enantioselectivity.

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