Long-Term Exposure Stability of Sol-Gel Immobilized Lipases in Organic Solvents

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Abstract: Stability of lipases from *Pseudomonas fluorescens* (Amano AK), *Burkholderia cepacia* (Amano PS) and *Candida antarctica* B (Cal B), immobilized by sol-gel entrapment and sol-gel entrapment combined with adsorption on Celite 545, was investigated by long-term incubation in seven different solvents. The immobilization has been carried out using a ternary silane precursors system consisted of phenyl-trimethoxysilane, vinyl-trimethoxysilane and tetramethoxysilane, at 1.6/0.4/1 molar ratio. The immobilized biocatalysts were incubated in various organic solvents for 7 days, at 25°C. The activity and enantioselectivity were assayed in the acylation reaction of 2-hexanol with vinyl acetate. The activity of the immobilized biocatalysts was not significantly altered by the tested organic solvents, while the enantioselectivity values of all immobilized enzymes were higher compared to the native ones. In the case of Cal B lipases the operational stability was excelent as well, the transesterification activity remaining unchanged during 14 reuse cycles.

Keywords: lipase, sol-gel entrapment, stability, organic solvent, reuse

1. Introduction

Biocatalysts are receiving more attention as an alternative to conventional catalysts because of several advantages such as high enantioselectivity, high catalytic efficiency, mild reaction conditions and low environmental impact. Especially biocatalysts, used for the synthesis of chiral compunds are starting to have a significant growth [1]. Lipases (EC 3.1.1.3) can be used as catalysts for various reactions such as amidation, esterification and transesterification of esters in anhydrous media for various substrates without changing their stereoselectivity and regioselectivity [2].

Usually, the substrates and/or products of lipases/esterases are not soluble in water and the industrial biotransformation processes may be carried out under harsh conditions such as extreme values of pH and temperature, high pressure, presence of salts, metals and organic solvents. Therefore a relevant property with potential in biotehnological applications is the tolerance of enzyme in organic solvents. The activity and stability of enzymes depend on the nature of the solvent (water miscible or immiscible, protic or aprotic, etc.) and the water content of the enzyme [3].

Since the stability of an enzyme in hydrophilic solvents can be reduced due to the solvent penetration effect on the surface of the enzyme, the stabilization of the enzyme in such solvents is an important issue for industrial applications [3]. Nowadays immobilization of enzymes and whole cells is a key issue for developing efficient and economically viable biocatalytical processes [4].

The aim of this study was to investigate the denaturation effect of organic solvents on native and immobilized *Pseudomonas fluorescens, Burkholderia cepacia* and *Candida antarctica* B lipases. The immobilization was carried out by sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption on Celite 545 (SGE-A).

2. Experimental

Lipase from *Candida antarctica* B (Cal B lipase) was the product of C-Lecta (Leipzig, Germany), *Pseudomonas fluorescens* (Amano AK) and *Burkholderia cepacia* (Amano PS) lipases were purchased from Aldrich. The silane precursors vinyl-trimethoxysilane (VTMOS) and phenyl-trimethoxysilane (PhTMOS) were purchased from Merck, while tetramethoxysilane (TMOS) was from Fluka. Other reagents used: 2-propanol (Merck), sodium fluoride (Fluka), 2-hexanol (Merck), vinyl acetate (Merck), were of analytical grade and have been used as purchased. Dodecane (Aldrich) was used as internal standard for quantitative gas-chromatographic analysis. The ionic liquid 1-octyl-3-methyl-imidazolium tetrafluoroborate (OmimBF$_4$), was a product of Fluka. *N*-Hexane, acetonitrile, toluene, acetone, tetrahydrofuran, *tert –* butanol, isooctane were purchased from Merck.

2.1. Immobilization by Sol-Gel Entrapment and Sol-Gel Entrapment combined with adsorption on Celite 545.

A microbial lipase suspension in TRIS/HCl 0.1 M, pH 8.0 buffer was stirred at room temperature for 30 min, centrifuged, and the supernatant used for immobilization. In a 4 mL glass vial, 1 mL of this lipase solution was mixed with 200 μL ionic liquid OmimBF$_4$, followed by addition of 100 μL 1M NaF solution, and 200 μL isopropyl alcohol. This mixture was kept for 30 min under continuous stirring for homogenization, and subsequently a tertiary mixture of

silane precursors PhTMOS, VTMOS and TMOS at 1.6/0.4/1 molar ratio (total 6 mmoles) was added. The mixture was stirred at room temperature until the gelation started. In the case of sol – gel entrapment combined with adsorption when the gelation started we added 0.5 g of Celite 545.

The obtained gel was kept for 24 h at room temperature to complete polymerization. The bulk gel was washed with isopropyl alcohol (7 mL), distilled water (5 mL), isopropyl alcohol again (5 mL) and *n*-hexane (5 mL), filtered, dried at room temperature for 24 h, and finally kept in a vacuum oven at room temperature for another 24 h. After that it was crushed in a mortar and kept in refrigerator.

2.2. Influence of organic solvents on stability

Native and immobilized lipases were incubated for 7 days at 25˚C in various organic solvents with different log P values: 2,2,4-trimethylpentane (also known as isooctane, log P 4.50), hexane (log P 4.00), toluene (log P 2.73), tetrahydrofuran (log P 0.46), *tert*-butanol (log P 0.35), acetonitrile (log P 0.25), and acetone (log P -0.24). As the literature data for log P of different organic compounds slightly differ, we selected a single source [5], excepting the log P value of isooctane that was taken from the work of Zhao et al. [6]. The solvent was removed with a pipette and after that the immobilized enzymes were washed two times with 1 ml of *n*-hexane and dried in the oven for one hour. The activity of the biocatalysts was determined in the acylation of 2-hexanol with vinyl acetate at 40˚C, in *n*-hexane.

The acylation reaction was performed in 4 mL capacity glass vials, charged with a mixture of 2-hexanol (0.5 mmole), vinyl acetate (1.5 mmole), internal standard (*n*-dodecane 15 μL), reaction medium (*n*-hexane, 1 mL) and free (5 mg) or immobilized lipase (25 mg).

The mixture was incubated using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40ºC (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). The conversion and enantiomeric excess of the product were assayed by gas-chromatography, on a Varian 450 instrument (Varian Inc., USA) equipped with flame ionization detector, using a 30 m x 0.25 mm Astec Chiraldex B-PM capillary column with 0.25 mm film thickness (Sigma-Aldrich, Supelco). The analysis conditions were: oven temperature from 100˚ to 140˚C with 10˚C/min heating rate, injector temperature 240˚C, detector temperature 280˚C, carrier gas (hydrogen) flow 1.2 mL/min. The reactions were usually run for 24 h. Conversions have been calculated based on the reacted alcohol, calculated by the internal standard method. The enantiomeric excess (e.e) was expressed as the ratio of the *(R)* and *(S)* ester product, calculated from the peak areas.

2.3. Multiple use of immobilized Cal B Lipase

The enzyme reutilization study was performed at 40˚C. The initial reaction system was set up as described for the acylation study. At the end of every reaction cycle, the product (upper liquid phase of the reaction mixture) was removed with a pipette, the remaining solid phase (native or immobilized lipase) was washed two times with n-hexane (2 mL), centrifuged at 15˚C and 5,000 rot/min, and the supernatant decanted. Subsequently, the same amounts of reagents (2-hexanol, vinyl acetate, and n-hexane) as for the initial reaction were added to the recovered enzyme, and the reaction was run under the same conditions.

2.4. Scanning electron microscopy (SEM)

The morphology of the immobilized preparates was studied by scanning electron microscopy (SEM) with a Quanta FEG 250 microscope (FEI, The Netherlands) equipped with EDAX analyzer. The analysis parameters were: pressure 1.5×10^{-2} Pa, resolution <10 nm at 3kV.

3. Results and Discussion

Enzymatic activity and enantioselectivity of enzymes are crucial factors in the enzymatic process and influence its potential applications [1]. On the other side, the efficiency of an enzyme in a synthetic reaction can be often overshadowed by the low operational stability in organic solvents. To increase the operational stability, three microbial lipases were immobilized through sol-gel entrapment and sol-gel entrapment combined with adsorption on Celite 545. The obtained hybrid materials were subjected to prolonged exposure in organic solvents with different polarities, to evaluate their potential as possible industrial biocatalysts.

The transesterification activities of all immobilized lipases was assayed in the acylation reaction of 2-hexanol with vinyl acetate, compared to the native enzyme (Table 1). As expected, the activities of the immobilized preparates were lower, but the highest value reached 36% of the native lipase activity in case of Cal B, an excellent result considering that the enzyme represents less than 5% of the solid material resulted after the immobilization process.

TABLE 1. *Transesterification reaction of 2-hexanol with vinyl acetate in n-hexane with immobilized lipases through sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption (SGE-A) at 40˚C, 24 h reaction time.*

Lipase	Immobilization method	C (%)	Activity*	e.e (%)	E
AK	native	40	1.536	70	9
	SGE-A	26	0.149	88	21
	SGE	21	0.130	90	24
PS	native	44	1.334	78	15
	SGE-A	37	0.248	86	22
	SGE	45	0.330	82	21
Cal B	native	31	1.044	98	152
	SGE-A	50	0.364	98	458
	SGE	50	0.378	98	458

*expressed as $\mu moles \cdot h^{-1} \cdot mg \ catalyst^{-1}$

To have an accurate measure of the effect of organic solvents on the immobilized lipases, the relative activity was calculated as a ratio of the transesterification activity of the biocatalyst after the incubation with an organic solvent and the transesterification activity without organic solvent incubation. Seven organic solvents with a broad polarity range were selected for this study, based on their utilization as reaction media for lipase-catalyzed reactions.

For Amano AK lipases immobilized through sol-gel entrapment (SGE, Fig 1a) the highest value of the relative activity was obtained after 7 days exposure in acetonitrile and the lowest for toluene. Amano AK lipase immobilized by sol-gel entrapment combined with adsorption (SGE-A) showed the lowest value of relative activity in acetonitrile and the highest value in *tert* - butanol. Undoubtedly, the relative activity values can not be correlated with the polarity of the solvent used for the stability study. Generally, polar protic solvents have a tendency to induce desolvation of the essential water near the active site of the enzyme, reducing the catalytic activity. As well, in some cases organic solvents may interact with the copolymer framework via H-bonding and dipolar interactions, which attenuates the lipase activity [7]. However, long-term stability in an organic solvent represents a different case, because it does not cause irreversible degradation of the enzyme and the required amount of water is supplied by the other components of the transesterification reaction system. It must be pointed out that the immobilized Amano AK preparates showed relative activity values above 1 following the incubation in several organic solvents (up to 1.4 in acetonitrile for the SGE preparate), meaning an activation effect. The sol-gel entrapment could lead to different amounts of water that remain in the matrix following the drying process. Whether the organic solvent removes this excess water, the activity will increase.

Unlike as in the case of Amano AK lipase, for the enzymatic preparations with Amano PS lipases the immobilization by the combined method resulted in higher values of relative activity compared to the sol-gel entrapment technique (Fig 1b). Tetrahydrofurane was the best incubation solvent for both SGE and SGE-A preparates, resulting 20-40% increase of the relative activity.

The immobilized biocatalysts with Cal B lipases showed slightly lower (less than 10%) relative activity values compared to those before the organic solvent exposure, regardless of the nature of the tested solvent. The long-term exposure stability of this lipase after immobilization was excellent in all tested solvents, therefore it can be used as catalyst for synthetic reactions in organic solvents with diverse polarities, according to the requirements of the reaction system.

Enantioselectivity is another important parameter for the practical application of the biocatalysis. In organic solvents it is influenced firstly by the water content and then by the temperature and the nature of the solvent [8].

The enantioselectivity of the studied reaction was expressed using the enantiomeric ratio (E) value which is considered the best parameter to characterize the enzyme-catalyzed kinetic resolutions, since it is based on the
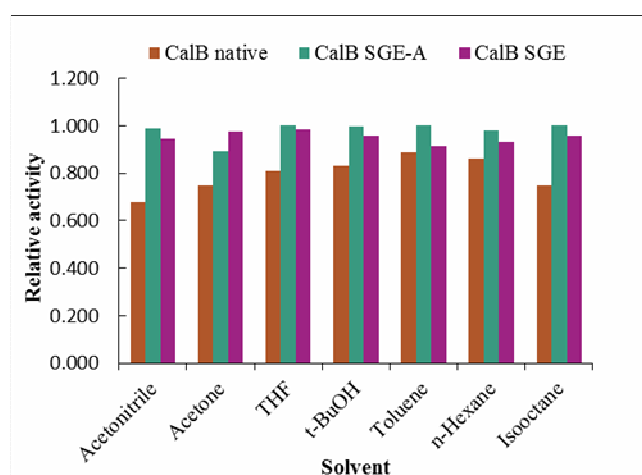
enantiomeric excess values at a function of the extent of conversion [9].
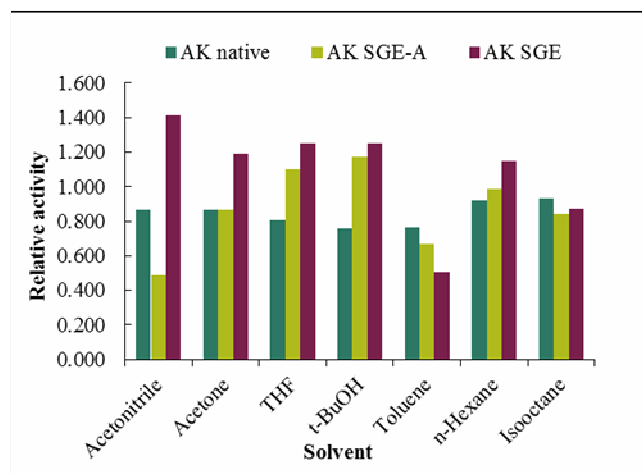
(a)
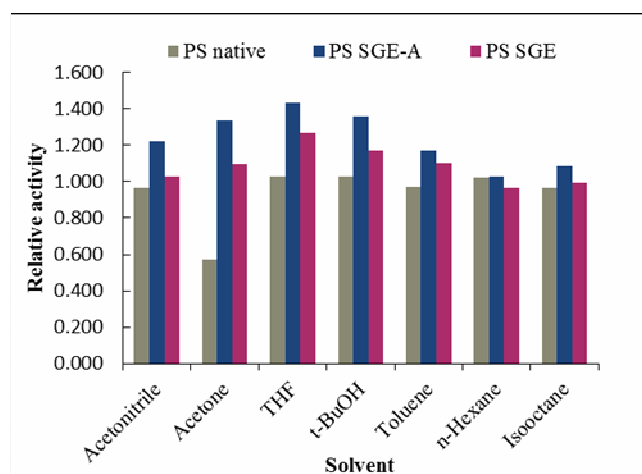
(b)

(c)

Figure 1. Influence of the solvent (7 days incubation at 25˚C) on the relative activity of native and immobilized (a) Amano AK, (b) Amano PS and (c) Cal B lipases by sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption (SGE-A), assayed for the acylation reaction of 2-hexanol with vinyl acetate in n-hexane at 40˚C and 24 h reaction time

The obtained enzymatic preparations showed high and constant values of enantiomeric ratio in all tested solvents, compared to the native lipases. The E values of the obtained biocatalysts were two times higher (for Amano AK and Amano PS preparates) or even five times higher (for Cal B preparates) than for the native lipases, as showed in Fig 2a-c.

For all lipases immobilized through the SGE-A method the highest values of the enantiomeric ratio (E) were obtained after incubation in acetonitrile, a polar aprotic solvent (log P 0.25), while in the case of SGE method the best values were obtained in the non-polar *n*-hexane (log P 4.0), excepting the Cal B preparate where the highest E value was obtained in acetonitrile. These results are correlated with those reported by Herbst *et al.*, that enantiomeric excess can decrease with decreasing hydrophilicity which means the higher the hydrophilicity, the higher the enantiomeric excess [8]. This can be assigned to the extraction of water molecules from the enzyme surface. The enzyme becomes more rigid, the discrimination between the two enantiomers is better leading to an increased enantioselectivity. In contrast to these findings, some studies showed an increasing enantiomeric excess with decreasing hydrophilicity of the solvent [8]. There are several reports dealing with the influence of the organic solvent used as reaction medium on the enantioselectivity of lipases. Our results show that even the contact between the enzyme and the solvent can increase the selectivity, probably favoring a sterical conformation of the enzyme that was preserved for the catalyzed reaction. It means that the enantioselectivity could be enhanced by incubation in a suitable solvent, even if this solvent is not appropriate as reaction medium for the forthcoming reaction.

Reuse of the immobilized biocatalysts

Because of the relatively high cost and processing requirements the use of enzymes at industrial scale is still limited. Multiple use of immobilized enzymes is important to make biocatalysts more feasible [7]. An enzyme can be reused until its activity decreases to less than 25% of the initial value [10].

We studied the reuse potential of the Cal B lipases immobilized through sol-gel entrapment and sol-gel entrapment combined with adsorption on Celite 545. The sol-gel matrix was obtained from the same ternary precursor mixture of PhTMOS:VTMOS:TMOS (at molar ratio 1.6/0.4/1), using [Omin]BF_4 as additive. The reaction was performed in *n*-hexane at 40˚C, for 24 hours. The relative activity of the native lipase after 6 reuse cycles decreased significantly, being only 27% of the initial value. There was no significant difference between the two immobilization methods, for both tested preparates the relative activity remained at about 80% (Fig 3).
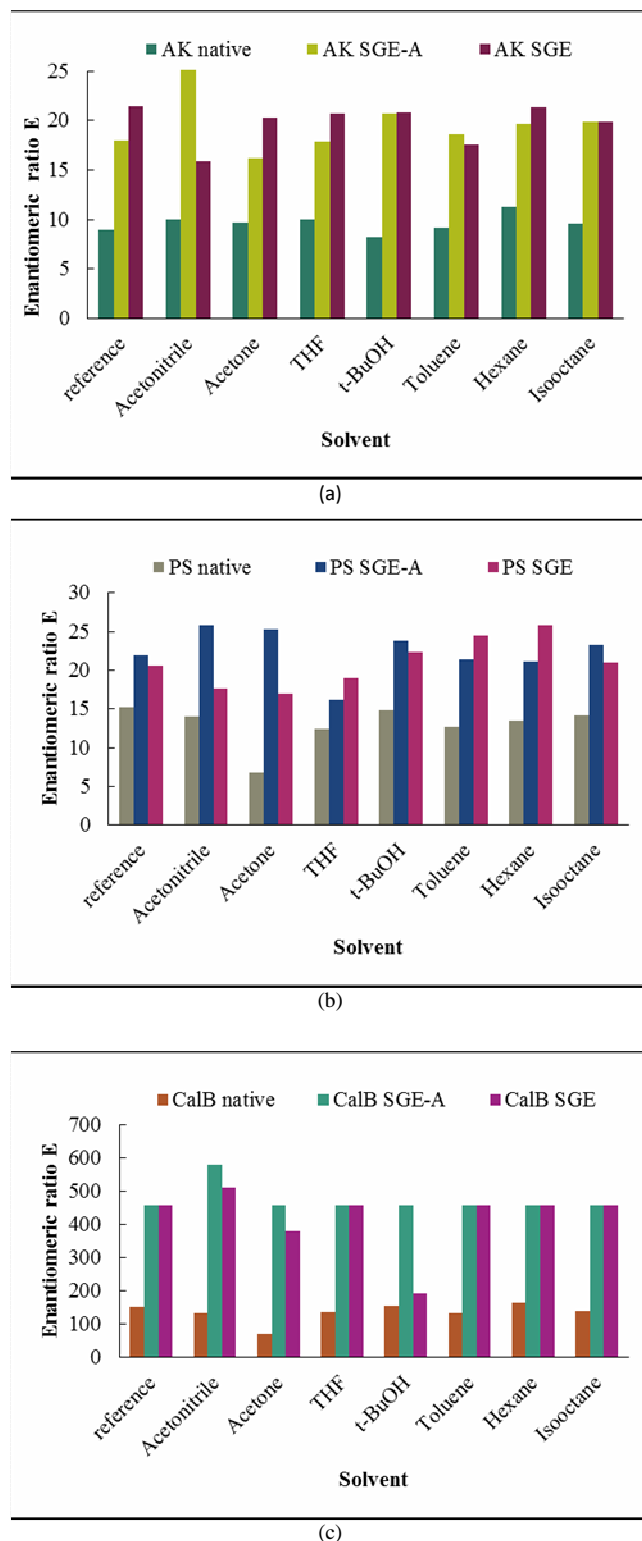
(a)

(b)

(c)

Figure 2. Influence of the solvent (7 days incubation at 25˚C) on the enantioselectivity of native and immobilized (a) Amano AK, (b) Amano PS and (c) Cal B lipases by sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption (SGE-A), assayed for the acylation reaction of 2-hexanol with vinyl acetate in n-hexane at 40˚C, at 24 h reaction time.
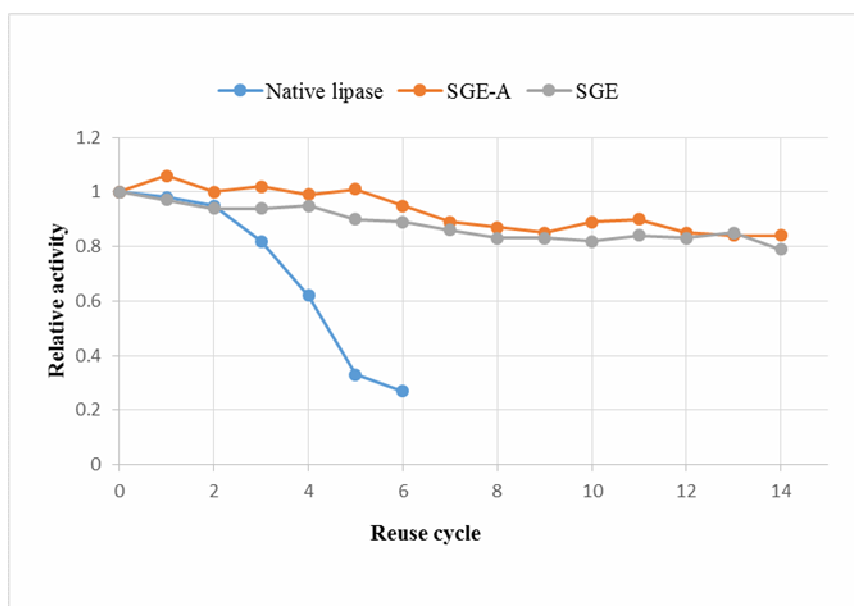
Figure 3. Influence of the enzyme reuse on the relative activity of native and immobilized lipase from Candida antarctica B in the acylation of 2-hexanol at 40˚C in n-hexane (native lipase, SGE-A – sol-gel and adsorption and SGE – sol-gel entrapment)

Scanning electron microscopy (SEM)

The SEM studies were carried out to examine the effect of solvents on the morphology of the sol-gel material used for the immobilization process before and after incubation in organic solvents. The information about the morphology can be correlated with the catalytic efficiency in the acylation reaction. In Figure 4 and Figure 5 are presented the SEM images at different magnifications of SGE preparate with PhTMOS, VTMOS and TMOS at 1.6/0.4/1 molar ratio before and after incubation for 7 days in THF.

The SEM images of the SGE preparate before incubation (Fig. 4) revealed particles with different sizes and blocky morphology with a smoother external shell. In cross-section (Fig 4. b) a single powder particle consists of

fine irregular and spherical granules agglomerated in a binder phase, presenting also a certain degree of porosity.

In the case of SGE preparate after incubation (Fig. 5) the SEM images consists of irregular particles with different sizes. In comparison to the biocatalyst before solvent incubation it can be noticed that the external surface of the biocatalysts is altered which might be attributed to the exposure within the solvent. Also the spherical granules are not present but instead, at higher SEM magnification (Fig.5. b), thin and irregular granules might be observed which are agglomerated in a binder phase. It looks like the solvent altered the initial inorganic matrix morphology. Nevertheless after incubation the preparate showed an insignificant modification of the transesterification reaction and enantioselectivity in the acylation of 2-hexanol.
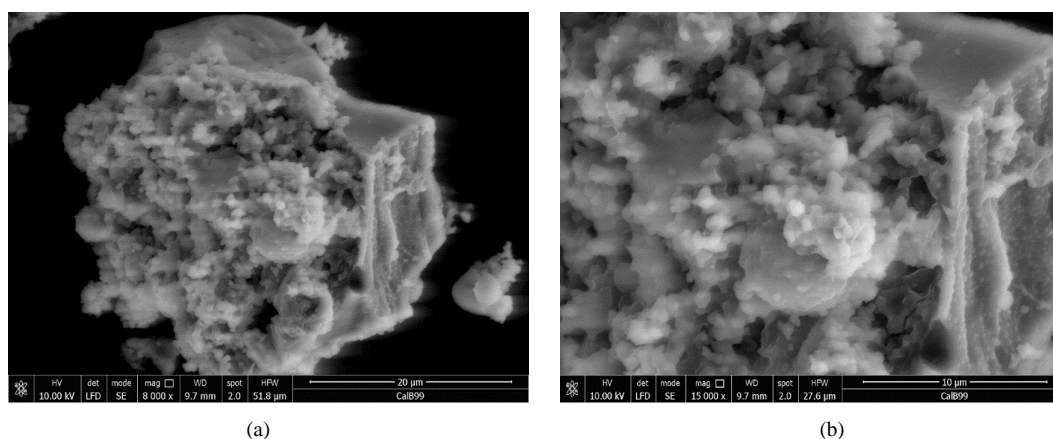

(a)

(b)

Figure 4. SEM micrographs at: (a) 8000 x and (b) 15000 x magnification of the sol-gel entrapped lipase from Candida antarctica B immobilized by sol-gel entrapment before incubation in organic solvent
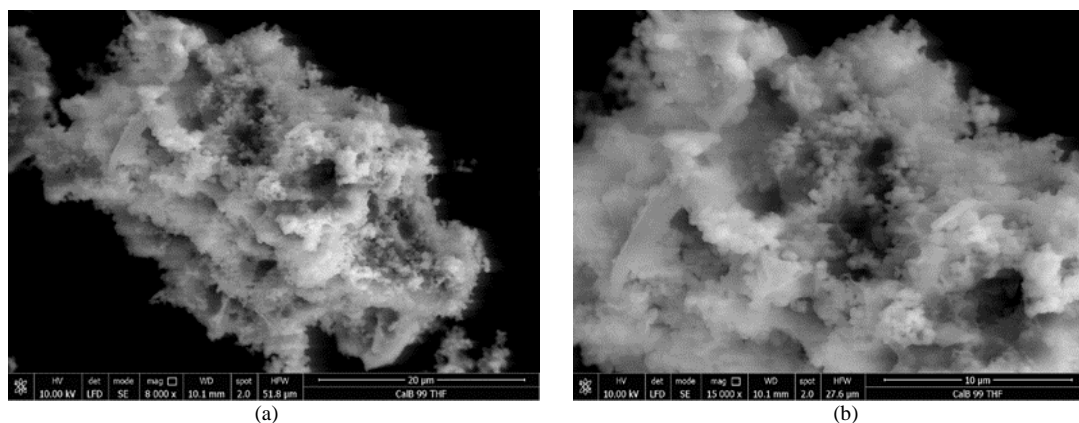
Figure 5. SEM micrographs at: (a) 8000 x and (b) 15000 x magnification of the sol-gel entrapped lipases from Candida antarctica B immobilized by sol-gel entrapment after incubation in organic solvent THF for 7 days.

4. Conclusions

In contrast to the native lipases, the immobilized biocatalysts tested showed high and constant values of enantiomeric ratio E in all tested solvents. The sol-gel matrix created around the enzyme protected the biocatalyst from the unfolding effect of the organic solvents, thus increasing the operational stability. Considering both activity and enantioselectivity the best biocatalysts for the studied reaction were those obtained with *Candida antarctica* B lipase. The immobilized biocatalysts also showed excellent stability during the reuse study. The SEM analysis showed a changed morphology of the biocatalyst without significant modification of the catalytic properties.

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