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High thermal stability of sol-gel entrapped lipases

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Abstract

Lipases from *Burkholderia cepacia* (Amano PS) and *Candida antarctica* B (CalB) were immobilized by sol-gel entrapment techniques, using a ternary silane precursors system with vinyl and phenyl non-hydrolyzable groups. The influence of temperature on the catalytic properties of native and immobilized lipases was investigated by incubating the biocatalysts for 24 h at temperatures between 40ºC and 80ºC, in n-hexane medium. A long term stability study was made with immobilized CaLB biocatalysts by incubation for 120 h in isooctane at 80°C. The acylation of aliphatic sec-alcohols by vinyl acetate was used as model reaction. Thermogravimetric analysis (TGA) was employed to evaluate the thermal stability and degradation of the immobilized biocatalysts.

Keywords: lipase, sol-gel, entrapment, thermostability

1. Introduction

Lipases (triacylglycerolacylhydrolase, E.C.3.1.1.3) have achieved a status of "indispensable catalysts" in a wide array of industrial applications, such as food technology (dairy products, bakery products, confectionaries, tea processing and flavor development), cosmetic industry, detergent industry, textile industry, pulp and paper industry, diagnostic and medical fields, synthesis of fine chemicals and pharmaceuticals, biodiesel production, synthesis of biodegradable polymers and waste–effluent treatment [1-4]. These comprehensive uses are mostly due to their activity, selectivity and stability characteristics.

Thermal stability is a major requirement for commercial enzymes, being critical for industrial applications, as thermal denaturation is a common cause of enzyme inactivation [5].

Biocatalyst thermostability allows a higher operation temperature, which is clearly advantageous because of a higher reactivity (higher reaction rate, lower diffusional restrictions), higher stability, higher process yield (increased solubility of substrates and products), lower viscosity and fewer contamination problems. These advantages surmount certain drawbacks arising from more severe requirements for materials, harder post-reaction inactivation, and restrictions in the case of labile substrates or products [3].

The most commonly used methods to overcome enzyme stability issues are the use of various immobilization techniques and resorting to enzyme mutagenesis (the combination of both is also possible) [6]. Immobilization aims at improving the operational stability of the enzyme and at enabling the use of continuous reactor systems combined with simpler separation of the biocatalyst from the reaction mixture, which makes catalyst recycling possible [7]. The utilization of immobilized enzymes became in the last period an emerging option for applications in food technologies, as well [8].

The sol–gel encapsulation in silica matrices is an immobilization technique that has been focused to

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prepare high-performance immobilized enzymes. The mild processing conditions in aqueous solution at room temperature, and controlled porous structure of sol–gel polymers ensure the captured enzymes highly active and essentially permanently entrapped [9]. Especially, the enzymes entrapped in sol–gels typically show improved resistance to thermal and chemical denaturation, as well as enhanced storage and operational stability [10-13].

In this work, lipases from *Burkholderia cepacia* (Amano PS) and *Candida antarctica* B (CalB) were immobilized by sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption on Celite 545 (SGE-A), using ternary silane precursors systems, with vinyl and phenyl non-hydrolyzable groups. We investigated the influence of temperature on the catalytic properties of native and immobilized lipases by sol-gel methods. The biocatalysts were incubated for 1 day at temperatures between 40ºC and 80ºC in n-hexane and for a long term stability study only with immobilized *Candida antarctica* B lipase preparates were incubated for 5 days in isooctane at 80°C. The acylation of aliphatic sec-alcohols by vinyl acetate, in n-hexane at 40ºC, was used as model reaction to evaluate the thermal stability. Thermogravimetry was also employed to evaluate the thermal stability and degradation of the immobilized biocatalysts.

2. Materials and Methods

2.1. Materials

Lipase from *Burkholderia cepacia* (Amano PS) was achieved from (Amano, Japan) and *Candida antarctica* B (CaLB lipase) was purchased from C-Lecta (Leipzig, Germany). Silane precursors vinyl-(VTMOS) and phenyl-trimethoxysilane (PhTMOS) were purchased from Merck and tetramethoxysilane (TMOS) from Fluka, Gemany. Other materials used: tris-(hydroxymethyl)-aminoethane (Loba Chemie, Germany), 2-propanol (Merck, Germany), sodium fluoride and 2-hexanol from (Fluka, Germany), 2-heptanol (Merck), 2-octanol, vinyl acetate , *n*-hexane (98%) were of analytical grade and have been used as purchased from Merck, Germany. n-decane and *n*-dodecane (Sigma-Aldrich, Germany) were used as internal standards for quantitative gas-chromatographic analysis. The ionic liquid 1-octyl-3-methyl-imidazolium tetrafluoroborate, [Omim]BF_4, was product of Fluka, Germany.

2.2. Immobilization by Sol-Gel Entrapment

A lipase suspension (120 mg/mL) 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, 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 (total 6 mmoles) was added. The mixture was stirred at room temperature until the gelation started. 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 finally *n*-hexane (5 mL), filtered, dried at room temperature for 24 h, and in a vacuum oven at room temperature for another 24 h. Finally, it was crushed in a mortar and kept in refrigerator.

2.3. Immobilization by Sol-Gel Entrapment and Adsorption

The immobilization protocol was identical to that described for the simple sol-gel entrapment, until the start of gelation, when 0.5 g Celite 545 was blended with the gelling mixture. Subsequently, the obtained solid preparate was processed as described above.

2.4. Acylation of secondary alcohols

Acylations were performed in 4 mL capacity glass vials, charged with a mixture of 2-hexanol, 2-heptanol or 2-octanol (0.5 mmole), vinyl acetate (1.5 mmole), internal standard (*n*-dodecane or *n*-decane, 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 × 0,25 mm Astec Chiraldex B-PM chiral column with 0.12 µm film thickness (Sigma Aldrich, Supelco). The

analysis conditions were: oven temperature from 100°C to 140°C with 10°C /min heating rate, injector temperature 240°C, detector temperature 280°C, carrier gas (hydrogen) flow 1.9 mL/min. The reactions were usually run for 24 h. Conversions have been calculated based on the internal standard method.

Transesterification activities were calculated at 24 h reaction time and expressed as the average 2-acetoxy-alcohol amount (in micromole) synthesized per hour by 1 mg of free or immobilized enzyme. The control reaction without enzyme did not give any product in the same conditions.

The enantiomeric excess of the resulted ester product (ee_p) was determined from peak areas of enantiomers, and the enantiomeric ratio (*E*) values were calculated based on conversion and $e.e_p$ values using the relation (1) [14]:

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

where *C* represent the conversion at 24 h.

All reactions have been run in duplicate and sampling was also made in duplicate. As the differences between the data for the same assay were less than 2%, average values have been calculated and presented in tables and figures.

2.5. Temperature stability

The native and immobilized lipases were incubated in n-hexane at different temperatures (40, 50, 60 and 70°C) for 24 h. In the next study, *Candida antarctica* B was incubated for 120 h days in isooctane at 80°C. After this, the solvent was removed by filtration and the remained solid phase (native or immobilized lipase) was washed two times with 2 mL hexane, centrifuged at 15°C and 5,000 rot min^{-1}, and the supernatant decanted. The activity of biocatalysts was determined in the acylation reaction of 2-hexanol, 2-heptanol and 2-octanol with vinyl acetate, as described above.

2.6. Thermal analysis (TGA/DTA)

Thermogravimetric measurements (TGA/DTA) were recorded using a TG 209 F1 Libra thermogravimetric analyzer (Netzsch, Germany) operating a resolution of 0.1 μg, in nitrogen atmosphere. Curves were recorded from 30 to 1000°C with a heating rate of 10°C min^{-1}. The average sample mass was 7.0 ± 0.15 mg in open alumina crucibles (average mass 190 ± 1.0 mg).

3. Results and Discussion

3.1. Thermal stability of immobilized lipases

Because the importance of thermostable lipases for different applications has been growing rapidly and as thermal stability is an essential property of an industrial enzyme, we studied the effect of temperature on the catalytic activity and enantioselectivity of native and sol-gel immobilized lipases. For this, lipases from *Burkholderia cepacia* (Amano PS) and *Candida antarctica* B (CalB) were immobilized by sol-gel entrapment (SGE) and sol-gel entrapment combined with adsorption on Celite 545 (SGE-A), using ternary silane precursors systems, particularly phenyl-trimethoxysilane/vinyl-trimethoxysilane/tetramethoxysilane (PhTMOS:VTMOS:TMOS at 1.6:0.4:1 molar ratio) and the ionic liquid 1-octyl-3-methyl-immidazolium tetrafluoroborate ($OmimBF_4$) was employed as additive.

The biocatalysts were incubated for 24 h at temperatures between 40ºC and 70ºC in n-hexane. The acylation of aliphatic sec-alcohols (2-hexanol, 2-heptanol and 2-octanol) by vinyl acetate, in n-hexane at 40ºC, was used as model reaction to evaluate the thermal stability in organic solvent.

The effect of temperature on the catalytic properties are presented in Tables 1 and 2 and Figures 1, 2 and 3.

Table 1. Influence of temperature on the activity of sol-gel immobilized lipase from *Burkholderia cepacia* (Amano PS).

Temperature [°C]	Activity (µmole·h^{-1}·mg^{-1})/ substrate/ biocatalyst								
	2-hexanol			2-heptanol			2-octanol		
	native	SGE-A	SGE	native	SGE-A	SGE	native	SGE-A	SGE
40	1.761	0.191	0.301	0.444	0.207	0.342	0.678	0.333	0.467
50	1.753	0.208	0.296	0.462	0.151	0.338	0.721	0.335	0.498
60	1.710	0.195	0.300	0.430	0.209	0.322	0.825	0.325	0.467
70	1.854	0.203	0.280	0.418	0.206	0.358	0.732	0.332	0.455

Table 2. Influence of temperature on the activity of sol-gel immobilized lipase from *Candida antarctica* B.

Temperature [°C]	Activity (µmole·h^{-1}·mg^{-1})/ substrate/ biocatalyst								
	2-hexanol			2-heptanol			2-octanol		
	native	SGE-A	SGE	native	SGE-A	SGE	native	SGE-A	SGE
40	0.762	0.358	0.357	0.587	0.341	0.355	0.612	0.390	0.391
50	0.895	0.346	0.355	0.590	0.373	0.366	0.667	0.408	0.394
60	0.836	0.354	0.336	0.486	0.362	0.379	0.668	0.391	0.397
70	0.801	0.345	0.359	0.515	0.390	0.365	0.632	0.308	0.380

As expected, after immobilization the values of catalytic activity were lower. Nevertheless, the immobilized biocatalysts showed an excellent thermal stability on the studied temperature range. Small variations of activity values can be assigned to the different distribution of the enzyme inside the sol-gel matrix.

Enzymatic preparates obtained with CalB showed the highest values of activity (activity > 0.340 µmoles/h/mg), excepting the acylation of 2-octanol using Amano PS biocatalysts. This could be probably due to a better affinity of this lipase for 2-octanol.

Enantioselectivity is another important parameter for practical applications of enzymatic biocatalysis. The values of enantiomeric ratio E were higher for the enzymatic preparates compared to native lipases, with no respect to the employed immobilization technique (Figures 1, 2 and 3).

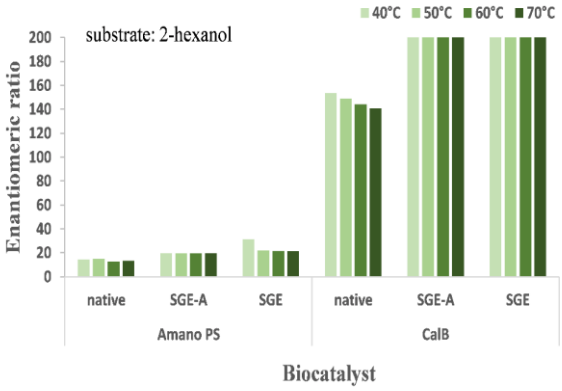

Figure 1. The influence of temperature on enantioselectivity of native and immobilized lipases by sol-gel entrapment (SGE) and by the combined method (SGE-A) in the enantioselective acylation of 2-hexanol in n-hexane at 40°C

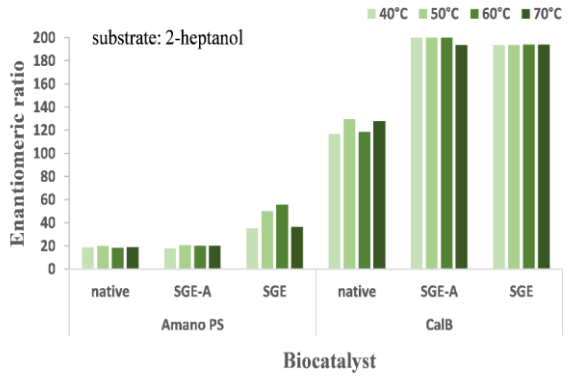

Figure 2. The influence of temperature on enantioselectivity of native and immobilized lipases by sol-gel entrapment (SGE) and by the combined method (SGE-A) in the enantioselective acylation of 2-heptanol in n-hexane at 40°C.

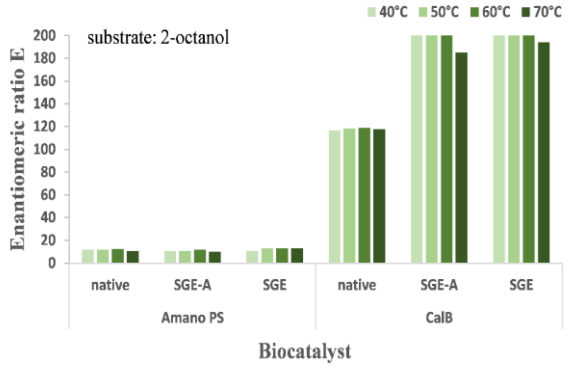

Figure 3. The influence of temperature on enantioselectivity of native and immobilized lipases by sol-gel entrapment (SGE) and by the combined method (SGE-A) in the enantioselective acylation of 2-octanol in n-hexane at 40°C.

The highest enantiomeric ratio values were obtained for CalB biocatalysts (E>200) for all investigated substrates, on the entire temperature range.

Regarding the immobilization technique, for both methods we obtained similar values of activity and enantioselectivity, excepting the enzymatic preparates obtained with lipase Amano PS by sol-gel entrapment SGE, which showed higher values of the studied characteristics.

When 2-octanol was used as substrate, higher values of activity and enantioselectivity compared to the other substrates were obtained for all the tested preparates, indicating an increasing substrate specificity for structures with longer saturated hydrocarbon chain.

3.2. Long term stability of immobilized *Candida antarctica* B lipaze

Since enzymatic preparates obtained with lipase from *Candida antarctica* B (CalB) showed the best thermal stability at the studied temperatures, we continued the study by incubating them at 80°C in isooctane up to 120 h (5 days).

After this, the biocatalysts have been tested in the enantioselective acylation of 2-hexanol with vinyl acetate at 40°C in n-hexane. The effect of temperature on the catalytic properties are presented in Figures 4 and 5.

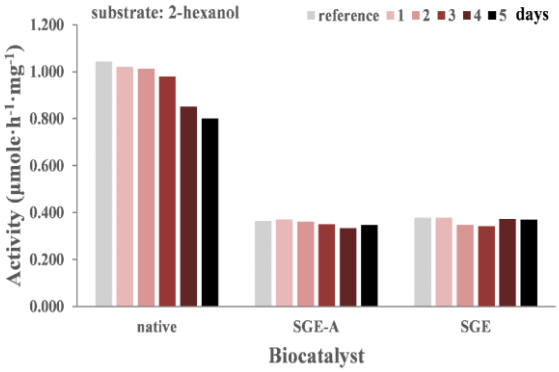

Figure 4. The influence of temperature (80ºC in isooctane for 5 days) on the catalytic activity of native and immobilized *Candida antarctica* B (CalB) by sol-gel entrapment (SGE) and by the combined method (SGE-A) in the enantioselective acylation of 2-hexanol in n-hexane at 40ºC.

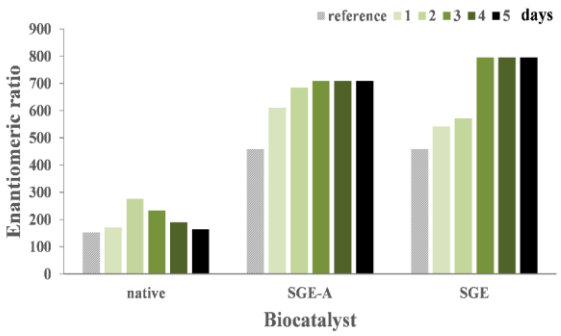

Figure 5. The influence of temperature (80ºC in isooctane for 5 days) enantioselectivity of native and immobilized Candida antarctica B (CalB) by sol-gel entrapment (SGE) and by the combined method (SGE-A) in the enantioselective acylation of 2-hexanol in n-hexane at 40ºC.

Even when the biocatalysts were incubated for 5 days at 80°C, the preparates obtained with CalB showed unchanged values for the catalytic activity, while the native lipase exhibited a decline to less than 25% of the initial activity (Figure 4). Also, the enantioselectivity of immobilized biocatalysts remained unchanged on the entire incubation period (E>200) (Figure 5).

3.3. Thermal analysis (TGA/DTA)

Thermogravimetry was also used to evaluate the thermal stability and degradation of the immobilized biocatalysts with *Candida antarctica* B. Figure 6 presents the TG images recorded from 30 to 1000°C with a heating rate of 10°C min^{-1} and Table 3 shows the thermal behaviour of the biocatalysts.

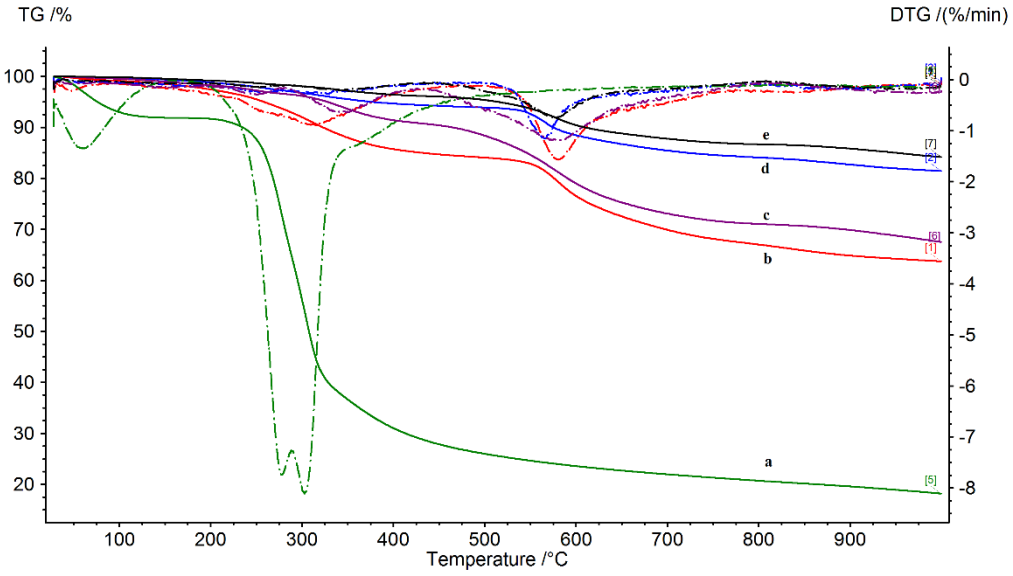

Figure 6. Thermograms (TG, continuous lines) and their derivatives (DTG, dotted lines) obtained for: (a) lipase from *Candida antarctica* B (native), green; (b) SGE, red; (c) SG matrix, magenta; (d) SGE-A, blue; (e) SG-A matrix, black

Table 3. Thermal behavior of the sol-gel immobilized biocatalysts

Preparate Code	Weight loss [%]			Residual mass [%]
	30-180°C	180-400°C	400-950°C	
native lipase	8.05	60.77	12.08	18.21
SGE	2.02	12.2	21.47	63.72
SG matrix	1.43	7.11	22.63	67.52
SGE-A	0.92	4.36	12.90	81.44
SG-A matrix	0.63	2.93	11.39	84.17

The weight loss curves of the sol-gel immobilized preparates were divided into three regions.

In region I from 30 to 180°C, the weight loss between 0.63 – 2.02% was associated to the loss of water and small organic molecules. As expected, the water content of native lipase was higher (around 8%).

Region II, in the range between 180 - 400°C, was related with the thermal decomposition of the organic part (protein), with a significant weight loss of 60.77 % for the native lipase, only a 5.09 % difference between SGE preparate and SG matrix and also a 1.43 % difference between SGE-A preparate and SG-A matrix.

In region III, the weight loss is associated with final thermal decomposition of the organic groups linked to the sol-gel matrix.

As seen in Figure 6, the TG curve of the native lipase was completely different in comparison to the immobilized biocatalysts, showing a significant total weight loss around 82%.

When the lipase was immobilized by sol-gel entrapment (SGE), the weight loss observed on recorded temperature range was around 36%, while for the lipase immobilized by sol-gel entrapment combined with adsorption (SGE-A) the weight loss was reduced (around 19%). The difference could be attributed to the adsorbent Celite, an inorganic material that has high thermal stability at elevated temperatures. The obtained results can be correlated with the excellent values of activity and enantioselectivity obtained for CalB preparates.

Thermal stability study demonstrate the possibility to maintain intact the catalytic properties of sol-gel entrapped enzymes on a longer period.

4. Conclusions

Increase of enzyme stability at higher temperatures represents one of the main advantages of immobilization. The study reveals good thermal stability of the immobilized biocatalysts in organic solvents. All immobilized biocatalysts showed higher enantiomeric ratio E values than the native enzyme, even at elevated temperatures. Excellent results were obtained for the preparates with lipase from *Candida antarctica* B incubated at 80ºC in isooctane for 5 days. Thermogravimetric analysis showed lower weight losses and better temperature stability of the materials obtained by sol-gel entrapment combined with adsorption (SGE-A).

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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