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Ioannis V Pavlidis, Kyriakos Tzafestas, Haralambos Stamatis

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Water-in-ionic liquid microemulsion-based organogels as novel matrices for enzyme immobilization

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4 **Water-in-ionic liquid microemulsion-based organogels as**
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8 **novel matrices for enzyme immobilization**
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13 Ioannis V. Pavlidis, Kyriakos Tzafestas, Haralambos Stamatis*

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15
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17
18 Laboratory of Biotechnology, Department of Biological Applications and
19
20 Technologies, University of Ioannina, 45110 Ioannina, Greece
21
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25 **Keywords:** lipase, immobilization, ionic liquids, microemulsions, organogels
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30 * Corresponding author:
31

32 H. Stamatis
33

34 Laboratory of Biotechnology,
35

36
37 Department of Biological Applications and Technologies, University Campus,
38

39
40 45110 Ioannina, Greece
41

42 telephone: +30-26510-07360; fax: +30-26510-07343;
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44 e-mail: hstamati@cc.uoi.gr
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49 **List of abbreviations**
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51 [bmim]BF₄: 1-butyl-3-methyl imidazolium tetrafluoroborate
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53 [bmim]PF₆; 1-butyl-3-methyl imidazolium hexafluorophosphate
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56 ATR: attenuated total reflectance
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58 AOT: bis-(2-ethylhexyl) sulfosuccinate sodium salt
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60 CalB: *Candida antarctica* lipase B

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3 Cvl: *Chromobacterium viscosum* lipase
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5 FT-IR: Fourier-transform infrared spectroscopy
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8 HPMC: (hydroxypropyl)methyl cellulose
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10 IL: ionic liquid
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12 MBGs: microemulsion-based organogels
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15 Tween 20: Polyethylene glycol sorbitan monolaurate
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17 w/IL: water-in-ionic liquid
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19 w/o: water-in-oil
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For Peer Review

Abstract

The use of water-in-ionic liquid microemulsion-based organogels (w/IL MBGs) as novel supports for the immobilization of lipase B from *Candida antarctica* and lipase from *Chromobacterium viscosum* was investigated. These novel lipase-containing w/IL MBGs can be effectively used as solid phase biocatalysts in various polar and non-polar organic solvents or ionic liquids, exhibiting up to 4.4-fold higher esterification activity compared to water-in-oil microemulsion-based organogels. The immobilized lipases retain their activity for several hours at 70°C, while their half life time is up to 25-fold higher compared to that observed in w/IL microemulsions. Fourier transform infrared spectroscopy data indicate that immobilized lipases adopt a more rigid structure, referring to the structure in aqueous solution, which is in correlation with their enhanced catalytic behaviour observed.

1. INTRODUCTION

Water-in-oil (w/o) microemulsions, also known as reverse micelles, have been widely reviewed as tool for achieving various enzymatic reactions in hydrophobic solvents [1-3]. These microheterogeneous media consist of aqueous nanodroplets dispersed in a non-polar organic phase stabilized by surfactants. Enzymes maintain their catalytic activity when hosted in these aqueous nanodroplets, catalyzing both synthetic and hydrolytic reactions [3, 4]. Reverse micelles have been associated with the idea of micro-reactors for enzymatic reactions, when substrates and/or products are lipophilic and low water content is desired [1, 2].

Interestingly, w/o microemulsions can be "gelled" by the addition of biopolymers, such as gelatin or cellulose derivatives, yielding a matrix suitable for enzyme immobilization [5-8]. The immobilization procedure can affect the catalytic behaviour of the entrapped enzymes, in terms of reaction rate, selectivity and stability, improving the catalytic performance of the immobilized enzymes [9]. The enzymes immobilized into microemulsion-based organogels (MBGs) retain their catalytic activity in several organic media, and therefore can be used for various biotransformations involving synthetic or natural substrates [5, 8, 10, 11]. Moreover, these enzyme-containing organogels overcome to a great extent the major problems which must be solved for the employment of a microemulsion system in industrial processes; most notably, the recovery of the products and the regeneration of the biocatalyst.

Nowadays, environmental demands necessitate the development of new type of green solvents and innovations towards more sustainable enzymatic processes. Room temperature ionic liquids (ILs), because of their specific physicochemical characteristics and their ability to dissolve compounds of different polarities, have

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3 shown great potential as an attractive alternative to conventional organic solvents, in
4 catalytic processes on both a laboratory and industrial scale. Recently the formation of
5 nano/micrometer-sized water droplets in ionic liquid continuous phase stabilized by
6 suitable surfactant (noted as w/IL microemulsions) was reported [12, 13]. These new
7 IL-based microemulsion systems combine the advantages of the ILs and those of the
8 organized nano-structure media and could be used as a novel “green” media for
9 biocatalytic reactions [14-16]. In a previous work we showed that w/IL
10 microemulsions formulated with Tween 20 and the hydrophobic ionic liquid
11 [bmim]PF₆ can be used for efficient entrapment of lipases [15]. Lipases entrapped in
12 these microheterogeneous systems exhibit high synthetic activity and significantly
13 high thermal and operational stability [15].
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29 In the present study we report the formation of MBGs formulated with w/IL
30 microemulsions and a biocompatible polymer such as (hydroxypropyl)methyl
31 cellulose (HPMC) and we investigate the application of these novel organogels as solid
32 supports for enzyme immobilization. The biocatalytic behaviour and the stability of
33 two commercial lipases, namely lipase B from *Candida antarctica* (CalB) and lipase
34 from *Chromobacterium viscosum* (Cvl), was investigated and compared to that
35 observed either in w/o MBGs, or in w/IL microemulsions. Lipases used in the present
36 work are widely employed in various biocatalytic processes [8, 20-22]. Their catalytic
37 behavior has been reported to differ, resulting from structural differences; Cvl has a
38 well-formed lid and presents interfacial activation, acting as a typical lipase, while
39 CalB has a very small lid and doesn't exhibit interfacial activation [23-27]. Particular
40 attention has been focused on the factors that affect the immobilized enzyme's
41 stability, as this is a critical factor when considering practical applications of the
42 immobilized enzymes in biocatalytic processes. Moreover, Fourier transform-infrared
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(FT-IR) spectroscopy was employed to investigate possible changes in enzyme structure upon immobilization and correlate with the biocatalytic behaviour of the immobilized enzymes.

2. MATERIALS AND METHODS

2.1. Materials

Lipases from *Candida antarctica* (lipase B, CalB), *Chromobacterium viscosum* (Cvl), *Candida rugosa* and *Rhizomucor miehei* were purchased from Fluka. Novozyme TL 100 L (*Thermomyces lanuginosa* lipase) was a generous gift from Novozymes. The ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF₆) was purchased from Alfa Aesar. Tween 20 (Polyethylene glycol sorbitan monolaurate) and 1-butyl-3-methylimidazolium tetrafluoroborate ([bmim]BF₄) were purchased from Fluka. (Hydroxypropyl)methyl cellulose (HPMC, 80-120 cP) and bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT) were purchased from Sigma. All other reagents were of analytical grade. The aqueous lipase solutions were prepared by dissolving lipase in 25 mM Tris-HCl buffer (pH 7.5).

2.2. Preparation of microemulsions and MBGs

In all cases a 25 mM Tris/HCl pH 7.5 buffer solution was used as aqueous phase. All microemulsion systems were prepared as described elsewhere [13]. The water-in-oil (w/o) microemulsions were prepared by adding appropriate amount of lipase in a 0.1 M AOT solution in isooctane with final molar hydration ratio w_o ($w_o = [H_2O]/[surfactant]$) equal to 10. Preliminary results showed that at $w_o = 10$ immobilized lipases exhibit high activity and stability (data not shown). The w/IL

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3 microemulsions were prepared by mixing buffer solution, Tween 20 and [bmim]PF₆
4 (35:50:15 weight ratio, except otherwise described).
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8 The MBGs were prepared by introducing appropriate amount of w/o or w/IL
9 microemulsion containing lipase to a second solution of HPMC in water. In a typical
10 immobilization procedure, 1 ml of microemulsion containing 190 µg of CalB or 200
11 µg of Cvl was gelled with 1 g of HPMC and 2 ml of water at 30°C. Mixtures were
12 vigorously shaken and stirred until homogeneous. Then, the organogel was overlaid
13 on a glass surface and incubated for 3 hours at 30 °C to yield the MBG. The
14 preparations were cut in small pieces of diameter from 0.2 to 0.5mm, washed twice
15 with *n*-hexane, air-dried and stored at 4 °C until used.
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27 **2.3. Lipase-catalyzed esterification reactions**

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29 The lipase-containing MBGs were used in various organic solvents and in
30 ionic liquids to catalyze a model reaction such as the esterification of lauric acid (0.1
31 M) with 1-butanol (0.2 M). Lipase-catalyzed reactions took place in capped vials
32 placed in an orbital shaker at 30 °C at 200 rpm, with 100 mg of MBG per 1 ml of
33 organic solvent or ionic liquid. At fixed time intervals aliquots were withdrawn
34 (extracted with equal volume of *n*-hexane when ionic liquid was used as reaction
35 medium) and were analyzed for ester content by gas chromatography (GC), using *n*-
36 decane as internal standard. All experiments were carried out in triplicate.
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49 Gas chromatography analysis was performed by a Shimatzu GC 17A device
50 (Tokyo, Japan), which was equipped with a β-DEX™ 120, fused silica capillary
51 column (30m x 0.25mm x 0.25µm film thickness, Supelco) and a Flame Ionization
52 Detector with helium as the carrier gas in a similar manner as described elsewhere
53 [15].
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2.4. Stability of free and immobilized lipases

The thermal and operational stability of CalB and Cvl immobilized in w/IL MBGs was investigated and compared to that observed in water and in w/IL microemulsions. The stability of the immobilized lipases was determined in isooctane over a 7 day period at temperatures up to 70 °C in the absence of substrates. After incubation, samples were withdrawn at regular time intervals in order to measure the remaining enzyme activity by monitoring the butyl laurate synthesis at 30 °C by GC, as described in Section 2.3. The stability of lipases in aqueous solution and w/IL microemulsions was monitored as described elsewhere [15].

The experimental results were elaborated with the series type deactivation model introduced by Henley and Sadana [28] ($E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$), where E, E₁ and E₂ are the three enzyme states having different specific activities, the parameters α_1 and α_2 are the ratio of specific activities E₁/E and E₂/E respectively, and k_1 and k_2 are first-order deactivation rate coefficients, respectively for E → E₁ and E₁ → E₂ steps.

The non-linear Levenberg-Marquadt regression method was applied to fit the residual activity data. In all cases, a good correlation between the experimental and the predicted values was found (correlation coefficient $r > 0.950$). The Newton-Raphson method was used to calculate the half-life times ($t_{1/2}$) from the two-step deactivation equation [28], using the parameter values calculated from the experimental data.

2.5. Structural Studies

IR spectra were measured using a Shimadzu FT-IR 8400 spectrometer (Tokyo, Japan) equipped with a deuterated triglycine sulfate detector, in the region of 4000-700 cm⁻¹. The spectrum acquisition (liquid samples were overlaid on a zinc selenide

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3 attenuated total reflectance accessory, immobilized enzymes were measured as 2 % wt.
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5 KBr pellets), the data analysis of the amide I region and the band assignment were
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7 performed as described in previous work [15]. The data were manipulated using
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9 WinSpec software (LISE-Faculte's Universitaires Notre-Dame de la Paix, Namur,
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11 Belgium).

18 3. RESULTS AND DISCUSSION

22 3.1. Formation of w/IL MBGs containing lipase

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24 In the present work we studied the formation of w/IL microemulsions-based
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26 organogels (MBGs) formulated with a biocompatible polymer such as
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28 (hydroxypropyl)methyl cellulose (HPMC) and we investigated the suitability of these
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30 novel MBGs as matrices for enzymes immobilization. It was found that MBGs can be
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32 formed with various w/IL microemulsions composed of [bmim]PF₆, Tween 20 and
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34 water, as described in our previous work [15]. HPMC forms w/IL MBGs with mass
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36 fraction of HPMC in the gels from 0.137 to 0.322 (prior to the drying step). It must be
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38 noted that MBGs formed at the above concentration range of HPMC were stable and
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40 maintained their structural integrity for several months at room temperature. Figure 1
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42 shows the effect of the mass fraction of HPMC in the gels on the reaction rate and
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44 conversion yield of the esterification of 1-butanol with lauric acid catalyzed by w/IL
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46 MBGs containing lipase B from *Candida antarctica* (CalB) in *n*-hexane. As can be
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48 seen, the higher synthetic activity of the immobilized enzyme was observed in MBGs
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50 with mass fraction of HPMC in the gels 0.240. Similar catalytic behaviour was
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52 observed from Cvl immobilized in w/IL MBGs (data not shown). This mass fraction
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54 was used in all cases studied in this work.
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As it can be seen from Table 1, various microbial lipases immobilized in w/IL MBGs exhibited up to 4.4-fold higher activity for the synthesis of butyl laurate compared to that observed in w/o MBGs at the same reaction conditions. Similar catalytic behaviour was observed for the esterification of fatty acids of different carbon chain length (C₈-C₁₈) with various primary alcohols (C₁-C₈) (data not shown). It is interesting to note that the esterification activity of CalB and Cvl immobilized in these novel w/IL MBGs was found to be up to 7 times higher than this observed when enzymes are immobilized in HPMC hydrogels, indicating that the presence of w/IL microemulsions is crucial for the preservation of the enzyme activity during the immobilization procedure. The hydrophobic ionic liquid used in the immobilization procedure could increase the accessibility of the hydrophobic reactants in the enzyme's microenvironment, resulting in higher esterification activity [29, 30].

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The highest enzyme concentration that can be achieved in w/IL MBGs is about 2.5mg/g, significantly higher than that reported for MBGs formulated with w/o microemulsions [5, 6]. This difference can be attributed to the larger aqueous phase of w/IL microemulsions compared to these of w/o microemulsions, in which higher amount of enzyme can be solubilized [5, 15]. Moreover, the esterification rate linearly increases as the amount of lipase in the w/IL MBGs increases up to 0.8 mg/g. However, at higher enzyme concentration, the dependence of the reaction rate on the enzyme concentration is not linear, probably due to mass transfer limitations, as described for other MBGs [11].

53 **3.2. Effect of system composition on lipase activity**

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The effect of the composition of the microemulsion used for the formation of w/IL MBGs on the esterification activity of the immobilized CalB is presented in Table 2. When the water content is constant (5% w/w), the activity of the immobilized

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3 lipase increases as the surfactant concentration increases. Similar catalytic behaviour
4 was observed in w/o MBGs [6]. An increase in surfactant concentration at constant
5 water content leads to increase of the number of microdroplets formed, which
6 probably facilitates the dispersion of enzyme in the gel matrix. On the other hand, at
7 constant surfactant concentration (50% w/w), the enzyme activity strongly depends on
8 the water content of the w/IL microemulsion system. In this case, the highest lipase
9 activity was observed at a molar ratio of water to surfactant (w_o) around 14. This
10 value is significantly lower than this reported for other w/o MBGs [6, 31-33]. This
11 difference could be attributed to the fact that lipases entrapped in w/IL
12 microemulsions exhibit their maximum catalytic activity in significantly lower w_o
13 values than those observed in other w/o microemulsions [15].
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30 **3.3. Effect of the reaction medium on lipase activity**

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32 It is well established that the nature of the reaction medium significantly
33 affects the catalytic behaviour of the immobilized enzymes and, in some extent, the
34 structure of the matrix. In previous studies it was reported that polar solvents (such as
35 acetone) affect the rigidity of w/o MBGs, leading to loss of incorporated surfactant and
36 to deactivation of immobilized enzyme [5]. In the present study the influence of the
37 reaction medium on the esterification activity of lipases immobilized in w/o and in
38 w/IL MBGs was studied using various polar and non-polar organic solvents and ionic
39 liquids as reaction medium. As it was found, w/IL and w/o MBGs maintained their
40 structural integrity in all solvents tested after incubation for several days at 30 °C. As
41 it can be seen in Table 3, the catalytic behaviour of both lipases immobilized in w/IL
42 MBGs depends significantly on the reaction medium. High esterification activity was
43 observed in non-polar organic solvents such as *n*-hexane and isooctane which is in
44 accordance to that reported previously for MBGs formulated with AOT or lecithin
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3 microemulsions [5]. Moreover, lipases immobilized in these novel w/IL MBGs keep
4 their activity in solvent free systems (1-butanol was used as substrate and reaction
5 medium) as well as in polar organic solvents (acetone, acetonitrile). Interestingly,
6 both lipases immobilized in w/IL MBGs exhibited their highest esterification activity
7 in [bmim]PF₆. On the other hand, the activity of CalB immobilized in w/o MBGs was
8 very low in both ionic liquids used, indicating that the entrapment of the lipase in
9 w/IL microemulsions prior the immobilization process significantly improves its
10 activity in such media.
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22 **3.4. Effect of incubation temperature on lipase activity**

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25 The effect of the temperature on the esterification activity of lipases
26 immobilized in w/IL MBGs as well as of enzyme entrapped in w/IL microemulsion
27 was studied using the esterification of lauric acid with 1-butanol. The reaction was
28 initiated by addition of the lipase-containing w/IL MBGs in the reaction solution
29 (isooctane for the immobilized enzymes, w/IL microemulsion for the free enzyme)
30 which had been pre-equilibrated to the desired temperature. For esterification
31 reactions in w/IL microemulsions, the substrates were dissolved in the pre-incubated
32 microemulsion system and then the enzyme was added as aqueous solution. Figure 2
33 shows the effect of the temperature on the esterification activity for the two enzyme
34 forms of CalB (entrapped in w/IL microemulsions and immobilized in w/IL MBGs).
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As it can be seen, the optimum temperature for the esterification activity of the CalB
entrapped in w/IL microemulsions was 50 °C, while at higher temperatures a decrease
of the esterification rate was observed. Significant modifications in the temperature
profile were observed after immobilization of CalB on w/IL MBGs since an increase
on the incubation temperature from 30 to 80 °C resulted in an increase in esterification
activity. Similar catalytic behavior was observed when a commercially available

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3 immobilized CalB, Novozyme 435[®], was used for the polymerization of ϵ -
4 caprolactone [34]. In the case of Cvl, although the optimum temperature value was
5 practically the same for the two enzyme forms (about 60 °C), the immobilized enzyme
6 retained higher fraction of its activity at temperatures up to the optimum value (data
7 not shown). The beneficial effect of immobilization on the catalytic behaviour
8 observed for both lipases could be attributed to increased thermal stability of the
9 immobilized enzymes, as discussed in the next paragraph.

10 11 12 **3.5. Lipase stability**

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Figure 3 depicts the deactivation curves of Cvl at 50 °C obtained in w/IL MBG, in w/IL microemulsion system and in water. As it can be seen, the stability of the lipase entrapped in w/IL microemulsions increases compared to that observed in water, which is in accordance to that reported previously [15]. Moreover, the lipase immobilization in w/IL MBGs further increases the lipase stability. The immobilized Cvl retained about 60 % of its initial activity after 5 days incubation in isooctane at 50 °C, while the non-immobilized lipase in aqueous solution or in w/IL microemulsion systems retained less than 5 %.

Table 4 shows the two-step deactivation kinetic constants and the half-life times for CalB and Cvl immobilized in MBGs formulated with w/IL microemulsions. As it can be seen, both immobilized enzymes are extremely stable. It must be noted that the half-life time of both immobilized lipases at 50 °C increases at least 150-fold compared to that observed in aqueous solution and 25-fold compared to that observed in w/IL microemulsions. These observations indicate that w/IL MBG matrix provides a protective environment for the lipases, especially at high incubation temperatures.

It is worth mentioning that at 30 °C an activation of both immobilized lipases was observed, as indicated from the α_1 values ($\alpha_1 > 1$ in these cases). It was proposed

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3 that the first unfolding step exposes to the protein's surface some unstable amino acid
4 residues and this probably provides flexibility to the lipase's structure (E_1) and
5 therefore higher activity [35]. This phenomenon is not observed at higher incubation
6 temperatures, where the inactivation of the enzyme is faster and the activity of the
7 intermediate (E_1) is lower than that of the native enzyme ($\alpha_1 < 1$).
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15 Both lipases immobilized in w/IL MBGs were recycled for the esterification
16 of 1-butanol with caprylic acid in isooctane. The reaction was performed for 3 h at 50
17 °C after which MBGs containing enzyme was removed from the reaction medium,
18 washed 5 times with isooctane and added to the fresh substrate solution. When ten
19 reaction cycles were completed (30 h of total operation), the residual activity of the
20 lipase was more than 50 %, as presented in Figure 4. Moreover, both lipases
21 immobilized at w/IL MBGs retained more than 80 % of their initial activity after 100
22 hours of total operational time at 30 °C, which was significantly enhanced compared
23 to that reported in previous studies [3, 6, 36], indicating that the immobilization of
24 both lipases onto these novel organogels significantly increases their operational
25 stability.
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41 **3.6. Structural studies**

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44 It is well established that the enzymes may undergo structural changes upon
45 immobilization [37, 38]. In the present study, FT-IR spectroscopy was applied in
46 order to monitor possible conformational changes of lipases during the
47 immobilization procedure. The secondary structure elements of lipases in aqueous
48 solution, entrapped in w/IL microemulsion system or immobilized in w/IL MBGs are
49 presented in Table 5. A difference between the structure of lipase in aqueous solution
50 and the crystallographic data was observed [26, 27]. This difference can be attributed
51 to the high enzyme concentration used in ATR FT-IR experiments (1.5mg/ml), which
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3 can lead to protein aggregation [39]. As it can be seen in Table 5, lipases entrapped in
4 w/IL microemulsions and immobilized in w/IL MBGs undergo some alterations in
5 their structure. More specifically, an increase in the β -sheet content is observed when
6 enzymes are entrapped in w/IL microemulsions compared to that observed in aqueous
7 solution, while no significant changes in the α -helix content were observed. The same
8 trend was observed when enzymes were immobilized in w/IL MBGs. In order to
9 further investigate the origin of the β -sheet content increase, we monitored the content
10 of the lipases in antiparallel β -sheets. The content of antiparallel β -sheets is an index
11 for intermolecular interactions, which lead to the formation of protein aggregates [40].
12 The increase in the β -sheet content observed upon immobilization is not proportional
13 to this of antiparallel β -sheets, so the increase is not due to intermolecular
14 interactions; on the contrary, in the case of Cvl the antiparallel β -sheet content
15 significantly decreases, indicating that less protein-protein interactions are present in
16 the immobilized preparation.

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These structural data presented in this work leads us to the assumption that the lipase may adapt a more rigid structure, referring to the structure in aqueous solution, which is in accordance to previous works [15, 41, 42]. This more rigid structure adopted from the immobilized enzymes results in enhanced thermal and operational stability as described before.

4. CONCLUDING REMARKS

In the present work we have shown that w/IL microemulsion-based organogels can be efficiently used as solid matrices for enzyme immobilization. These novel lipase-containing w/IL MBGs are more biocompatible, compared to well known w/o MBGs used so far for enzyme immobilization. The operational and storage stability of

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3 immobilized lipases was significantly enhanced when immobilized in these MBGs,
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5 indicating that the w/IL microemulsion systems provide a protecting environment for
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7 the enzymes. This observation was confirmed by conformational studies, which
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9 indicate that the lipases immobilized in w/IL MBGs tend to adopt a more rigid
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11 structure compared to that observed in water or in w/IL microemulsions. The high
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13 enzyme loading which can be attained in these MBGs, among with the enhanced
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15 stability, the reusability even at relatively high incubation temperatures suggest that
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17 these novel lipase-containing w/IL MBGs can be effectively used for biocatalytic
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19 processes in various polar and non-polar organic solvents or ionic liquid-based media.
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Conflict of interest

The authors have declared no conflict of interest.

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5. REFERENCES

- [1] Stamatis, H., Xenakis, A., Kolisis, F. N., Bioorganic reactions in microemulsions: The case of lipases. *Biotechnol. Adv.* 1999, 17, 293-318.
- [2] Orlich, B., Schomäcker, R., Enzyme catalysis in reverse micelles, in: Scheper, T. (Ed.), *Advances in Biochemical Engineering / Biotechnology*, Springer-Verlag, Berlin, Heidelberg 2002, pp. 185-209.
- [3] Rees, G. D., Robinson, B. H., Esterification reactions catalyzed by *Chromobacterium viscosum* lipase in CTAB-based microemulsion systems. *Biotechnol. Bioeng.* 1995, 45, 344-355.
- [4] Crooks, G. E., Rees, G. D., Robinson, B. H., Svensson, M., *et al.*, Comparison of hydrolysis and esterification behavior of *Humicola lanuginosa* and *Rhizomucor miehei* lipases in AOT-stabilized water-in-oil microemulsions .1. Effect of pH and water content on reaction kinetics. *Biotechnol. Bioeng.* 1995, 48, 78-88.
- [5] Delimitsou, C., Zoumpantioti, M., Xenakis, A., Stamatis, H., Activity and stability studies of *Mucor miehei* lipase immobilized in novel microemulsion-based organogels. *Biocatal. Biotransformation* 2002, 20, 319-327.
- [6] Jenta, T. R. J., Batts, G., Rees, G. D., Robinson, B. H., Biocatalysis using gelatin microemulsion-based organogels containing immobilized *Chromobacterium viscosum* lipase. *Biotechnol. Bioeng.* 1997, 53, 121-131.
- [7] Zoumpantioti, M., Parmaklis, P., De María, P. D., Stamatis, H., *et al.*, Esterification reactions catalyzed by lipases immobilized in organogels: Effect of temperature and substrate diffusion. *Biotechnol. Lett.* 2008, 30, 1627-1631.
- [8] Zoumpantioti, M., Stamatis, H., Xenakis, A., Microemulsion-based organogels as matrices for lipase immobilization. *Biotechnol. Adv.* 2010, 28, 395-406.

1
2
3 [9] Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., *et al.*,
4
5 Improvement of enzyme activity, stability and selectivity via immobilization
6
7 techniques. *Enzyme Microb. Technol.* 2007, 40, 1451-1463.

8
9
10 [10] Dave, R., Madamwar, D., *Candida rugosa* lipase immobilized in Triton-X100
11
12 microemulsion based organogels (MBGs) for ester synthesis. *Proc. Biochem.* 2008,
13
14 43, 70-75.

15
16
17 [11] Stamatis, H., Xenakis, A., Biocatalysis using microemulsion-based polymer gels
18
19 containing lipase. *J. Mol. Catal. : B Enzym.* 1999, 6, 399-406.

20
21
22 [12] Gao, Y., Li, N., Zheng, L. Q., Zhao, X. Y., *et al.*, A cyclic voltammetric
23
24 technique for the detection of micro-regions of bmimPF₆/Tween 20/H₂O
25
26 microemulsions and their performance characterization by UV-Vis spectroscopy.
27
28 *Green Chem.* 2006, 8, 43-49.

29
30
31 [13] Gao, Y. N., Han, S. B., Han, B. X., Li, G. Z., *et al.*, TX-100/water/1-butyl-3-
32
33 methylimidazolium hexafluorophosphate microemulsions. *Langmuir* 2005, 21, 5681-
34
35 5684.

36
37
38 [14] Moniruzzaman, M., Kamiya, N., Goto, M., Biocatalysis in water-in-ionic liquid
39
40 microemulsions: A case study with horseradish peroxidase. *Langmuir* 2009, 25, 977-
41
42 982.

43
44
45 [15] Pavlidis, I. V., Gournis, D., Papadopoulos, G. K., Stamatis, H., Lipases in water-
46
47 in-ionic liquid microemulsions: Structural and activity studies. *J. Mol. Catal. B:*
48
49 *Enzym.* 2009, 60, 50-56.

50
51
52 [16] Zhou, G. P., Zhang, Y., Huang, X. R., Shi, C. H., *et al.*, Catalytic activities of
53
54 fungal oxidases in hydrophobic ionic liquid 1-butyl-3-methylimidazolium
55
56 hexafluorophosphate-based microemulsion. *Colloids Surf. B: Biointerfaces* 2008, 66,
57
58 146-149.
59
60

1
2
3 [17] Chen, B., Miller, E. M., Miller, L., Maikner, J. J., *et al.*, Effects of macroporous
4 resin size on *Candida antarctica* lipase B adsorption, fraction of active molecules, and
5 catalytic activity for polyester synthesis. *Langmuir* 2007, 23, 1381-1387.
6
7

8
9 [18] Pepin, P., Lortie, R., Influence of water activity on the enantioselective
10 esterification of (R,S)-ibuprofen by *Candida antarctica* lipase B in solventless media.
11
12 *Biotechnol. Bioeng.* 1999, 63, 502-505.
13
14

15 [19] Talukder, M. M. R., Zaman, M. M., Hayashi, Y., Wu, J. C., *et al.*, Pretreatment
16 of *Chromobacterium viscosum* lipase with acetone increases its activity in sodium bis-
17 (2-ethylhexyl) sulfosuccinate (AOT) reverse micelles. *J. Chem. Technol. Biotechnol.*
18
19 2005, 80, 1166-1169.
20
21
22
23
24
25

26 [20] Gotor-Fernández, V., Busto, E., Gotor, V., *Candida antarctica* lipase B: An ideal
27 biocatalyst for the preparation of nitrogenated organic compounds. *Adv. Synth. Catal.*
28
29 2006, 348, 797-812.
30
31
32

33 [21] Anderson, E. M., Larsson, K. M., Kirk, O., One biocatalyst - many applications:
34 The use of *Candida antarctica* B-lipase in organic synthesis. *Biocatal.*
35
36 *Biotransformation* 1998, 16, 181-204.
37
38
39

40 [22] Shah, S., Sharma, S., Gupta, M. N., Biodiesel preparation by lipase-catalyzed
41 transesterification of Jatropha oil. *Energy Fuels* 2004, 18, 154-159.
42
43
44

45 [23] Martinelle, M., Holmquist, M., Hult, K., On the interfacial activation of *Candida*
46 *antarctica* lipase A and B as compared with *Humicola lanuginosa* lipase. *Biochim.*
47
48 *Biophys. Acta* 1995, 1258, 272-276.
49
50
51

52 [24] Verger, R., 'Interfacial activation' of lipases: Facts and artifacts. *Trends in*
53
54 *Biotechnology* 1997, 15, 32-38.
55
56
57
58
59
60

- 1
2
3 [25] Melo, E. P., Taipa, M. A., Castellar, M. R., Costa, S. M. B., *et al.*, A
4 spectroscopic analysis of thermal stability of the *Chromobacterium viscosum* lipase.
5
6 *Biophysical Chemistry* 2000, 87, 111-120.
7
8
9
10 [26] Lang, D., Hofmann, B., Haalck, L., Hecht, H. J., *et al.*, Crystal structure of a
11 bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 angstrom
12 resolution. *J. Mol. Biol.* 1996, 259, 704-717.
13
14
15
16
17 [27] Uppenberg, J., Öhrner, N., Norin, M., Hult, K., *et al.*, Crystallographic and
18 molecular-modeling studies of lipase B from *Candida antarctica* reveal a
19 stereospecificity pocket for secondary alcohols. *Biochemistry* 1995, 34, 16838-16851.
20
21
22
23 [28] Henley, J. P., Sadana, A., Categorization of enzyme deactivations using a series-
24 type mechanism. *Enzyme Microb. Technol.* 1985, 7, 50-60.
25
26
27
28 [29] Petkar, M., Lali, A., Caimi, P., Daminati, M., Immobilization of lipases for non-
29 aqueous synthesis. *J. Mol. Catal. B: Enzym.* 2006, 39, 83-90.
30
31
32
33 [30] Tzialla, A. A., Pavlidis, I. V., Felicissimo, M. P., Rudolf, P., *et al.*, Lipase
34 immobilization on smectite nanoclays: Characterization and application to the
35 epoxidation of α -pinene. *Bioresour. Technol.* 2010, 101, 1587-1594.
36
37
38
39 [31] Nagayama, K., Katakura, R., Hata, T., Naoe, K., Imai, M., Reactivity of *Candida*
40 *rugosa* lipase in cetyltrimethylammonium bromide microemulsion–gelatin complex
41 organogels. *Biochem. Eng. J.* 2008, 38, 274-276.
42
43
44
45 [32] Nagayama, K., Tada, K., Naoe, K., Imai, M., *Rhizopus delemar* lipase in
46 microemulsion-based organogels: Reactivity and rate-limiting study. *Biocatal.*
47 *Biotransformation* 2003, 21, 321-324.
48
49
50
51 [33] Soni, K., Madamwar, D., Ester synthesis by lipase immobilized on silica and
52 microemulsion based organogels (MBGs). *Proc. Biochem.* 2001, 36, 607-611.
53
54
55
56
57
58
59
60

- 1
2
3 [34] Kumar, A., Gross, R. A., *Candida antartica* lipase B catalyzed polycaprolactone
4 synthesis: Effects of organic media and temperature. *Biomacromolecules* 2000, 1,
5 133-138.
6
7
8
9
10 [35] Carvalho, C. M. L., Cabral, J. M. S., Aires-Barros, M. R., Cutinase stability in
11 AOT reversed micelles: System optimization using the factorial design methodology.
12 *Enzyme Microb. Technol.* 1999, 24, 569-576.
13
14
15 [36] Zoumpantioti, M., Karali, M., Xenakis, A., Stamatis, H., Lipase biocatalytic
16 processes in surfactant free microemulsion-like ternary systems and related
17 organogels. *Enzyme Microb. Technol.* 2006, 39, 531-539.
18
19
20 [37] Palomo, J. M., Muoz, G., Fernández-Lorente, G., Mateo, C., *et al.*, Interfacial
21 adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads):
22 Immobilization, hyperactivation and stabilization of the open form of lipases. *J. Mol.*
23 *Catal. B: Enzym.* 2002, 19-20, 279-286.
24
25
26 [38] Menea, B., Herrero, M., Rives, V., Lavrenko, M., *et al.*, Favourable influence of
27 hydrophobic surfaces on protein structure in porous organically-modified silica
28 glasses. *Biomaterials* 2008, 29, 2710-2718.
29
30
31 [39] Palomo, J. M., Fuentes, M., Fernández-Lorente, G., Mateo, C., *et al.*, General
32 trend of lipase to self-assemble giving bimolecular aggregates greatly modifies the
33 enzyme functionality. *Biomacromolecules* 2003, 4, 1-6.
34
35
36 [40] Mukherjee, S., Chowdhury, P., Gai, F., Infrared study of the effect of hydration
37 on the amide I band and aggregation properties of helical peptides. *J. Phys. Chem. B*
38 2007, 111, 4596-4602.
39
40
41 [41] De Diego, T., Lozano, P., Gmouh, S., Vaultier, M., *et al.*, Understanding
42 structure - stability relationships of *Candida antartica* lipase B in ionic liquids.
43 *Biomacromolecules* 2005, 6, 1457-1464.
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 [42] Griebenow, K., Klibanov, A. M., On protein denaturation in aqueous-organic
4 mixtures but not in pure organic solvents. *J Am. Chem. Soc.* 1996, *118*, 11695-11700.
5
6
7
8
9
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11
12
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Figure legends

Figure 1: Effect of mass fraction of HPMC (prior to the drying step) in the w/IL MBGs on the esterification activity of immobilized CalB in *n*-hexane.

Figure 2: Effect of the reaction temperature on the esterification activity of CalB entrapped in w/IL microemulsions (●) and immobilized in w/IL MBGs (■).

Figure 3: Thermal deactivation curves of Cvl in aqueous system (■), entrapped in w/IL microemulsions (▲), and immobilized in w/IL MBGs (▼) at 50°C. Symbols correspond to the experimental results and lines correspond to the predicted deactivation curve.

Figure 4: Reusability of CalB and Cvl immobilized in w/IL MBGs at 50°C in isooctane.

Table 1: Effect of microemulsion type on the esterification activity of immobilized lipases onto MBGs at 30°C in isooctane.

Lipase	Reaction rate (mM/h)	
	w/o MBG	w/IL MBG
<i>Candida antarctica</i> lipase B	7.5 ± 0.3	8.1 ± 0.3
<i>Candida rugosa</i> lipase	not determined	0.4 ± 0.0
<i>Chromobacterium viscosum</i> lipase	3.7 ± 0.2	16.4 ± 0.8
<i>Rhizomucor miehei</i> lipase	1.1 ± 0.1	1.9 ± 0.1
<i>Thermomyces lanuginosa</i> lipase	13.1 ± 0.7	13.7 ± 0.6

Table 2: Effect of w/IL microemulsion composition on the esterification activity of immobilized CalB onto MBGs. Standard deviation in all cases was less than 5%.

Weight Ratio (%)				Reaction Rate (mM/h)
[bmim]PF ₆	Tween 20	Aqueous	w _o	(% Conversion after 24 h of incubation)
47.5	50.0	2.5	3.4	4.6 (78.2)
45.0	50.0	5.0	6.8	5.1 (80.8)
40.0	50.0	10.0	13.7	10.8 (100.0)
35.0	50.0	15.0	20.5	8.1 (100.0)
30.0	50.0	20.0	27.3	7.9 (77.8)
20.0	50.0	30.0	40.9	5.8 (88.6)
10.0	85.0	5.0	4.0	9.3 (89.0)
20.0	75.0	5.0	4.6	6.0 (75.0)
40.0	55.0	5.0	6.2	5.2 (81.9)
60.0	35.0	5.0	9.7	4.1 (79.8)
80.0	15.0	5.0	22.8	0.5 (17.4)

Table 3: Effect of the reaction medium on the esterification activity (mM/h) and the 24 hour-conversion (in the brackets, %) of lipases immobilized in MBGs. Standard deviation in all cases was less than 7%.

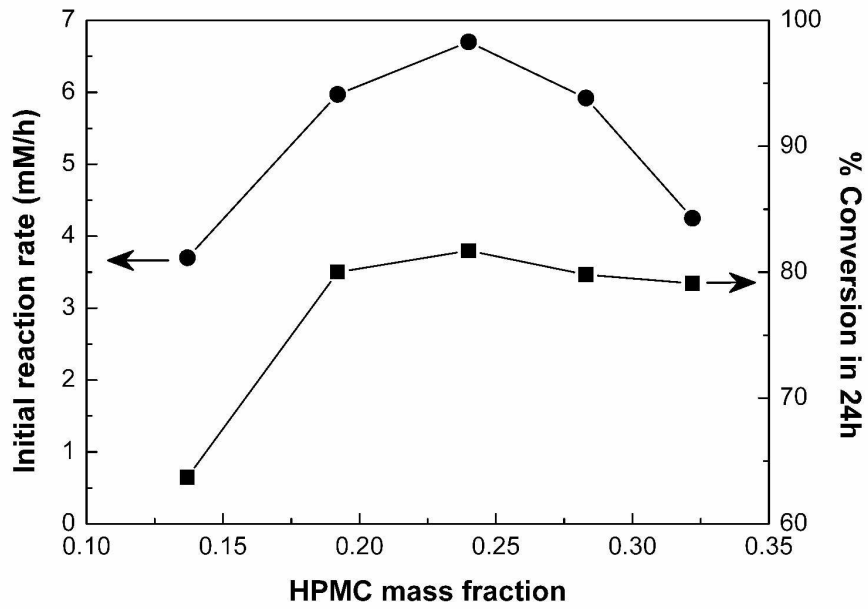
Reaction medium	Log P	w/IL MBG		w/o MBG
		Cvl	CalB	CalB
Isooctane	4.0	16.4 (100.0)	8.1 (100.0)	7.5 (80.5)
<i>n</i> -Hexane	3.4	7.9 (84.2)	6.7 (81.7)	6.9 (80.6)
Isopropyl ether	1.9	4.7 (64.7)	4.8 (66.4)	5.8 (68.4)
<i>tert</i> -Butanol	1.45	7.7 (33.6)	3.8 (41.6)	1.4 (19.3)
1-Butanol	0.80	8.6 (47.4)	2.5 (18.7)	0.6 (16.0)
Acetone	-0.23	1.7 (16.9)	0.8 (10.3)	0.5 (19.1)
Acetonitrile	-0.33	7.9 (87.2)	14.7 (90.3)	10.1 (79.9)
[bmim]PF ₆	-2.38	25.7 (100.0)	23.0 (100.0)	0.9 (61.9)
[bmim]BF ₄	-2.44	1.1 (13.3)	1.3 (49.2)	0.1 (3.0)

Table 4: Deactivation kinetic constants and half-life time as predicted from the two-step deactivation model described in Section 2.4. In all cases $\alpha_2 = 0$. The correlation coefficient r was higher than 0.950 in all cases.

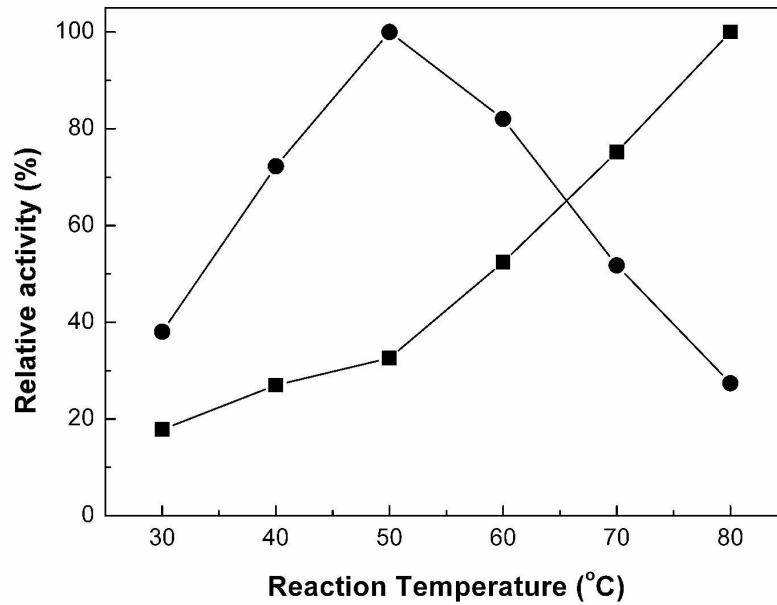
Lipase	Temperature (°C)	α_1	k_1 (h ⁻¹)	k_2 (h ⁻¹)	$t_{1/2}$ (h)
CalB	30	1.612	1.085	0.003	391
	50	0.748	2.056	0.002	202
	70	0.510	1.830	0.030	2
Cvl	30	1.263	0.006	0.006	319
	50	0.670	0.451	0.002	149
	70	0.431	0.662	0.026	3

Table 5: Quantitative estimation (%) of the secondary structure elements of lipases in water, entrapped in w/IL microemulsions and immobilized in w/IL MBGs calculated by FT-IR analysis in amide I region.

Lipase	System	α -helix	β -sheet	Other	<i>Antiparallel β-sheet</i>
CalB	Aqueous	24.0	29.0	47.0	8.5
	w/IL microemulsion	25.7	33.4	40.9	8.7
	w/IL MBGs	21.0	42.0	37.0	11.7
Cvl	Aqueous	22.0	37.5	40.5	25.9
	w/IL microemulsion	25.1	39.6	35.2	11.5
	w/IL MBGs	21.8	53.1	25.2	5.1



Effect of mass fraction of HPMC (prior to the drying step) in the w/IL MBGs on the esterification activity of immobilized CalB in n-hexane.
1155x808mm (150 x 150 DPI)



Effect of the reaction temperature on the esterification activity of CalB entrapped in w/IL microemulsions (●) and immobilized in w/IL MBGs (■).
1187x839mm (150 x 150 DPI)

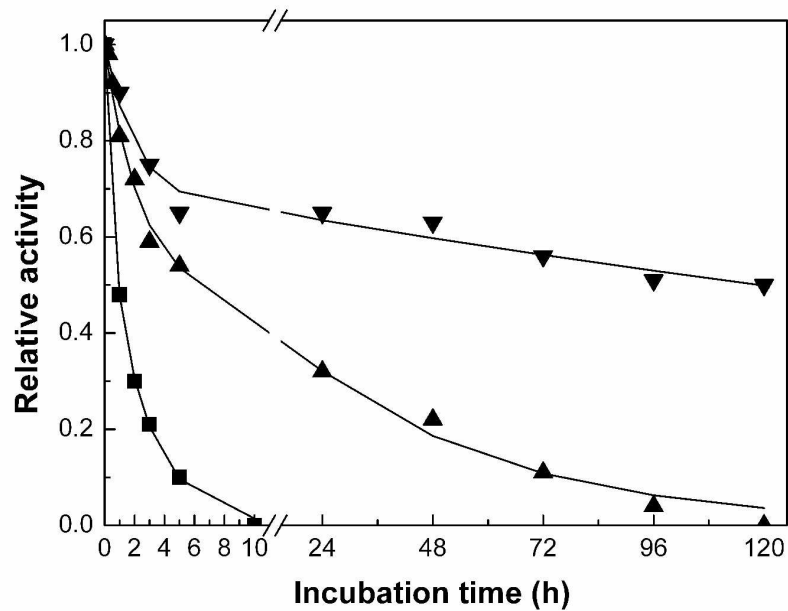
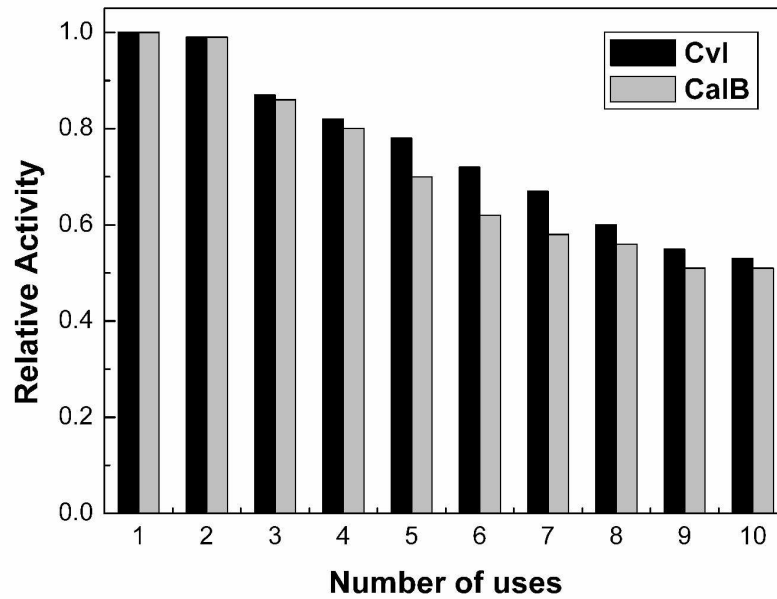


Figure 3: Thermal deactivation curves of Cvl in aqueous system (■), entrapped in w/IL microemulsions (▲), and immobilized in w/IL MBGs (▼) at 50°C. Symbols correspond to the experimental results and lines correspond to the predicted deactivation curve.
1147x806mm (150 x 150 DPI)



Reusability of CalB and Cvl immobilized in w/IL MBGs at 50°C in isooctane.
1147x806mm (150 x 150 DPI)