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Journal of Molecular Catalysis B: Enzymatic 35 (2005) 93–99
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Generation of lipase-containing static emulsions in silicone spheres for synthesis in organic media

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Received 30 December 2004; received in revised form 21 April 2005; accepted 2 May 2005
Available online 7 July 2005

Abstract

Because of the broad versatility of lipases as biocatalysts, interest has for some years been focused on the improvement of the economy of processes using these enzymes, especially by appropriate immobilisation. In this study, a method was developed to emulsify aqueous solutions of lipase A of *Candida antarctica* (CALA) and lipase of *Thermomyces lanuginosa* (TLL) in silicone elastomers yielding elastic beads. The persistent water-organic interface created by this static emulsion enabled an improved performance of the immobilised lipases due to the well known fact that from a kinetic point of view these enzymes show a higher efficiency in biphasic than in monophasic systems. The entrapped lipases catalysed the esterification of octanol and caprylic acid in hexane with an activity that, related to the free enzyme, was enhanced about 31-fold for CALA and 250-fold for TLL. Comparison to the activity of the same enzymes in sol–gels revealed that for CALA immobilisation in static emulsion was the only method yielding active biocatalysts, whereas activation of TLL was in the same range in static emulsion and sol–gels. However, apparent activity of TLL in static emulsion was considerably higher than in sol–gels due to the feasible high enzyme loading. The results indicate that immobilising lipases as static emulsion is a technique suitable for biotechnological application. Moreover, a transfer to enzymes of other classes seems possible.
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Keywords: Ester synthesis; Lipase; Immobilisation; Silicone; Emulsion

1. Introduction

Lipases are efficient catalysts which enable a number of esterifications, transesterifications and hydrolysis reactions with high chemo-, regio- and enantioselectivity, accepting a broad range of even synthetic substrates with vast industrial importance. They are therefore the most frequently used biocatalysts in the production of (chiral) fine- as well as bulk-chemicals.

Transformations with lipases can be performed in monophasic, either aqueous or organic media [1], or in a combination of both, i.e. biphasic systems [2]. In order to improve process economy, investigations have for some years been focused on the development of technologies for the continuous or repetitive use and easy recovery of lipases. In this context, immobilisation was performed by various methods, most often by physical adsorption on or covalent binding to solid supports [3,4], and by entrapment in organic or inorganic polymers [5–10].

The diverse methods of immobilisation show general differences in their effects on biocatalysts: Adsorption usually has a low impact on activity, but resulting immobilisates suffer from poor operational stability due to the weak binding forces and subsequent easy release of enzyme [5]. Vice versa, covalent binding can improve operational stability, but often decreases activity [5]. Entrapment is frequently combined with limitations in mass-transfer, but can nevertheless be superior to binding when working in organic solvents, as the formation of stable aqueous compartments protects the entrapped biocatalysts from detrimental effects of the solvent phase [10,11]. Moreover, supply of a favourable microenvironment for the biocatalyst is possible due to the huge

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doi:10.1016/j.molcatb.2005.05.006

diversity of available inorganic or organic entrapment matrices [7,8]. Many inorganic materials e.g. offer the possibility to control balance of hydrophobicity/hydrophilicity [9], average pore size or pore size distribution [12].

Regarding lipases, entrapment in alkyl-substituted organic silane precursors has so far led to the most active immobilisates. The obtained sol–gels even show a significantly higher activity than free dispersed enzyme [5,12,13]. On the background that lipases are activated by hydrophobic-hydrophilic interfaces [14] this is not surprising as they probably interact with hydrophobic matrices in a similar manner. Additional to direct activation, the hydrophobic sol–gels facilitate mass-transfer of hydrophobic substrates and products in the immobilisates [5]. Consequently, development in lipase immobilisation has concentrated on entrapment in optimised sol–gels. Sol–gels, however, are limited in their industrial application due to their high brittleness and small size. They are most often produced and applied as powders, though bead or cube shape can also be achieved, if an appropriate support is used during sol–gel formation [15,16]. Special requirements for technical use of immobilisates in bioreactors, however, include distinct diameters and confined compressibility in packed-beds, or elasticity and separation in stirred tanks. As matrices with hydrophobicity similar to sol–gels, but superior mechanical properties silicones have recently been investigated [17–20]. However, up to date only few lipases were successfully immobilised in this kind of material, and compared to sol–gels lipase activation was only moderate [17–22].

In this study, we present a novel technique for the immobilisation of lipases which creates a stable emulsion of lipase-containing aqueous solution in bead-shaped silicone elastomers. Thus, a persistent water/organic interface is created which improves performance of the immobilised enzymes due to the well-known fact that lipases show a higher efficiency in biphasic than in monophasic systems [2,23,24]. The method was evaluated in comparison to immobilisation in sol–gels, investigating the esterification of octanol and caprylic acid in hexane as a model for industrially more important esterifications. As representative catalysts lipase A of *Candida antarctica* and lipase of *Thermomyces lanuginosa* were investigated.

2. Material and methods

2.1. Material

Liquid preparation of lipase A from *Candida antarctica* (CALA; Novozym 735, protein content 1.8% (w/w); lipolytic activity against tributyrin 372 kU/g) was a generous gift from NovoNordisk (Kopenhagen, Denmark). Lyophilised lipase of *Thermomyces lanuginosa* (TLL; Chirazyme L-8, protein content 35.3% (w/w); lipolytic activity 3180 kU/g) was obtained from Roche Diagnostics (Mannheim, Germany). Both enzymes were used without further purification. Sylgard® 184 and Syl-Off® 4000 were received from DowCorning (Manchester, UK). Iso-butyltrimethoxysilane (i-BTMS) was purchased from ABCR (Karlsruhe, Germany). Polyvinyl alcohol (PVA; MW 10-98), octyl octanoate, caprylic acid, octanol, hexane, and other chemicals, all in reagent grade, were obtained from Fluka (Taufkirchen, Germany).

2.2. Esterification

Lipase activity was determined for the esterification of 50 mM caprylic acid and 50 mM n-octanol in n-hexane. 20 mM of n-decane were present as internal standard for GC analysis. In order to evaluate the dependence of the reaction on water, up to 120% (v/v) of potassium phosphate buffer (0.1 M; pH 7.0) were added to 3 mL of substrate solution containing free enzyme (20 μL of CALA and 20 mg of TLL). For esterification with immobilised lipases, 200 mg of silicone spheres were suspended in 3 mL (CALA) or 6 mL (TLL) of substrate solution, and 100 mg (CALA) or 20 mg (TLL) of sol–gels were suspended in 3 mL of substrate solution. The reaction vessels were incubated under vigorous shaking at room temperature. After separation from the biocatalysts, samples of 50 μL were withdrawn from the organic phase and analysed with GC/FID (60 °C for 1 min, heating with 25 °C/min up to 200 °C, kept for 4 min; carrier gas N_2 1 mL/min; column: FFAP-CW, 25 m, ID 0.25 mm, d_f 0.25 μm; CS-Chromatographie, Langerwehe, Germany; retention times: 1.34 min for n-decane; 4.00 min for octanol; 5.96 min for octyl octanoate; 6.21 min for caprylic acid). Activity of all enzyme preparations was determined from initial rates of esterification under exactly identical conditions.

2.3. Immobilisation of lipase in sol–gel

For entrapment of lipases in sol–gel 400 μL of a lipase-containing solution (protein concentration: 4.4–17.8 mg/mL of CALA and 2.7–7.5 mg/mL of TLL in 0.1 M potassium phosphate buffer, pH 7.0) were mixed with 50 μL of an aqueous solution of sodium fluoride (1 M), 100 μL of a solution of 4% (w/v) polyvinyl alcohol (PVA, 15.000 Da) in water, 100 μL isopropyl alcohol, 480 μL iso-butyltrimethoxysilane, 440 μL propyltrimethoxysilane (PTMS) and 148 μL tetramethoxysilane (TMOS). Subsequent treatment was carried out as described by Reetz et al. [5].

2.4. Immobilisation of lipase in silicone spheres

Silicone rubbers were formed by homogenising a mixture of 4 g of Sylgard® 184 component A (α,ω-divinyl terminated polydimethylsiloxan), 0.4 g of component B (copolymer of methylhydrosiloxane and dimethylsiloxane), 44 mg of Syl-Off® 4000 (bis(1,3-divinyl-1,1,3,3-tetradisiloxane)platin(0)) and 2 mL of chloroform. In this mixture 1 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 4.4–17.8 $mg_{Protein}$/mL of CALA or 22.1–88.3 $mg_{Protein}$/mL of TLL was emulsified. The emulsion was mixed on an

overhead-shaker for 20–30 min and subsequently dispersed in 150 mL of a temperated (45 °C) and stirred solution of 4% (w/w) polyvinyl alcohol (PVA; MW 10-98) in water for 2–3 h. The silicone spheres were separated from the PVA solution by filtration and washed with 250 mL of deionised water. For further use they were stored at room temperature in hexane.

2.5. *Characterisation of silicone immobilisates*

2.5.1. *Entrapment efficiency*

The yield of silicone spheres was determined as the ratio of total amount of used material (neglecting the volatile chloroform) and total mass of obtained spheres. Enzyme release during vulcanisation was quantified by measuring protein content [25] and lipolytic activity in the PVA-hardening bath. Lipolytic activity was measured at room temperature in an emulsion of 50 mM tributyrin in 1 mM Tris–HCl buffer (pH 7.5; 0.1 M $CaCl_2$; 0.1 M NaCl) by titrating 50 mM NaOH to a constant pH after addition of the lipase-containing sample. The amount of enzyme inside the silicone spheres was calculated from the difference between initial amount of enzyme and enzyme released during bead formation. The amount of entrapped water was determined as the difference in weight after drying a defined quantity of silicone spheres in a weighed beaker at 60 °C for approximately one week. The stability of immobilisates in repetitive use was demonstrated by incubating 200 mg of immobilisates in 3 mL (CALA) or 6 mL (TLL) of substrate solution (composition as stated above) in four reaction cycles. Between separate cycles the immobilisates were washed with hexane until no remaining substrate or product was detectable in the solvent.

2.5.2. *Swelling in hexane*

Swelling of silicone spheres with a diameter of more than 1 mm was quantified by evaluating a two-dimensional digital picture with the ImageTool® software (Version 3.0, available free of charge from the University of Texas Health Science Center in San Antonio, Texas, USA) before and after incubating in hexane for 60 min. To generate a contrast for this optical analysis, the spheres were transferred into a petri dish filled with a solution of 0.1% (w/v) methylene blue in water. The petri dish was placed on a translucent plexi-glass plate above a light source. The swelling of silicone spheres with a diameter of less than 1 mm was determined by placing a defined amount of spheres in a measuring flask and measuring the total volume before and after swelling in hexane. During this measurement the space between the beads in the measuring flask was minimised by compression with a stamp.

2.5.3. *Compressibility and abrasion*

For characterisation of mechanical properties a single silicone sphere with a diameter of 2–4 mm was placed on a balance and slowly compressed by a pressure plate. The force acting on the plate and its displacement were recorded. Resistance against abrasion was evaluated by shaking 300 mg of silicone spheres (diameter 2–4 mm) with 8 g of glass-beads (0.3 mm in diameter) in a closed vessel containing 15 mL of hexane for one week. After this, the surface of the spheres was optically inspected. Additionally, the beads were weighed to determine loss of material.

3. Results and discussion

3.1. *Generation and properties of a static emulsion*

Silicone elastomers incorporating a persistent emulsion of water were generated by vulcanising a mixture of siloxane prepolymers in which water was previously emulsified. Prerequirement for maintaining this emulsion in the final elastomer was continuous shaking until an increase in viscosity indicated that vulcanisation was proceeding. Vulcanisation was affected at a water content above 25% (w/w) in the mixture of siloxane prepolymers, all silicone elastomers investigated in this study were therefore formed at a water content of 22.5% (w/w). Spherical shape of the silicone elastomers (Fig. 1) was achieved when the emulsion of water in siloxane prepolymers was dropped in an aqueous solution of PVA as described by Hilgers [26] after vulcanisation had proceeded to a notable degree (after 10 to 30 min). The average diameter of the resulting beads depended on the stirring intensity of the PVA solution. With the setup used in this study a maximum bead diameter of 4 mm was accessible, but for experiments mostly beads in a size range of 0.5 mm to 2 mm at an average size distribution of approximately ±40% were employed.

The total yield of silicone beads ranged between 90 and 95% of the initially applied material, the loss being ascribed to the viscosity of the prevulcanised silicone mixture which caused strong adhesion to the reaction vessel. Of the water initially emulsified in the mixture of siloxane prepolymers,

Fig. 1. Silicone spheres with lipase-containing static emulsion. Bead diameter was up to 4 mm, depending on the stirring intensity of the PVA-hardening bath during preparation.

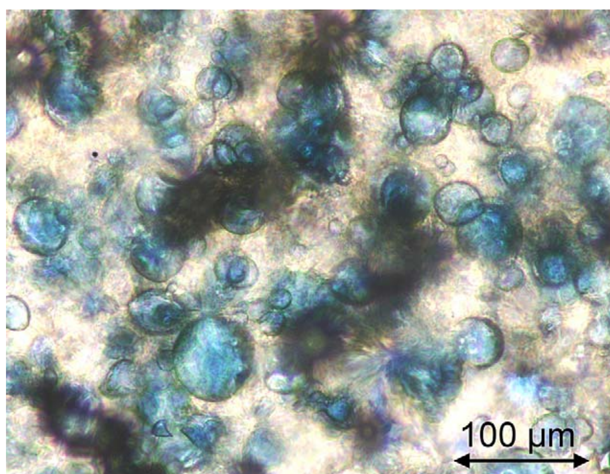

Fig. 2. Microscopic cross section of a cured silicone elastomer with an entrapped emulsified aqueous phase (dyed with 0.1% (w/v) methylene blue for demonstration). Diameter of the aqueous droplets ranged between 5 and 60 μm.

$83.7 \pm 0.3\%$ were entrapped in the elastomer. This was determined from the maximum loss of weight which occurred at incubation of silicone spheres at 60 °C to constancy of weight. The loss of water could be explained by coalescence with the PVA solution during hardening of the elastomer. On the other hand, the chosen experimental conditions might not have enabled complete evaporation of entrapped water, whereby the detected water content would have been lower than the actual one. This assumption is supported by the finding that entrapment of dissolved protein in the static emulsion ranged between 89 and 96% of applied protein and thus was highly efficient. The size of the emulsified aqueous droplets in silicone was estimated to range between 5 and 60 μm in diameter (Fig. 2). It was not possible to further reduce this size by adding tensides, such as PVA or gum Arabic, to the emulsion.

In hexane, silicone spheres incorporating a static emulsion swelled by a factor of 2 when more than 1 mm in diameter and by a factor of 2.5 when smaller. This observation is in accordance with the swelling of silicone elastomers in organic solvents that was already reported by Gill et al. [17] and Ragheb et al. [18]. In the study of Gill et al. [17] the volume increase was 60–230%, Ragheb et al. [18] did not give distinct values. The effect can be ascribed to the expansion of the polymeric network by the influx of organic solvent. Thus, the observed difference between small and large silicone spheres might be caused by a different degree of polymerisation, if not by inaccuracies of the measurement techniques. It can be supposed that the swelling of silicone elastomers in organic solvents is advantageous for mass transfer of hydrophobic substrates and products. Interestingly, the silicone spheres used in this work turned out to be firmer after swelling than before.

Incorporating a static emulsion did obviously not affect known elasticity of silicone elastomers, i.e. a breaking point under strain was not observed and the initial shape was repetitively regained. Even under very drastic conditions, at vigorous shaking of suspended silicone spheres in the presence of glass beads for one week [27], mechanical resistance was very high: only $3.4 \pm 0.9\%$ of the original bead weight were lost. From this findings can be expected that the here described static emulsion will easily fulfill the requirements of long-term use in biotechnological applications, as the employed processes do usually not subject a stress as extreme and enduring as in our investigation.

3.1.1. Lipase activity in static emulsions

The entrapment of lipase A from *Candida antarctica* (CALA) and lipase from *Thermomyces lanuginosa* (TLL), as static emulsions in silicone spheres resulted in a considerable activation of both enzymes. This is documented by the activity of the immobilised lipases which, in comparison to free enzyme in hexane (relative activity, Table 1), was enhanced by factor 31 for CALA and factor 250 for TLL.

A maximum apparent activity of $0.5\,\mathrm{U/g_{Immob.}}$ and $33\,\mathrm{U/g_{Immob.}}$ was achieved for CALA and TLL, respectively. The linear relationship between the apparent activity of CALA and protein content of the immobilisates indicates that for this enzyme an increase of apparent activity can still be achieved by increasing the amount of entrapped enzyme. In contrast, maximum loading of immobilisates with TLL was obtained at a protein content between 3.7 and $5.6\,\mathrm{mg/g_{Immob.}}$, which is obvious from the constant apparent activity of TLL at protein loadings of $5.6 - 14.9\,\mathrm{mg/g_{Immob.}}$ (Table 1).

Since sol–gels are up to date the by far most efficient matrices for the entrapment and activation of lipases, the here presented novel method was evaluated in comparison to sol–gel preparations which were produced according to Reetz et al. [5]. In this sol–gels an activation of TLL by factor 260, which is comparable to published data [21], was achieved, whereas CALA revealed dramatically low catalytic activity (Table 1). This latter result was unexpected because Reetz et al. [5] had previously reported a low, but distinct activation of lipase from *C. antarctica* in sol–gels. However, as these authors did not specify the enzyme preparation used for their experiments, their investigation might refer to a mixture of lipase B and A which is customary in many commercial preparations. In that case, an enhancement of activity of lipase B would have led to an overall enhancement of activity even if lipase A was not active at all, and would thus explain the contradiction to the here presented results. In this context, it is remarkable that the entrapment in a static emulsion is, to our knowledge, the first recorded method of a successful immobilisation of CALA.

Regarding the performance of the static emulsion in the immobilisation of TLL, activation in comparison to the free enzyme was of factor 250 (Table 1) and thus in the same range as after entrapment in sol–gels. Admittedly, this has to be relativised for the "second generation" of sol–gels [21] which has a clearly higher activating effect on lipases than the here investigated "first generation". Mass transfer

Table 1
Esterification activity of CALA and TLL after immobilisation in sol–gels and static emulsions, respectively (average activities were determined for five different preparations per enzyme loading)

Enzyme	Immobilised protein[a] (mg/g)	Apparent activity ($U/g_{Immob.}$)	Specific activity ($U/mg_{Protein}$)	Relative activity[b]
CALA				
Static emulsion	0.7	0.14 ± 0.03	0.18 ± 0.04	31.1 ± 7.4
	1.5	0.22 ± 0.09	0.14 ± 0.06	24.1 ± 9.8
	2.3	0.36 ± 0.13	0.15 ± 0.06	26.8 ± 10.0
	3.2	0.49 ± 0.13	0.16 ± 0.04	27.1 ± 7.4
Sol–gel	2.7	0	0	0
	5.0	0	0	0
	10.4	0.034	0.004	0.6
TLL				
Static emulsion	3.7	22.1 ± 10.7	5.85 ± 2.89	250.9 ± 123.9
	5.6	31.6 ± 9.6	5.60 ± 1.70	240.1 ± 42.9
	7.4	33.2 ± 6.8	4.36 ± 0.96	187.0 ± 41.2
	11.1	31.7 ± 7.2	2.81 ± 0.64	120.5 ± 27.4
	14.9	32.9 ± 19.1	2.20 ± 1.25	94.3 ± 53.6
Sol–gel	1.6	9.5 ± 1.6	6.07 ± 0.99	260.5 ± 42.5
	2.9	9.1 ± 0.8	3.11 ± 0.36	133.2 ± 15.2
	4.4	8.6 ± 1.5	1.97 ± 0.47	84.6 ± 20.0

[a] The amount of immobilised protein was calculated by taking into account entrapment efficiency and maximum yield of immobilisate. Entrapment efficiency ranged between $92.5 \pm 3.5\%$ of applied protein.
[b] The relative activity is defined as ratio of activities of immobilised and free enzyme ($activity_{Immob.}/activity_{Free}$).

limitation, however, is obviously lower in static emulsions than in sol–gels: At different protein loadings the apparent activity of TLL in sol–gels remains constant, while the specific activity decreases with increasing protein content. Apparently, maximum enzyme loading is exceeded at very low concentrations, and subsequently only a low apparent activity can be achieved (9.5 $U/g_{Immob.}$). In static emulsions higher protein loading is feasible and results in a significantly higher apparent activity of TLL (33.2 $U/g_{Immob.}$). At present, this advantage can be overridden by the increase in apparent activity that can be obtained with optimised sol–gel materials [12,15,21,22], but nevertheless demonstrates the promising potential of static emulsions with regard to technical application. After all, extensive optimisation has not yet been performed for this novel method of lipase immobilisation.

The good performance of static emulsions with regard to the activity of CALA and TLL is probably a result of the persistent two-phases and large water-organic interfaces present in the immobilisates. Turner et al. [2] as well as Louwrier et al. [23] pointed out that from a kinetic point of view esterification with lipases is more effective in liquid-liquid biphasic systems than in microaqueous systems, even if the water activity is close to one. This is due to the extraction of the reaction product by the surrounding organic phase and probably to a favourable conformation of the enzyme at water-organic interfaces [2,23]. Additionally, the interaction with the interface can lead to an enrichment of lipases and thus more enzyme molecules contribute to the overall activity in biphasic systems [28–30]. The beneficial effect of biphasic systems was also observed for CALA and TLL in our study when the native enzymes were used for esterification in hexane at increasing amounts of water (Fig. 3a and b): At a water content of 90% (v/v) the activity of CALA and TLL was still 14-fold and 37-fold, respectively, higher than in water-free media. In static emulsions, lipase activity decreased significantly when immobilisates were stored under conditions which allowed the evaporation of water

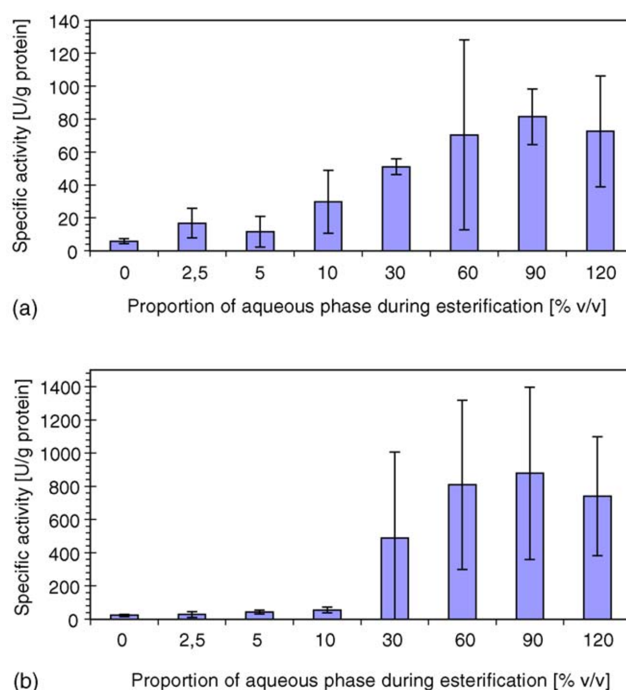

Fig. 3. Influence of different amounts of water in hexane (up to 120% v/v) on the esterification of octanol and caprylic acid. Experiments were carried out five times for each enzyme. The large standard deviation is probably due to not exactly identical emulsions.

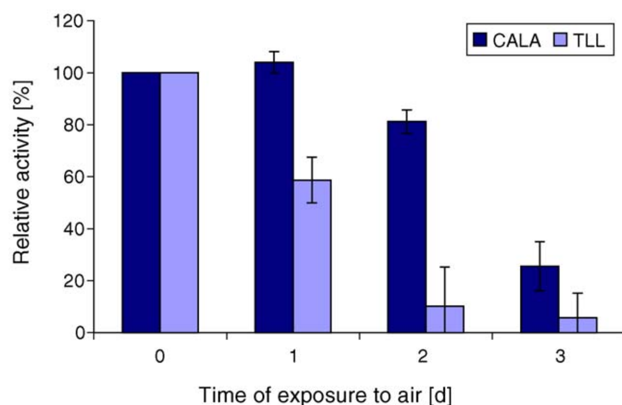

Fig. 4. Influence of the exposure of static emulsions with an initial water content of 22.5% (v/v) to air at room temperature on the catalytic activity of entrapped CALA (3.2 $mg_{Protein}/g_{Immob.}$) and TLL (7.3 $mg_{Protein}/g_{Immob}$). Experiments were repeated three times.

(Fig. 4). In contrast, no activity loss was detected when immobilisates were stored in hexane. The excellent stability of the static emulsion was shown in four repetitive reaction cycles where lipase activity remained at a constant value.

4. Conclusion

With the here presented persistent emulsification of CALA and TLL in silicone spheres a new and promising immobilisation method has been developed which allows enhancement of enzyme activity along with enzyme loadings and matrix properties suitable for technical use. The good results obtained with two different lipases indicate suitability for a broad range of lipases. At first sight, this assumption seems to be contradicted by the work of Ragheb et al. [18], who reported a decrease in activity of lipase from *Candida rugosa* in silicone elastomers when water was added to the non-vulcanised matrix. However, the immobilisation technique applied in the respective study probably allowed phase separation and thus no static emulsion was achieved. Moreover, Wang et al. [19] reported successful entrapment of aqueous solutions of α-amylase and glucose oxidase in membrane-shaped elastomers, which is a strong hint that the static emulsions developed in the here presented study could be applied not only to the immobilisation of lipases, but also to enzymes of other classes.

In contrast to non-static, thermodynamically stable micro-emulsions [31], which have been successfully applied to biocatalytic reactions before, difficulties with downstream processing at the end of the production process [32] are overcome. This might also be achieved using micro-emulsions stabilised with hydrogels [32–34], but the use of hydrophobic material for generating our static emulsion shall certainly have a beneficial effect on transfer of hydrophobic compounds. Moreover, mechanical stability of silicones is much higher than of hydrogels, and the use of tensides, which can have a negative influence on entrapped enzymes [6], is unnecessary. Different from silicone matrices formerly used for enzyme immobilisation [17–20], silicone elastomers used in this study are of spherical shape – a shape which might be advantageous for both, large-scale application and large-scale production of immobilisates.

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