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Highly efficient one pot dynamic kinetic resolution of benzoins with entrapped *Pseudomonas stutzeri* lipase

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Abstract

The immobilisation of lipase from *Pseudomonas stutzeri* (Lipase TL®) by different entrapment protocols (sol–gel, static emulsion-silicone and direct entrapment in silicone spheres) is described for the first time. As this not very common commercial lipase has been recently reported as able to catalyse the dynamic kinetic resolution of benzoins (1,2-diaryl-2-hydroxyethanone structures) combined with a transition metal catalyst, although suffering a deactivation at high temperatures, the different immobilisation methodologies were tested with the aim of enhance lipase activity and stability in the above mentioned process. The enzyme immobilisation by silicone spheres entrapment was the most appropriate method, resulting in a considerable activation of this lipase. Furthermore, the high stability of this immobilised lipase at 60 °C, allowed the development of a “one pot” benzoin DKR process, reaching high conversions in short time, with a 30-fold increase of the productivity of the process due to the possibility of recycling and reuse of the catalyst.

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1. Introduction

The demand for enantiomerically pure compounds, strongly influenced by pharmaceutical industry, has rapidly increased in recent years. Enzymatic kinetic resolution (KR) of racemic mixtures has proven to be a useful method to obtain chiral intermediates [1]. Recently, the coupling of enzymes and metal catalyst in a dynamic kinetic resolution (DKR) process has overcome the intrinsic limitation of KR of the maximum theoretical yield at 50%, making possible the complete transformation of racemic substrates to single enantiomeric products [2–6].

As it is well known, the application of lipases in the resolution of racemic mixtures is widely spread, due to their regio-, stereo- and chemoselectivity and their stability in organic solvents. The mostly employed lipase for DKR of secondary alcohols, CAL B from *Candida antarctica* [6] has been applied to a wide range of substrates, but this enzyme usually displays a low activity for secondary alcohols having bulky substituents on both sides of the stereocentre [7]. Recently, we have reported the DKR of a special type of secondary alcohol, such as benzoins [8] (1,2-diaryl-2-hydroxyethanone structures) catalysed by Lipase TL® (from *Pseudomonas stutzeri*) coupled to an in situ racemisation of the remnant substrate through a ruthenium catalysed redox process (Shvo’s catalyst) obtaining high conversions and excellent enantiomeric excess. This unusual lipase seems to be able to catalyse the resolution of these α-hydroxyketones [9–12], which could not be carried out with other more “classical” lipases, such as CALB, although recently this enzyme has been described to be able to catalyse the DKR of allylic alcohols which are subsequently oxidised to acyloins acetates [13].

In fact, chiral α-hydroxyketones as benzoins are useful building blocks for the synthesis of different heterocycles and

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biologically active compounds [14–18]. Thus, several biocatalytical methods have been described for the synthesis of homochiral benzoins, such as the reduction of α-diketones [19–21] or a fungal deracemisation [22]. On the other hand, enantiomerically pure (*R*)-benzoins can be enzymatically synthesized by a carboligation catalysed by thiamine diphosphate dependent enzymes [23–27], while the antipode (*S*)-benzoins could be reached by above mentioned Lipase TL® catalysed DKR [8].

It is well established that enzyme properties have to be usually improved before being applied in an industrial process [28]. In this sense, immobilisation of lipases can be a simple solution to enhance enzyme activity, selectivity and stability [29,30]. Furthermore, this methodology allows the enzyme separation from the reaction products by a simple filtration and its subsequent recycling and reuse without significant loss of activity. A plethora of protocols for lipase immobilisation have been described, by either physical adsorption on solid supports, covalent binding or by entrapment in organic or inorganic polymers [31]. These different methods of immobilisation present different effects on the lipase [32].

Most of lipases show the so-called interfacial activation, when the higher catalytic activity is observed only in the presence of a hydrophobic interphase [33,34]. This fact has been related to the presence of a mobile amphipatic polypeptide chain (lid) which covers the active site when the enzyme is in its inactive form. In a hydrophobic environment, the lipase suffers a conformational change, the lid is displaced and the active site is exposed to the medium, increasing the lipase activity. It has been shown that the immobilisation of lipases using hydrophobic materials promotes the stabilisation and activity of these enzymes [29,35]. Although as far as we know there are not any data about Lipase TL® structure, the good results obtained by these methodologies moved us to immobilise this enzyme by entrapment in hydrophobic supports, by sol–gel (alkyl-substituted organic silane precursors) [32,36–38], silicone elastomer spheres [39–42] and static emulsion silicone [43] spheres entrapment.

Thus, in this paper we present the immobilisation of lipase from *P. stutzeri* by the three different entrapment methods, comparing their activity and stability versus free enzyme. This lipase suffers a deactivation when incubated at 50°, so the DKR of benzoins had to be carried out in a sequential methodology [8]. The compatibility of the most active immobilised derivative with Shvo's catalyst was studied in order to perform one pot DKR of benzoins, obtaining higher yields and maximum enantiomeric excess than those obtained with the native enzyme. Furthermore, the immobilised lipase could be used several times, therefore increasing the efficacy of the DKR.

2. Results and discussion

2.1. Benzoin kinetic resolution

Lipase TL®-catalysed benzoin kinetic resolution (KR) was chosen as test reaction to measure the activity of both immobilised and free enzyme (Scheme 1). This reaction has been previously described in detail [8].

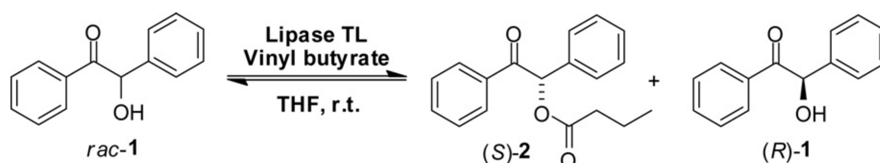
2.2. Free and immobilised lipase activity

Protein content in lipase commercial powder was determined to be 34%, according to Bradford assay [44]. Different quantities of Lipase TL® powder were used as catalyst in the benzoin KR in order to determine lipase specific activity. Results are shown in Table 1.

Sol–gel immobilisation was carried out in two different ways, following the classical methodology described by Reetz et al. [32]. In the first one, *iso*-butyltrimethoxysilane (iBTMS), propyltrimethoxysilane (PTMS) and tetramethoxysilane (TMOS) were added as a component of the sol–gel matrix, obtaining a derivative named LipTL-SG1. Nevertheless, Reetz et al. [36] pointed out that an increased hydrophobicity in the silicon oxide matrix leads to higher enzyme activity. Therefore, the entrapment of Lipase TL® was performed in a different sol–gel derived solely from (iBTMS) and (TMOS), obtaining a new derivative named LipTL-SG2. As a standard comparison, 300 mg of each sol–gel matrix were used as catalyst in the benzoin kinetic resolution (Table 1) and higher lipase activity was observed for LipTL-SG2 as it was expected. Therefore, this methodology was selected to carry out further experiments to determine specific activity (Table 1).

Static emulsion-silicone entrapment was carried out as described by Buthe et al. [43], rendering a derivative named LipTL-SE. Some experiments to determine lipase activity of this immobilised enzyme after different drying times were conducted, and the results are shown in Fig. 1. As it can be seen, the time needed for drying the static-emulsion silicone spheres strongly influenced on the enzyme activity, showing the maximum lipase activity when spheres were dried at 40 °C for 7 h.

Finally, immobilisation of lipase in silicone elastomer spheres was performed in a similar manner by simply adding the crude lipase to the mixture of siloxane prepolymers, as described [40], for obtaining the derivative named LipTL-SS. Spheres were subsequently dried overnight (12 h) at 40 °C. For this methodology, any substantial activity variation with shorter or longer drying times was not observed. Specific activity was determined for benzoin KR as described above for other immobilised derivatives, and the results are shown in Table 1.



Scheme 1. Benzoin (**1**) kinetic resolution catalysed by Lipase TL®.

Table 1
Compared activity of commercial Lipase TL® vs. immobilised derivative in the kinetic resolution of benzoin

	Catalyst (mg)	Protein (mg)[a]	Conversion 24 h (%)	ee_p (%)	Enzyme activity (U)	Specific activity (mU/mg prot.)[b]
Commercial Lip TL	15	5.10	49	99	0.327	
	25	8.50	49	99	0.567	
	35	11.90	49	99	0.823	62.7
	50	17.00	49	99	0.966	
	75	25.50	49	99	1.645	
LipTL-SG1	300	0.58	17	99	0.017	
LipTL-SG2	100	0.18	9	99	0.016	
	200	0.36	19	99	0.032	87.8
	300	0.54	28	99	0.048	
LipTL-SE	100	0.06	7	99	0.002	
	200	0.12	9	99	0.005	
	300	0.17	24	99	0.011	60.8
	350	0.20	25	99	0.011	
LipTL-SS	100	0.77	40	99	0.096	
	200	1.53	43	99	0.240	
	250	1.91	47	99	0.259	139.0
	300	2.30	49	99	0.330	
	350	2.68	49	99	0.359	

[a] According to Bradford methodology for the crude enzyme and the enzymatic loading for the immobilised derivatives (see Section 2).
[b] Experimental error 5%.

In all cases, lipase enantioselectivity was maintained, obtaining ee_p higher than 99%.

As can be observed from Fig. 2, the entrapment of Lipase TL® in silicone spheres resulted in a twofold activation compared to the crude enzyme. The immobilisation in sol–gel matrix (LipTL-SG2) showed a slight activation, which could not be obtained for LipTL-SE; this fact is probably caused because in this last methodology the benzoin must diffuse inside the water phase contained in the sphere [43], therefore resulting in a limited access of the substrate, which solubility in water is really low. As in all cases the excellent enantioselectivity was maintained, the entrapment in silicone spheres was selected as the best methodology, so that LipTL-SS was tested in the DKR of benzoin.

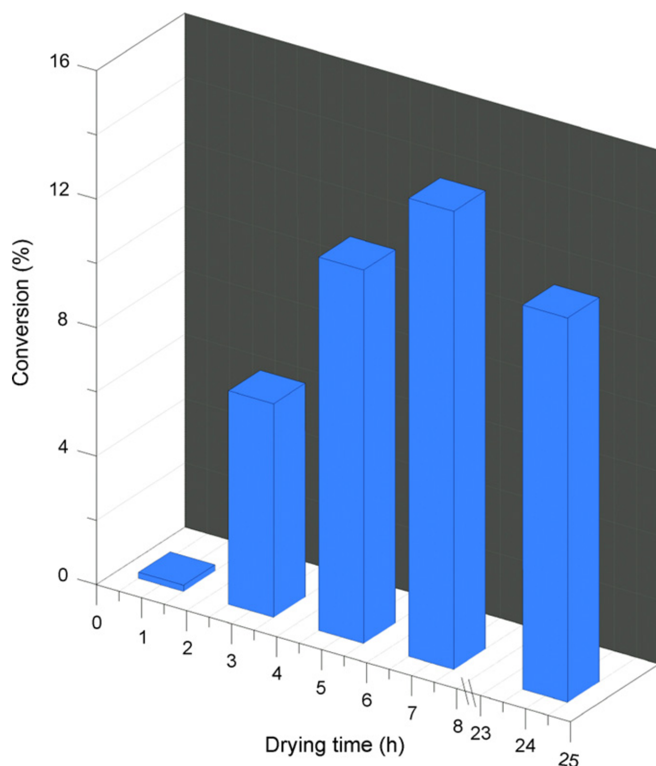


Fig. 1. Conversion (%) after 23 h reaction catalysed by 250 mg of static emulsion-silicone spheres dried for different periods of time. *Experimental conditions*: 30 mg of rac-**1** (0.14 mmol) were dissolved in 1.5 mL of THF and 250 mg LipTL-SE and vinyl butyrate (150 μL, 0.99 mmol) were added. The mixture was shaken at room temperature, and analysed by HPLC as indicated in Section 2.

2.3. Temperature optimisation for benzoin kinetic resolution catalysed by Lipase TL® entrapped in silicone elastomer spheres (LipTL-SS)

As higher amount of immobilised lipase did not show a notable increase of enzyme activity, as shown in Fig. 3, 300 mg of immobilised enzyme in 1.5 mL of substrate solution was selected as the most adequate proportion to catalyse enantioselective benzoin transesterification at room temperature. In order to study if the lipase powder distribution inside the silicone beads was homogeneous enough, benzoin KR catalyzed by 300 mg of LipTL-SS was repeated three times, finding out no substantial activity differences considering the experimental error.

As the native lipase suffers a deactivation when incubated at 50 °C [8], which is further accentuated at 60 °C, the kinetic resolution reaction was carried out at those temperatures (50 and 60 °C) in order to study the stability and activity of LipTL-SS (Fig. 4). As can be seen from Fig. 4, the reaction at 60 °C proceeded faster than the standard conditions at room temperature, with no signs of any thermal deactivation under the reaction conditions, therefore showing how the lipase entrapment had increased the enzymatic stability.

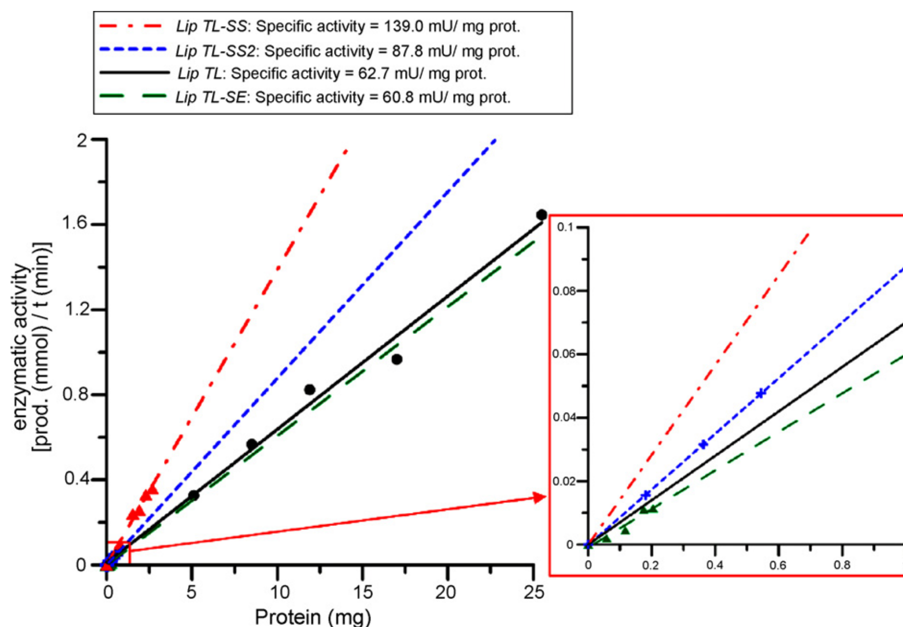


Fig. 2. Specific activity of Lip TL and immobilised Lip TL.

2.4. Dynamic kinetic resolution catalysed by LipTL-SS

Once the optimal conditions were selected for the kinetic resolution, and provided that there was no enzymatic deactivation at 60 °C, the dynamic kinetic resolution was the next logical step to confirm the real capability of the entrapped enzyme. Nevertheless, in order to avoid the described interference of acetaldehyde produced from vinyl esters during KR with the hydrogen transfer catalyst [45], thrifluoroethyl butyrate was used as acyl donor (Scheme 2). The mixture was stirred in a closed vessel at 60 °C, not observing any significant difference when carrying out the reaction under reflux. After

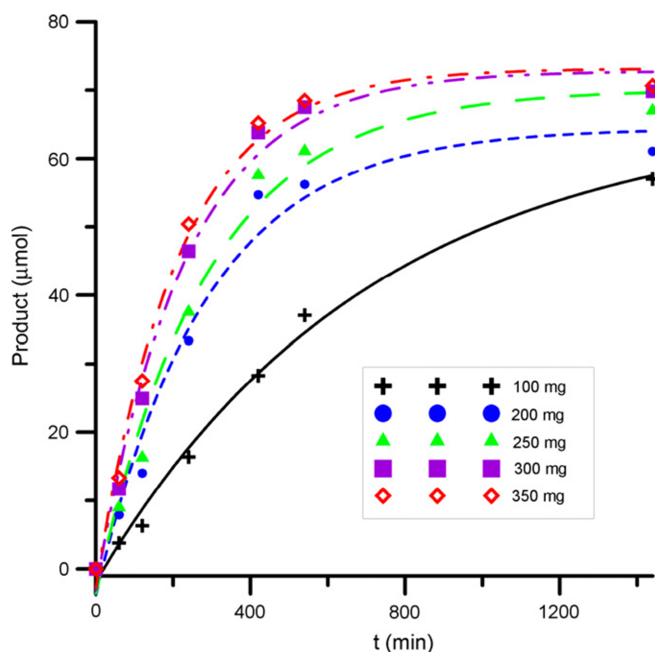


Fig. 3. Benzoin kinetic resolution catalysed by different amounts of LipTL-SS. *Experimental conditions*: 30 mg of rac-**1** (0.14 mmol) were dissolved in 1.5 mL of THF and different amounts of LipTL-SS and vinyl butyrate (150 μL, 0.99 mmol) were added. The mixture was shaken at room temperature, and analysed by HPLC as indicated in Section 2.

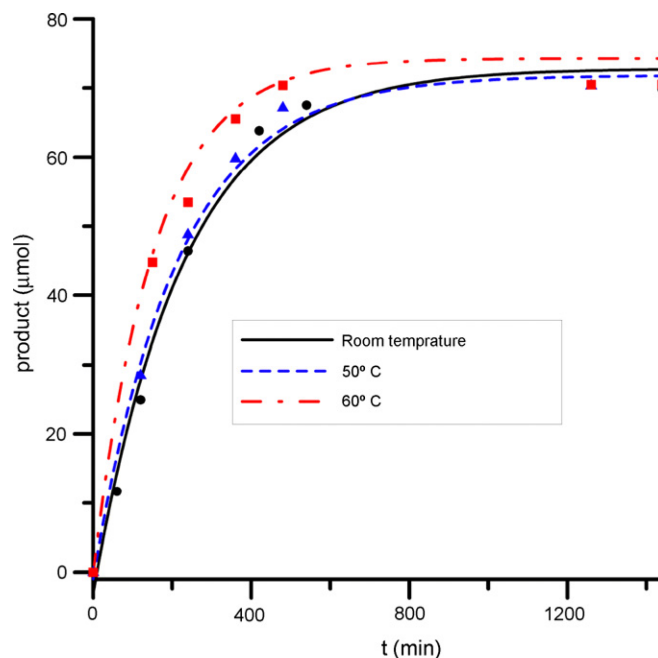
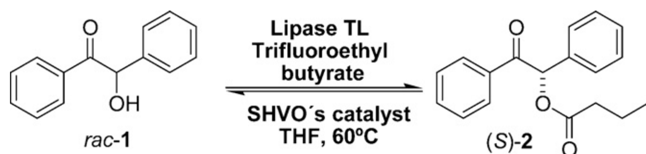


Fig. 4. Benzoin KR catalysed by LipTL-SS at different temperatures. *Experimental conditions*: 30 mg of rac-**1** (0.14 mmol) were dissolved in 1.5 mL of THF and 300 mg of LipTL-SS and vinyl butyrate (150 μL, 0.99 mmol) were added. The mixtures were shaken at room temperature, 50 °C and 60 °C, and analysed as indicated in Section 2.



Scheme 2. Benzoin DKR catalysed by Lipase TL®.

Table 2
Repetitive DKR of benzoin with trifluoroethyl butyrate catalysed by LipTL-SS

Catalyst	Conversion (%)	ee_p	Productivity[a]
Commercial LipTL	92[b]	99	2
LipTL-SS (Cycle 1)	87	99	15
LipTL-SS (Cycle 2)	81	99	29
LipTL-SS (Cycle 3)	80	99	43
LipTL-SS (Cycle 4)	78	99	56

[a] mg of product/mg protein.
[b] $T = 50\,^{\circ}C$ (data taken from Hoyos et al. [8]).

reaching maximum conversion, spheres were washed with THF until no remaining substrate or product was detectable in the solvent and another DKR cycle was started. The results are shown in Table 2, expressed in terms of productivity (considered as mg of product obtained by mg of protein present in the catalyst). As can be seen, this value was enhanced from 2 (commercial enzyme, only one possible catalytic cycle due to the enzyme deactivation [8]) to 56 mg product/mg protein by recycling the derivative four times. This 23-fold increase is therefore caused by the combination of the increase in the specific activity of the enzyme and the smaller amount of protein present in the derivative compared to the native enzyme. As can be seen, the stability has been significantly increased, because the immobilised lipase can be reused at 60 °C, while commercial LipTL is slowly deactivated at 50 °C, and the addition of an extra amount of enzyme is required to finish the DKR process [8].

3. Conclusions

The immobilisation of Lipase TL® in a hydrophobic material by silicone elastomers entrapment resulted in a considerable activation of this enzyme, possibly due to an enhanced mass transfer of hydrophobic compounds like benzoin and the stabilisation of the lipase in its active form, while commercial lipase suffers a deactivation when incubated at 50 °C. After immobilisation, temperatures up to 60 °C have been applied without a significant loss of activity. Silicone immobilised lipase is stable at 60 °C and it is possible to reuse it at least four times without significant loss of activity.

Due to the enhancement of Lipase TL® activity and stability, a "one pot" benzoin DKR has been developed (best results with free lipase are obtained in three steps). Highest conversion can be reached in shorter time than using free lipase (20 h vs. 48 h), and the catalyst can be reused, therefore enhancing nearly 30 times the productivity after the 4th cycle, going from 2 to 56 mg product/mg protein.

4. Experimental

4.1. General

Lipase TL® from *P. stutzeri* was a generous gift from Meito & Sangyo Co., Ltd.® Shvo's catalyst (1-hydroxytetraphenylcyclopentadienyl(tetraphenyl-2,4-cyclopentadien-1-one)-μ-hydrotetracarbonyldiruthenium (II), 98%) was obtained from Strem Chemicals Inc. (Newburyport, MA, USA). Sylgard®184 and Syl-Off®4000 were received from Dow Corning. *Iso*-butyltrimethoxysilane (iBTMS), tetramethyltrimethoxysilane (TMOS) and propyltrimethoxysilane (PTMS) were purchased from Aldrich.

HPLC analyses were performed with a chiral column Chiracel OD-H at room temperature, using HPLC (mobile phase of *n*-hexane/2-propanol, 90/10 at a flow rate of 1 mL/min).

NMR spectra were recorded on a Bruker AC-250. Chemical shifts (δ) are reported in parts per million (ppm) relative to $CHCl_3$ (^1H: δ 7.27 ppm) and $CDCl_3$ (^{13}C: δ 77.0 ppm).

4.2. Lipase immobilisation

4.2.1. Immobilisation of Lipase TL® in a sol–gel matrix

LipTL-SG1: The entrapment of lipase in sol–gel was carried out according to the methodology described by Reetz et al. [32]. Four hundred microliters of Lipase TL® solution (15 mg of commercial powder/mL, equivalent to 5.1 mg protein/mL) were added to a Falcon tube containing a mixture of aqueous sodium fluoride (100 μL, 1 M), polyvinyl alcohol (200 μL, 4% (w/w) in water; MW 15,000 Da) and 200 μL isopropyl alcohol. The solution was shaken and 480 μL *iso*-butyltrimethoxysilane (iBTMS, 2.5 mmol), 440 μL propyltrimethoxysilane (PTMS, 2.5 mmol), and 148 μL tertramethoxysilane (TMOS, 1 mmol) were added. The mixture was vigorously shaken until it formed a nearly homogeneous solution and warmed up. Subsequently, it was placed in an ice bath for 5 min and dried overnight in the opened tube. The white solid was washed out with isopropyl alcohol, and finally it was filtered and washed again with water and hexane. Enzyme loading was determined as 95%.

LipTL-SG2: The procedure was the same as the one described above for sol–gel 1, adding 960 μL of iBTMS (5 mmol) and 148 μL of TMOS (1 mmol) to form the sol–gel matrix. The loading efficiency of lipase was found to be 92%.

4.2.2. Immobilisation of Lipase TL® in static emulsion silicone spheres, LipTL-SE

This methodology was carried out as described by Buthe et al. [43], adding 1 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mg/mL of Lipase TL® to the mixtures of silicone components. Same amount of spheres were dried at 40 °C for different periods of time to study the influence of loss of water in lipase activity.

Enzyme loading was determined as 93%.

4.2.3. Immobilisation of Lipase TL® in silicone spheres, LipTL-SS

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), 40 mg of Syl-Off®4000 (bis (1,3-divinyl-1,1,3,3-tetradisiloxane)platin(0)) and 1 mL of hexane. A 105 mg of Lipase TL® powder were added to this mixture and it was vigorously shaken until viscosity increased. It was dropped in an aqueous solution of polyvinyl alcohol (PVA, 4% (w/w) in water, MW 10–98, 150 mL) and stirred at 45 °C for 2 h. Silicone spheres were filtered and washed with water before being dried at 40 °C overnight, and then stored at room temperature.

The loading efficiency was determined as 96%.

4.3. Protein determination

Protein content in crude Lipase TL® powder was determined following the Bradford methodology [44], which indicated that the protein percentage of the crude lipase was 34%.

Loss of enzyme during immobilisation process was quantified by measuring protein content [44] in the water used to wash the sol–gel matrix and in the PVA solution in the case of silicone spheres. The amount of immobilised lipase was calculated from the difference between initial amount of enzyme and enzyme released during the immobilisation process.

4.4. Lipase activity

Lipase activity was determined for the kinetic resolution of racemic benzoin: (30 mg, 0.14 mmol) in THF (1.5 mL) and vinyl butyrate as acyl donor (180 μL, 1.42 mmol). Different amounts of free lipase and immobilised lipase were added, and the mixtures were stirred at room temperature. Conversion and enantiomeric excess were determined by HPLC analysis.

4.5. Benzoin dynamic kinetic resolution catalysed by LipTL-SS

Thirty milligrams of benzoin (0.14 mmol) were dissolved in THF (1.5 mL). Shvo's catalyst (6 mg, 5.5×10^{-3} mmol), LipTL-SS (300 mg) and thrifluoroethyl butyrate (150 μL, 0.99 mmol) were added to the solution and the mixture was stirred at 60 °C. Conversion and enantiomeric excess were determined by HPLC analysis. After 20 h the reaction was finished and the mixture was filtered. LipTL-SS was washed with THF until no remaining product was detected by HPLC analysis, and these recycled spheres were used in a new benzoin DKR process.

Acknowledgements

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