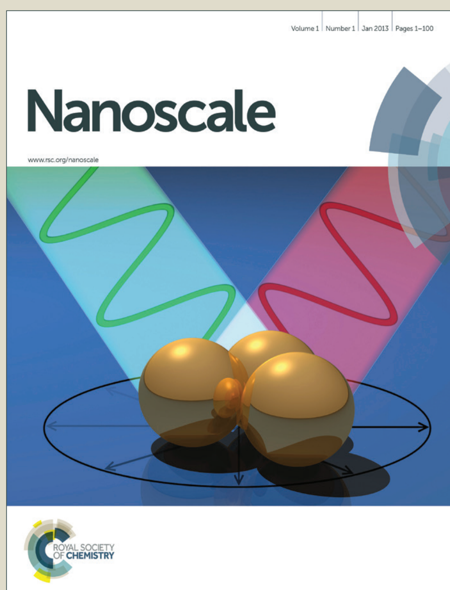
Nanoscale

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/nanoscale

Journal Name RSCPublishing

REVIEW

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Increasing the Activity and Enantioselectivity of Lipases by Sol-Gel Immobilization: Further Advancements of Practical Interest

Patrick Tielmann,[a] Hans Kierkels,[b] Albin Zonta,[a] Adriana Ilie,[a,c] and Manfred T. Reetz[a,c,*]

The entrapment of lipases in hydrophobic silicate matrices formed by sol-gel mediated hydrolysis of $RSi(OCH_3)_3/Si(OCH_3)_4$ as originally reported in 1996 has been improved over the years by a number of modifications. In the production of second-generation sol-gel lipase immobilizates, a variety of additives during the sol-gel process lead to increased activity and enhanced stereoselectivity in esterifying kinetic resolution. Recent advances in this type of lipase immobilization is reviewed here, in addition to new results regarding the sol-gel entrapment of the lipase from *Burkholderia cepacia.* It constitutes an excellent heterogeneous biocatalysts in the acylating kinetic resolution of two synthetically and industrially important chiral alcohols, *rac*-sulcatol and *rac-trans*-2-methoxycyclohexanol. The observation that the catalyst can be used 10 times in recycling experiments without losing significant activity or enantioselectivity demonstrates the practical viability of the sol-gel approach.

1 Principles and Early Results

Many different enzymes are available to the practicing organic chemist and biotechnologist for a variety of different transformations.[1-4] One of the milestones in the application of enzymes as catalysts in synthetic organic chemistry was the discovery that numerous enzymes can be used in non-aqueous media, allowing transformations of synthetic interest to be performed that are not possible in the natural aqueous environment.[5-6] An example of significant synthetic importance is the use of lipases (EC 3.1.1.3) as catalysts in organic solvents. These enzymes are the most often used biocatalysts in synthetic organic chemistry, catalyzing the hydrolysis of carboxylic acid esters in aqueous medium or the reverse reaction (esterification) as well as transesterification in organic solvents.[7-16] When working in organic media, nucleophiles other than alcohols can be used, e.g., amines or H_2O_2 affording amides or peroxy carboxylic acid, respectively. Numerous examples involving enantioselectivity in the production of chiral alcohols, amines and carboxylic acids have been reported, as for example in the kinetic resolution of chiral amines by the BASF process.[17] Lipases are structurally and mechanistically characterized by a so-called lid. When hydrophobic substrates interact with certain hydrophobic regions of a lipase, the lid opens and thus exposes the binding pocket and the catalytic triad composed of serine, histidine and aspartate in a process called interfacial activation.[7-16,18,19]

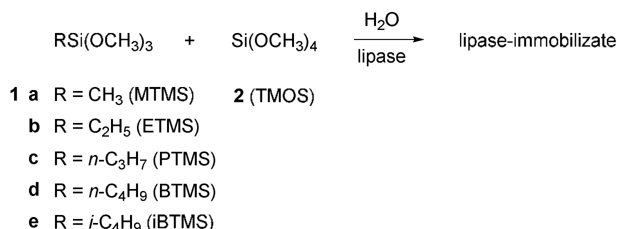
When performing reactions in organic solvents, commercially available lipase powders are often employed.[5-16] Inspite of the obvious advantages in such simple protocols, several drawbacks need to be considered, primarily the considerably reduced lipase activities relative to those observed in aqueous medium, and the extreme difficulty in recycling the enzyme. For real (industrial) applications of lipases some form of immobilization is necessary which allows for efficient separation and re-use of the enzyme, biocatalyst recyclization over many cycles of application being ideal. Moreover, a highly desirable feature of immobilization would be increased activity and perhaps even enhanced enantioselectivity. Several approaches have been described as summarized in the recent monograph edited by Guisan.[20] In our work we focused on sol-gel encapsulation, which is reviewed here. New data is also presented which lends support to previous conclusions.

Sol-gel encapsulation has proven to be a particularly easy and effective way to immobilize many different types of enzymes.[21] Following isolated reports describing immobilization of enzymes in silicates,[22,23] it was the seminal work of Avnir and co-workers which led to the generalization of the sol-gel technique for encapsulating enzymes.[21,24,25] Sol-gel methods involve the acid- or base-catalyzed hydrolysis of tetraalkoxysilanes $Si(OR)_4$.[26,27] Mechanistically, the silane-precursor undergoes hydrolysis and cross-linking condensation with formation of an SiO_2 matrix in which the enzyme is trapped. This type of encapsulation works well for a number of

Nanoscale Accepted Manuscript

enzymes.[21,24,25] However, in the case of lipases, materials were obtained which showed disappointingly low enzyme activities, as measured by the rate of a model reaction involving the esterification of lauric acid by *n*-octanol in *iso*-octane as solvent.[28] Only 5-10% activity relative to the traditional use of the respective lipase powder was observed, equivalent to relative rates of 0.05 to 0.1.

In our early research we speculated that the micro-environment in SiO_2 may be too polar. Therefore we tested mixtures of alkylsilanes of the type $RSi(OCH_3)_3$ (**1**) or polydimethylsiloxane (PDMS) having non-hydrolyzable lipophilic alkyl groups (R) and $Si(OCH_3)_4$ (**2**) (Scheme 1).[28] This strategy was considered because the original silicon oxide matrix is now endowed with a hydrophobic character, which can facilitate or simulate a type of interfacial activation of the entrapped lipase. Basic catalysts such as NaF were used for the sol-gel process, because such conditions lead to large pores in the silicate matrix.



Scheme 1 Encapsulation of lipases in hydrophobic sol-gel materials.[28-31,36]

Indeed, dramatically improved relative lipase activities typically amounting to 200-8800% were observed in the model reaction involving the esterification of lauric acid with *n*-octanol. This corresponds to an enhancement of relative enzyme activity by a factor ranging from 2 to 88 with respect to the traditional use of the corresponding lipase powder (lyophilizate).[28,29] Relative activity is defined as [*v*(immobilized lipase)/*v*(commercial lipase)], where *v* is the initial rate of the reaction in each case. A pronounced increase in thermal stability was also observed. In most cases the optimal ratio of $RSi(OCH_3)_3$ to $Si(OCH_3)_4$ turned out to be about 5:1, although it was not possible to present an experimental protocol which is completely general. Usually $CH_3Si(OCH_3)_3$ was used as the precursor and polyvinyl alcohol (PVA) as an additive, the latter possibly acting as a stabilizer of the lipase.[28,29]

Recycling of these first-generation heterogeneous biocatalysts was also possible. After 30 consecutive runs, only ≈15% decrease in activity was observed, probably due to partial loss from the outer surface of the support.[29] This underscores the robustness of these heterogeneous biocatalysts. By performing the sol-gel process in the presence of a porous solid carrier such as Celite® 577, heterogeneous biocatalysts can be obtained having high mechanical stability as well.[30,31] Accordingly, the lipase-containing sol-gel particles are caught and fixed in the large pores of the robust solid carrier. In early work that followed the original study,[28] several useful modifications were introduced including variation of the drying procedure.[32-35] A comprehensive review of sol-gel encapsulation of lipases has not appeared, although the basic principles were summarized in a 2012 review.[36]

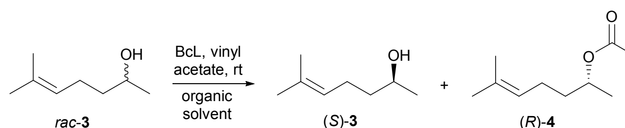
Aside from developing immobilization techniques, other researchers reported the positive effects of additives such as surfactants, salts or crown ethers on the activity and/or enantioselectivity of lipases in solution.[37-42] We therefore repeated our original sol-gel immobilization of lipases, this time in the presence of additives. This led to the second-generation sol-gel encapsulated lipases, which are highly improved heterogeneous biocatalysts for use in organic solvents.[43] Moreover, they were found to exhibit enhanced enantioselectivity in the acylating kinetic resolution of several chiral alcohols, although systematic investigations regarding this aspect were not strived for, nor was optimization performed. These questions are addressed in the present treatise by presenting new data which help to define guidelines on how to perform optimal sol-gel lipase immobilization. Recent contributions by other groups are likewise included.

2 A New Case Study Leading to General Guidelines

As already alluded to, a general procedure for entrapping lipases in hydrophobic sol-gels does not exist,[43] and it is unlikely that this will be achieved. However, recommendations on how to proceed in a given case are possible, as illustrated here using the lipase from *Burkholderia cepacia* (BcL).

2.1 Kinetic Resolution of (±)-Sulcatol Using BcL as Catalyst

The aliphatic secondary alcohol sulcatol occurs as a single enantiomer (*S*)-**3** in the beetles *Gnathotrichus sulcatus* and *Gnathotrichus retusus*, where it serves as a sexual pheromone.[44] Moreover, it is an important chiral building block for the synthesis of other biologically active natural products,[45,46] which makes an efficient kinetic resolution protocol for (±)-sulcatol desirable (Scheme 2). Alternative approaches to the synthesis of this compound have been reported.[47-50]



Scheme 2 Kinetic resolution (±)-sulcatol of (*rac*-**3**).

Several successful approaches to this kinetic resolution have been described in the literature applying such lipases as porcine pancreas lipase,[51-54] *Candida antarctica* lipase B[55] or *Burkholderia cepacia* lipase[56,57] (formerly *Pseudomonas cepacia*). A systematic investigation of the influence of different solvents on the enantioselectivity of *Burkholderia cepacia* lipase in the kinetic resolution of (±)-sulcatol was also described.[58] Accordingly, the enantioselectivity as measured by the selectivity factor (*E*-value)[59] varies significantly as a function of the solvent (e.g., hexane (28.5), cyclohexane (10.9), benzene (38), tetrahydrofuran (24.9) and methyl *tert*-butyl ether (12).[58]

Nanoscale Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012

Preliminary experiments using our second-generation sol-gel immobilizates appeared promising.[60] Therefore, we performed a systematic investigation of the kinetic resolution of (±)-sulcatol (*rac*-**3**) by testing various sol-gel immobilizates of *Burkholderia cepacia* lipase (BcL) in methyl *tert*-butyl ether (MTBE) and THF as solvents. Both solvents had been shown to induce notable changes in enantioselectivity.[58] Likewise considered is the influence of the alkyl groups in the precursor silane **1**, different additives (18-crown-6, KCl, methyl-*β*-cyclodextrin and Tween® 80) and an additional solid carrier (Celite® 577) upon which the sol-gel lipase immobilizates can be supported for additional mechanical stability.[30] The results are summarized in Tables 1 and 2.

Table 1 Acylating kinetic resolution of *rac*-**3** using *n*-propyl-substituted sol-gel immobilizates of *Burkholderia cepacia* lipase. All reactions were performed twice (see brackets). MTBE : methyl *tert*-butyl ether; THF : tetrahydrofuran.

Enzyme/ Additive	Solvent	Quantity (mg)	Time (h)	Conversion (%)	*ee* ((*S*)-alcohol) (%)	*ee* ((*R*)-ester) (%)	*E*-value
free enzyme	MTBE	50	48	37	41	72	9
				37	42	72	9
	THF	200	48	22	26	92	29
				24	28	91	29
immobilizate none	MTBE	10	8	48	62	68	10
				48	63	67	9
	THF	20	48	43	67	90	40
				41	64	92	44
immobilizate 1 mmol KCl	MTBE	10	8	40	49	74	11
				39	47	73	10
	THF	20	48	29	38	93	37
				32	44	92	38
immobilizate 0.5 mmol 18-crown-6	MTBE	10	8	38	45	74	10
				40	47	72	10
	THF	20	48	22	26	94	43
				26	34	93	38
immobilizate 20 mg methyl-*β*-cyclodextrin	MTBE	10	8	44	53	69	9
				44	55	70	10
	THF	20	48	38	56	92	44
				41	63	91	39
immobilizate 120 mg Tween® 80	MTBE	10	8	51	67	66	9
				47	61	69	10
	THF	20	48	38	56	92	42
				40	61	91	40

Nanoscale Accepted Manuscript

Table 2 Acylating kinetic resolution of *rac*-**3** using *iso*-butyl-substituted sol-gel immobilizates of *Burkholderia cepacia* lipase. All reactions were performed twice (see brackets).

Additive/Support	Solvent	Quantity (mg)	Time (h)	Conversion (%)	*ee* ((*S*)-alcohol) (%)	*ee* ((*R*)-ester) (%)	*E*-value
none/none	MTBE	10	48	45	54	68	9
				40	47	70	9
	THF	20	72	19	21	92	29
				27	33	92	34
none/Celite® 577	MTBE	10	48	25	25	73	8
				17	15	73	8
	THF	20	72	9	9	89	19
				12	13	95	42
60 mg Tween® 80	MTBE	10	24	45	56	69	10
				48	62	67	9
	THF	30	72	41	62	89	32
				43	66	89	34
60 mg Tween® 80/ Celite® 577	MTBE	10	24	39	43	68	8
				32	34	71	8
	THF	20	72	24	29	92	31
				31	40	91	32
2 mmol 18-crown-6	MTBE	10	24	42	50	70	9
				43	52	69	9
	THF	30	72	35	49	92	40
				33	46	93	40
2 mmol 18-crown-6/ Celite® 577	MTBE	10	48	49	63	67	10
				54	62	72	12
	THF	20	72	16	17	92	27
				21	25	94	43
40 mg methyl-β-cyclodextrin	MTBE	10	24	27	27	74	9
				39	45	72	9
	THF	30	72	33	46	91	33
				34	48	92	38

These heterogeneous reactions are reproducible and allow for several important conclusions. Firstly, all lipase immobilizates show a significantly higher activity in the acylation of (±)-sulcatol (*rac*-**3**) than the commercial lyophilized *Burkholderia cepacia* lipase powder. For instance, using 10 mg of a *n*-propyl-modified sol-gel biocatalyst, corresponding to 0.4 mg of lipase, conversion reached the desired 50% after only 8 h in MTBE, whereas 50 mg of the free enzyme resulted in only 37% conversion after 48 h. It is thus clear that sol-gel entrapment results in a pronounced increase in catalytic activity relative to the use of a non-immobilized enzyme powder. Secondly, the activity of the *n*-propyl-substituted sol-gel biocatalyst proved to be much higher than that of the analogous *iso*-butyl-derived immobilizate (to reach the same conversion the reaction time had to be increased to 24-48 h in MTBE). Thirdly, the kinetic resolution proceeded much faster using MTBE as solvent compared to THF (Table 1 and 2), which holds true for the free enzyme as well (Table 1). Although additional experiments regarding possible differences in enzyme stability as function of the two solvents were not performed, we do not believe that such an effect is operating. If partial decomposition had occurred, a change in activity/enantioselectivity would have resulted. For instance, applying 200 mg of the lyophilized enzyme powder resulted in a conversion of 23% after 48 h in THF, while 50 mg enzyme in MTBE gave 37% conversion. Figure 1 gives an overview of the enantioselectivity observed in the kinetic resolution of *rac*-**3** using *n*-propyl-substituted sol-gel immobilizates. The same applies to Figure 2, which refers to the experiments using *iso*-butyl-substituted sol-gel biocatalysts and Celite® 577 as an additional support.

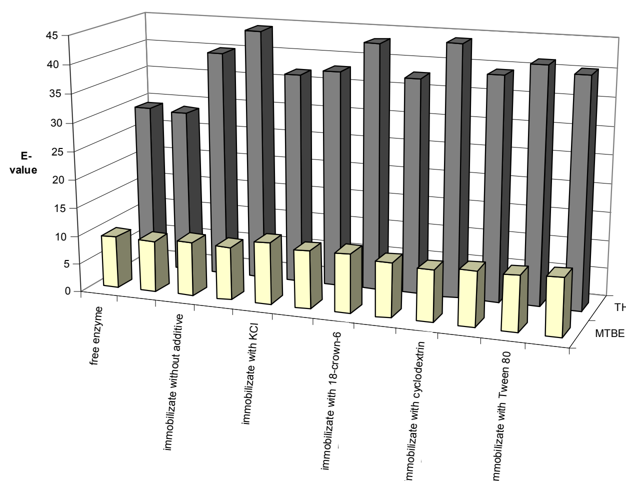


Fig. 1 Enantioselectivities obtained in the kinetic resolution of *rac*-sulcatol (*rac*-**3**) with *n*-propyl-substituted sol-gel immobilized *Burkholderia cepacia* lipase in two different solvents. Every second column represents the results of the reproduction of the enzymatic reaction.

Nanoscale Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

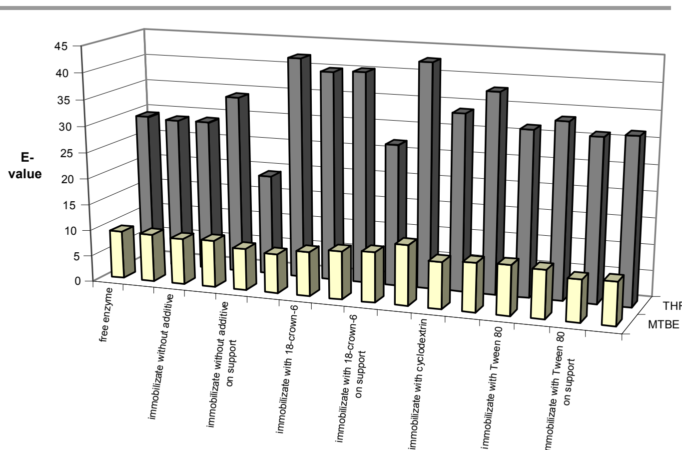


Fig. 2 Enantioselectivities obtained in the kinetic resolution of racemic sulcatol (*rac*-**3**) with *iso*-butyl-substituted sol-gel immobilized preparations of *Burkholderia cepacia* lipase in two different solvents. Celite® 577 was used as solid support. Every second column represents the results of the reproduction of the enzymatic reaction.

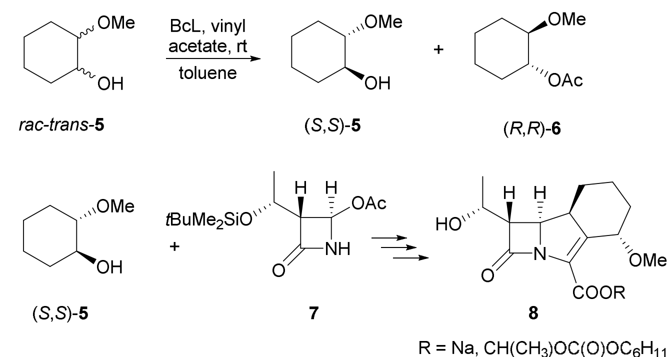
For the *n*-propyl-substituted sol-gels (Figure 1), a moderate solvent effect on enantioselectivity was observed, THF being substantially better than MTBE. While the free enzyme in THF delivers an *E*-value of up to 29, the immobilized biocatalyst leads to *E*-values of up to 41, depending upon the exact composition. The hydrophobic silicate matrix itself plays the most important role in altering the enantioselectivity, whereas the additives induce only small effects. The reaction in MTBE results in an *E*-value of approximately 10, irrespective of the composition.

The situation changes remarkably upon switching to the *iso*-butyl-derived immobilizates (Figure 2). In this case, the enzyme preparations without additives show hardly any increase in enantioselectivity. Upon including additives during the sol-gel process, the entrapped enzymes show markedly enhanced *E*-values depending on the type of additive. For example, the immobilizate prepared in the presence of 18-crown-6 leads to an enantioselectivity of $E = \sim 40$, while the immobilizate with Tween® 80 shows no enhancement. As before the tested immobilized enzymes do not show any increase in enantioselectivity when the reaction is performed in MTBE.

Taking the results together, it is clear that immobilization can indeed result in increased enantioselectivity and activity. Interestingly, the less optimal choice of the *iso*-butyl groups can be overcome by some additives, while there was no additivity observed in case of the *n*-propyl-substituted sol-gel materials. Since in both cases the highest obtained enantioselectivities level off around *E*-values of 40–45, it seems that this represents a kind of an upper limit for enhancing the enantioselectivity in this particular kinetic resolution. Finally, the solvent also plays a substantial role in this kinetic resolution reaction. While the reaction proceeds much faster but with lower enantioselectivity in MTBE, the lower reaction rate in THF is still high enough for practical purposes while ensuring high enantioselectivity. One possible explanation might be that the binding pocket of the enzyme is differently occupied by the two solvents. During catalysis some THF molecules may still reside in the catalytic pocket, inducing additional steric interactions.

2.2 Kinetic Resolution of *rac-trans*-2-Methoxycyclohexanol Using BcL as Catalyst

The kinetic resolution of *rac-trans*-2-methoxycyclohexanol (*rac-trans*-**5**) (Scheme 3) served as a second test system. Obtaining this compound in enantiopure form, especially the enantiomer (*S,S*)-**5**, is of industrial interest since this chiral compound is an important intermediate in the synthesis of novel tricyclic *β*-lactam antibiotics such as sanfetrinems (**8**), patented by *GlaxoWellcome* in 1991.[61,62]



Scheme 3 Kinetic resolution of *rac-trans*-2-methoxycyclohexanol (*rac-trans*-**5**) with formation of (*S,S*)-**5** and its known transformation into the antibiotic **8**.

Several enzymatic approaches for the enantioselective synthesis of (*S,S*)-**5** have been developed. As early as 1989 Schneider et al. reported on a highly enantioselective hydrolytic kinetic resolution of the acetate derived from *rac-trans*-**5** using the lipase *Pseudomonas sp*.[63] Other groups have also described the kinetic resolution of 2-methoxycyclohexanol-derived esters,[64,65] but these approaches require the preparation of the esters prior to performing the hydrolytic kinetic resolution reaction. Enantioselective acylation of the starting alcohol avoids the additional esterification step, a strategy that has been implemented by several groups.[66,67] The industrial process employing *Candida antarctica* lipase B (Novozyme 435) and vinyl acetate as the acylating agent deserves special attention, because the reaction essentially stops at 50–55% conversion after 10 hours delivering (*S,S*)-**5** in >98% enantiomeric purity.[66,67]

Since our initial attempts to apply sol-gel immobilized *Burkholderia cepacia* lipase in the kinetic resolution of *rac-trans*-**5** proved to be promising, we embarked on a systematic study using different immobilizates, the focus being on the activity and recyclability of the enzyme preparations. In doing so, only the *n*-propyl-derived sol-gel materials were considered, because they had been shown to be most active in preliminary experiments. Here, the relative activity could be increased to a factor of 160 by sol-gel immobilization without compromising the high enantioselectivity. In order to establish a GC-based analytical system for the determination of activity and enantiomeric excess (*ee*) of substrate and product, the racemic

Nanoscale Accepted Manuscript

acylated *trans*-2-methoxycyclohexanol was prepared by published procedures.[64-67]

Subsequently we investigated the kinetic resolution of *rac-trans*-**5** using vinyl acetate as the acylating agent and various immobilizates of *Burkholderia cepacia* lipase as the heterogeneous biocatalysts (Figure 3 and Table 3).

Figure 3 shows that in all cases the desired product (*R,R*)-**6** was obtained with >99% *ee* after 18 h. The corresponding *ee* of the substrate (*S,S*)-**5** was found to vary somewhat, depending upon the nature of the immobilizate (up to 93% *ee*). This can be attributed primarily to the different activity of the immobilizates and therefore to the %-conversion, rather than to true differences in stereoselectivity. Table 3 shows that in all cases *E*-values considerably higher than 100 were observed. A more detailed investigation of the initial reaction rate corroborated the conclusion that the immobilizate containing Tween® 80 leads to the highest activity. Compared to the free enzyme in powder form, the reaction proceeds 205-times faster. However, the enzyme preparation without additive led to a relative activity of 179, which shows that the major positive effect originates from the actual encapsulation in the hydrophobic silicate matrix.

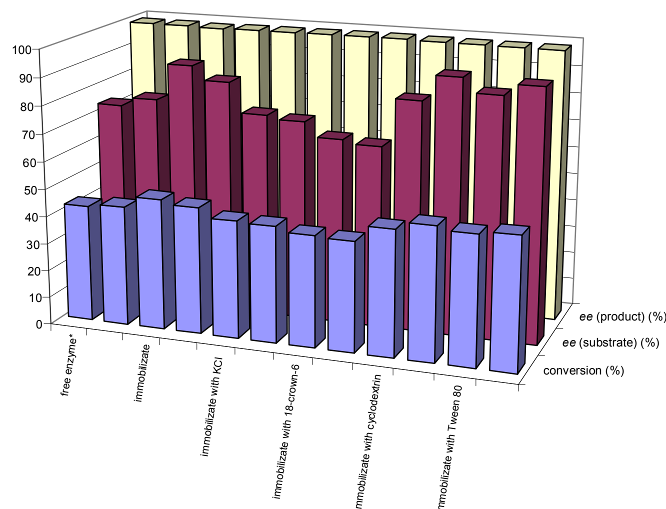
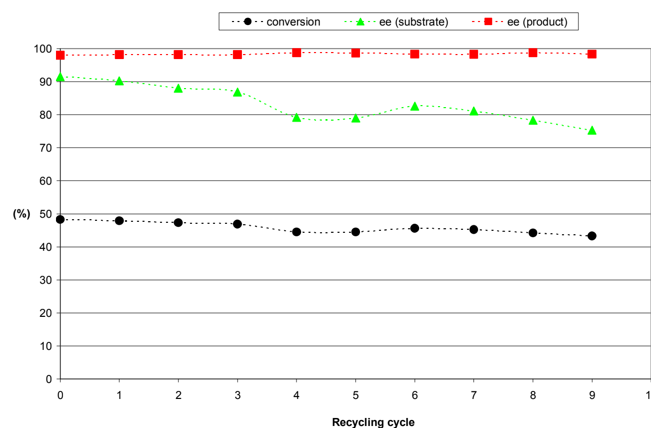


Fig. 3 Results of the kinetic resolution of *rac-trans*-**5** using different *n*-propyl-substituted sol-gel preparations of *Burkholderia cepacia* lipase after 18 h (48 h and tenfold increase in quantity in the case of the free enzyme for comparison reasons). Every second column represents the results of the reproduction of the enzymatic reaction.

Table 3 Results of the kinetic resolution of *rac-trans*-**5** using *n*-propyl-substituted sol-gel immobilizates of *Burkholderia cepacia* lipase.

Enzyme Additive	Quantity (mg)	Time (h)	Conversion (%)	*ee* ((*S,S*)-alcohol) (%)	*ee* ((*R,R*)-ester) (%)	*E*-value
free enzyme	100	48	43 44	73 77	99 99	>100
immobilizate none	10	18	48 46	90 85	99 99	>100
immobilizate 1 mmol KCl	10	18	43 42	74 73	99 99	>100
immobilizate 0.5 mmol 18-crown-6	10	18	41 40	68 66	99 99	>100
immobilizate 20 mg methyl-*β*-cyclodextrin	10	18	46 48	84 93	99 99	>100
immobilizate 120 mg Tween® 80	10	18	47 48	88 92	99 99	>100

Having highly active enzyme preparations in hand, the important question of recyclability of the sol-gel biocatalysts was addressed. For this purpose the lipase immobilizate without additive and the one with Tween® 80 were used in the kinetic resolution of *rac-trans*-**5** and recycled nine times. In these studies the initial reaction mixtures were shaken for 3 h and analyzed by GC. Then the enzyme preparations were recovered by filtration, washed with toluene (three times) and pentane (once), and dried for 24 h at room temperature before starting the reaction again. In order to maintain comparability, the amount of substrate was adjusted to the amount of recovered biocatalyst. The results are summarized in Figures 4 and 5 and Table 4.



This journal is © The Royal Society of Chemistry 2012
Nanoscale Accepted Manuscript

Fig. 4 Conversion and *ee*-values in the kinetic resolution of *rac-trans*-**5** using the lipase immobilizate without additive upon nine recycling cycles.

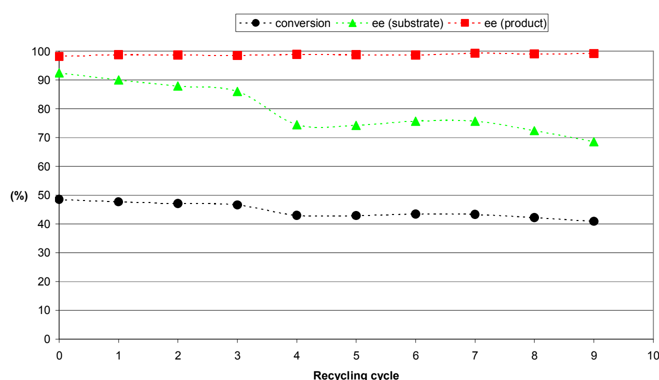


Fig. 5 Conversion and *ee*-values in the kinetic resolution of *rac-trans*-**5** using the lipase immobilizate with Tween® 80 upon nine recycling cycles.

It is remarkable that in both cases only a slight decrease in activity was observed after 10 cycles (conversion of ≈49% decreases to 40-42% under standard conditions). Moreover, the *ee* of the product (*R,R*)-**6** remained constant at about 99%, although the analytical procedure was not exact enough to detect possible decreases from e.g. 99.5% to 99.0%. The decrease in enantioselectivity of the substrate (*S,S*)-**5** is not due to a decrease in selectivity of the biocatalyst. Rather, it has to do with the fact that the conversion has not reached the ideal 50% under the reaction conditions in later cycles.

Table 4 Results of the recycling of the *n*-propyl-substituted sol-gel immobilizate of *Burkholderia cepacia* lipase in the kinetic resolution of *rac-trans*-**5**.

Cycle	Without additive			With 120 mg Tween® 80		
	Conversion (%)	*ee* ((*S*,*S*)-alcohol) (%)	*ee* ((*R*,*R*)-ester) (%)	Conversion (%)	*ee* ((*S*,*S*)-alcohol) (%)	*ee* ((*R*,*R*)-ester) (%)
1	48	92	98	49	93	98
2	48	90	98	48	90	99
3	47	88	98	47	88	99
4	47	87	98	47	86	99
5	45	79	99	43	75	99
6	45	79	99	43	74	99
7	46	83	98	43	76	99
8	45	81	98	43	76	99
9	44	78	99	42	72	99
10	43	75	98	41	69	99

3 Experimental Details Serving as a Guide

3.1 General remarks

All solvents and reagents were used as received without further purification. The lipase from *Burkholderia cepacia* (Amano PS) was purchased from Amano Pharmaceutical Co. *rac*-Sulcatol (*rac*-**3**) and 2-methoxycyclohexanol (*cis/trans*-mixture) (**5**) were purchased from Aldrich and vinylacetate from Fluka. Gas chromatographic (GC) analyses were performed in the Department of Chromatography, Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr, Germany. The following GC conditions were applied for the preparative separation of the isomers of 2-methoxycylohexanol: retention time (min): *cis*-2-methoxycyclohexanol (14.8), *trans*-2-methoxycyclohexanol (16.7), HP 6890, column 30 m CW 20 M/G 104, H_2 0.6 bar, 230 °C, 5 min 60 °C iso, 60–240 °C, 4 °C/min, 15 min 240 °C iso, 350 °C. The general procedure for immobilizing *Burkholderia cepacia* lipase was performed according to the second generation protocol[43] with additives as listed in Tables 1-3.

3.2 Kinetic resolution of *rac*-sulcatol (*rac*-3)

In a 10 mL flask an enzyme preparation (see Tables 1 and 2) was suspended in 4 mL of methyl *tert*-butyl ether or 4 mL THF and then treated with *rac*-**3** (14.7 µL; 12.4 mg; 0.1 mmol) and vinyl acetate (44.4 µL; 41.5 mg; 0.5 mmol). The reaction mixture was shaken at room temperature with 250 min^{-1}. At different time intervals samples of 60 µL were taken for GC analysis (Tables 1 and 2).

3.3 Kinetic resolution of *rac-trans*-2-methoxycyclohexanol (*rac-trans*-5)

In a 10 mL flask an enzyme preparation (see Table 3) was suspended in 4 mL of toluene and then treated with *rac-trans*-2-methoxycyclohexanol (*rac-trans*-**5**) (130 µL; 132 mg; 10 mmol) and vinyl acetate (184 µL; 171 mg; 20 mmol). The reaction mixture was shaken at room temperature with 250 min^{-1}. At different time intervals samples of 60 µL were taken for GC analysis (Table 3).

3.4 Recycling of immobilized *Burkholderia cepacia* lipase in the kinetic resolution of (*rac-trans*-5)

In a 25 mL flask the lipase-containing sol-gel (150 mg) was suspended in 10 mL of toluene and treated with *rac-trans*-**5**

Nanoscale Accepted Manuscript

(325 µL; 330 mg; 2.5 mmol) and vinyl acetate (460 µL; 429 mg; 5.0 mmol). The reaction mixture was shaken at room temperature with 250 min for 3 h. Then samples of 70 µL were taken and diluted with 200 µL of toluene. After centrifugation for 2 min at 13000 min^{-1}, 150 µL of supernatant was analyzed by GC. To recover the immobilizates the reaction mixture was filtered and subsequently washed three times with 10–15 mL of toluene and once with 10–15 mL of pentane. After drying for 24 h at room temperature, the recycled sol-gel biocatalyst was again used in the kinetic resolution of *rac-trans*-**5**. In this case and in all other cycles the substrate and solvent quantities were adjusted to the amount of recovered enzyme preparation. The reaction was performed a total of ten times using the same sample of immobilizate.

4 Other Contributions to Sol-Gel Entrapment of Lipases

As already pointed out, several early modifications of this approach to immobilize lipases deserve mention.[32-35] These include variation of the drying procedure, leading to the generation of aerogels as first demonstrated by Pierre.[35]

Numerous recent applications demonstrate once more that the technique of lipase entrapment by the sol-gel process based on the use of hydrophobic silicon precursors is easy to apply, efficient and cheap. A few key studies are listed here.[68-79] In rare comparative studies, the superiority of the sol-gel lipase immobilization relative to other approaches was demonstrated.[79,80] The reader is referred to pertinent review articles.[30,80-82] The sol-gel technique was also fine-tuned by adding chiral template substrates or substrate analogs for bioimprinting.[83,84] Sol-gel lipase encapsulates have been cleverly applied in continuous-flow and micro reactors.[30,85-87] In conventional applications the separation of the heterogeneous biocatalysts from the products poses no problems by simple filtration, but magnetic separation is possible if magnetite powder is added during the sol-gel encapsulation,[88] a procedure that has been used occasionally in more recent studies.[80,81] Finally, although most applications of sol-gel lipase immobilizates generally involve esterification or transesterification in standard organic solvents, ionic liquids or super critical CO_2, they can also be used in hydrolysis reactions, although at lower rates.[89]

5 General Conclusions and Perspectives

In most cases regarding the use of enzymes as (bio)catalysts in synthetic organic chemistry or biotechnology some sort of immobilization is necessary.[3,20,82] In ideal systems this allows for recyclability, but also in increased thermal stability and activity as well as enhanced stereoselectivity. Turning a water-soluble natural enzyme into a heterogeneous catalyst will certainly reduce activity if the latter is used in aqueous medium. However, if the enzyme-catalyzed transformation needs to be performed in conventional organic solvents, ionic liquids or supercritical CO_2, then the use of immobilized enzymes relative to the application of the respective enzyme powder has enormous advantages. A prime example is the immobilization of lipases as catalysts in esterification or transesterification, stereoselectivity often being the focus of interest.[1-6] A variety of techniques have been used successfully for a long time.[3-5,80-82] In more recent years the sol-gel procedure as reviewed here has emerged as a particularly efficient procedure. The present contribution summarizes progress in this field and includes some useful guidelines for upcoming applications.

In future work it can be expected that the combination of sol-gel encapsulation and directed evolution of stereoselective lipases[90-92] or the alternative rational design of mutants by site-directed mutagenesis[93,94] will offer further practical perspectives for this important class of enzymes.

Acknowledgements

We thank the Max-Planck-Society for generous financial support.

Notes and references

[a,*] Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 45470 Mülheim/Ruhr, Germany, e-mail: reetz@mpi-muelheim.mpg.de.
[b] DSM Research, P. O. Box 18, 6160 DM Geleen, The Netherlands.
[c] Fachbereich Chemie, Philipps-Universität, Hans-Meerwein-Strasse, 35032 Marburg, Germany.

† *Dedicated to the pioneer of sol-gel enzyme immobilization, Professor David Avnir, on the occasion of his 65th birthday.*

1 K. Drauz, H. Gröger and O. May, *Enzyme Catalysis in Organic Synthesis*, 3rd edn., Vol. I–III, VCH, Weinheim, 2012.
2 K. Faber, *Biotransformations in Organic Chemistry*, 6th edn., Springer, Berlin, 2011.
3 A. Liese, K. Seelbach and C. Wandrey, *Industrial Biotransformations*, Wiley-VCH, Weinheim, 2006.
4 M. T. Reetz, *J. Am. Chem. Soc.*, 2013, **135**, 12480–12496.
5 A. M. Klibanov, *Nature,* 2001, **409**, 241–246.
6 G. Carrea and S. Riva, *Angew. Chem., Int. Ed.,* 2000, **39**, 2226–2254.
7 Early review of lipases: R. D. Schmid and R. Verger, *Angew. Chem., Int. Ed.*, 1998, **37**, 1608–1633.
8 K.-E. Jaeger, B. W. Dijkstra and M. T. Reetz, *Annu. Rev. Microbiol*., 1999, **53**, 315–351.
9 U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, Wiley-VCH, Weinheim, Germany, 1999.
10 S. Naik, A. Basu, R. Saikia, B. Madan, P. Paul, R. Chaterjee, J. Brask and A. Svendsen, *J. Mol. Catal. B: Enzym.,* 2010, **65**, 18–23.
11 D. Sharma, B. Sharma and A. K. Shukla, *Biotechnology*, 2011, **10**, 23–40.
12 A. Baldessari, *Methods Mol. Biol.,* 2012, **861**, 445–456.
13 B. Andualema and A. Gessesse, *Biotechnology*, 2012, **11**, 100–118.
14 A. Bassegoda, S. Cesarini and P. Diaz, *Comput. Struct. Biotechnol.*, 2012, **2**, e201209005.
15 X. Song, X. Qi, B. Hao and Y. Qu, *Eur. J. Lipid Sci. Technol*., 2008, **110**, 1095–1101.

Nanoscale Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

16 M. Paravidino, P. Böhm, H. Gröger and U. Hanefeld, *Hydrolysis and Formation of Carboxylic Acid Esters*, in *Enzyme Catalysis in Organic Synthesis*, 3rd edn., eds. K. Drauz, H. Gröger and O. May, Wiley-VCH, Weinheim, Germany, 2012, pp. 251–362.
17 F. Balkenhohl, K. Ditrich, B. Hauer and W. Ladner, *J. Prakt. Chem.,* 1997, **339**, 381–384.
18 A. M. Brzozowski, U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar and L. Thim, *Nature,* 1991, **351**, 491–494.
19 H. van Tilbeurgh, M.-P. Egloff, C. Martinez, N. Rugani, R. Verger and C. Cambillau, *Nature,* 1993, **362**, 81–820.
20 J. M. Guisan, *Immobilization of Enzymes and Cells*, in *Methods in Biotechnology*, 2nd edn., Humana Press, Totowa, 2013, Vol. 1051.
21 D. Avnir, S. Braun, O. Lev and M. Ottolenghi, *Chem. Mater.,* 1994, **6**, 1605–1614.
22 P. Johnson and T. L. Whateley, *J. Colloid Interface Sci.,* 1971, **37**, 557–563.
23 M. Glad, O. Norrlöw, B. Sellergren, N. Siegbahn and K. Mosbach, *J. Chromatogr.*, 1985, **347**, 11–23.
24 D. Avnir, *Acc. Chem. Res.,* 1995, **28**, 328–334.
25 D. Avnir, T. Coradin, O. Lev and J. Livage, *J. Mater. Chem*. 2006, **16**, 1013–1030.
26 L. L. Hench and J. K. West, *Chem. Rev.,* 1990, **90**, 33–72.
27 C. J. Brinker and G. W. Scherer, *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*, Academic Press, Boston, 1990.
28 M. T. Reetz, A. Zonta and J. Simpelkamp, *Angew. Chem., Int. Ed. Engl.,* 1995, **34**, 301–303.
29 M. T. Reetz, A. Zonta and J. Simpelkamp, *Biotechnol. Bioeng.,* 1996, 49, 527–534.
30 M. T. Reetz, A. Zonta, J. Simpelkamp and W. Könen, *Chem. Commun.,* 1996, 1397–1398.
31 M. T. Reetz, *Adv. Mater.,* 1997, **9**, 943–954.
32 J. Livage, *C. R. Acad. Sci., Ser. IIb: Mec., Phys., Chim., Astron.,* 1996, **322**, 417–427.
33 I. Gill, *Chem. Mater.,* 2001, **13**, 3404–3421.
34 W. Jin and J. D. Brennan, *Anal. Chim. Acta,* 2002, **461**, 1–36.
35 A. C. Pierre, *Biocatal. Biotransform.,* 2004, **22**, 145–170.
36 M. T. Reetz, *Practical Protocols for Lipase Immobilization Via Sol-Gel Techniques,* in *Immobilization of Enzymes and Cells; Methods in Biotechnology*, ed. J. M. Guisan, 2nd edn., Humana Press, Totowa, 2013, Vol. 1051, pp. 241–254.
37 D. N. Reinhoudt, A. M. Eendebak, W. F. Nijenhuis, W. Verboom, M. Kloosterman and H. E. Schoemaker, *J. Chem. Soc., Chem. Commun.,* 1989, 399–400.
38 D.-J. van Unen, J. F. J. Engbersen and D. N. Reinhoudt, *Biotechnol. Bioeng.,* 2002, **77**, 248–255.
39 K. Griebenow, Y. D. Laureano, A. M. Santos, I. M. Clemente, L. Rodríguez, M. W. Vidal and G. Barletta, *J. Am. Chem. Soc.,* 1999, **121**, 8157–8163.
40 Y. L. Khmelnitsky, S. H. Welch, D. S. Clark and J. S. Dordick, *J. Am. Chem. Soc.,* 1994, **116**, 2647–2648.
41 Y.-Y. Liu, J.-H. Xu and Y. Hu, *J. Mol. Catal. B: Enzym.,* 2000, **10**, 523–529.
42 F. Theil, *Tetrahedron,* 2000, **56**, 2905–2919.
43 M. T. Reetz, P. Tielmann, W. Wiesenhöfer, W. Könen and A. Zonta, *Adv. Synth. Catal.,* 2003, **345**, 717–728.
44 K. Mori, *Tetrahedron,* 1989, **45**, 3233–3298.
45 K. Mori and H. Kikuchi, *Liebigs Ann. Chem.,* 1989, 1267–1269.
46 S. Liang and L. A. Paquette, *Tetrahedron: Asymmetry,* 1990, **1**, 445–452.
47 S.-L. Chen, Q.-H. Hu and T.-P. Loh, *Org. Lett.,* 2004, **6**, 3365–3367.
48 A. Arnone, P. Bravo, W. Panzeri, F. Viani and M. Zanda, *Eur. J. Org. Chem.,* 1999, 117–127.
49 S. G. Davies and G. D. Smyth, *J. Chem. Soc., Perkin Trans. I,* 1996, 2467–2477.
50 S. G. Davies and G. D. Smyth, *Tetrahedron: Asymmetry,* 1996, **7**, 1005–1006.
51 A. Belan, J. Bolte, A. Fauve, J. G. Gourcy and H. Veschambre, *J. Org. Chem.,* 1987, **52**, 256–260.
52 T. M. Stokes and A. C. Oehlschlager, *Tetrahedron Lett.,* 1987, **28**, 2091–2094.
53 T. Sugai, O. Katoh and H. Ohta, *Tetrahedron,* 1995, **51**, 11987–11998.
54 E. Vänttinen and L. T. Kanerva, *Tetrahedron: Asymmetry,* 1995, **6**, 1779–1786.
55 F. Secundo, G. Ottolina, S. Riva and G. Carrea, *Tetrahedron: Asymmetry,* 1997, **8**, 2167–2173.
56 Y. Kita, Y. Takebe, S. Murata, T. Naka and S. Akai, *Tetrahedron Lett.,* 1996, **37**, 7369–7372.
57 Y. Kita, Y. Takebe, K. Murata, T. Naka and S. Akai, *J. Org. Chem.,* 2000, **65**, 83–88.
58 K. Nakamura, M. Kinoshita and A. Ohno, *Tetrahedron,* 1995, **51**, 8799–8808.
59 Definition of the selectivity factor *E* in kinetic resolution: C.-S. Chen and C. J. Sih, *Angew. Chem., Int. Ed. Engl.,* 1989, **28**, 695–707.
60 P. Tielmann, Dissertation, Ruhr-Universität Bochum, Germany, 2002.
61 B. Tamburini, A. Perboni, T. Rossi, D. Donati, D. Andreotti, G. Gaviraghi, R. Carleso and C. Bismara, EP0416953A2, 1991.
62 B. Tamburini, T. Rossi, D. Donati, G. Gaviraghi and G. Tarzi, *Recent Advances in the Chemistry of anti-Infective Agents*, eds. P. H. Bently and R. Ponsford, Royal Society of Chemistry, Cambridge, 1993, pp. 21–35.
63 K. E. Laumen, D. Breitgoff, R. Seemayer and M. P. Schneider, *J. Chem. Soc., Chem. Commun.,* 1989, 148–150.
64 H. Hönig and P. Seufer-Wasserthal, *Synthesis,* 1990, 1137–1140.
65 D. Basavaih and P. R. Krishna, *Tetrahedron,* 1994, **50**, 10521–10530.
66 P. Stead, H. Marley, M. Mahmoudian, G. Webb, D. Noble, Y. T. Ip, E. Piga, T. Rossi, S. Roberts and M. J. Dawson, *Tetrahedron: Asymmetry,* 1996, **7**, 2247–2250.
67 P. M. Jackson, S. M. Roberts, S. Davalli, D. Donati, C. Marchiroro, A. Perboni, S. Proviera and T. Rossi, *J. Chem. Soc., Perkin Trans. I,* 1996, 2029–2030.
68 S. Nanda and A. I. Scott, *J. Mol. Catal. B.: Enzym*., 2004, **30**, 1–12.
69 A. Tomin, D. Weiser, G. Hellner, Z. Bata, L. Corici, F. Péter, B. Koczba and L. Poppe, *Process Biochem.*, 2011, **46**, 52–58.
70 D. Weiser, Z. Boros, G. Hornyanszky, A. Toth and L. Poppe, *Process Biochem.*, 2012, **47**, 428–434.
71 H. Noureddini, X. Gao and R. S. Philkana, *Bioresource Technol.*, 2005, **96**, 769–777.
72 A. Ursoiu, C. Paul, C. Marcu and F. Peter, *World Acad. Sci. Eng. Technol.*, 2011, **76**, 70–74.

Nanoscale Accepted Manuscript

73 S. Sayin, E. Yilmaz and M. Yilmaz, *Org. Biomol. Chem*., 2011, **9**, 4021–4024.
74 E. Yilmaz and M. Sezgin, *Appl. Biochem. Biotechnol*., 2012, **166**, 1927–1940.
75 M. Päiviö, P. Perkiö and L. T. Kanerva, *Tetrahedron: Asymmetry*, 2012, **23**, 230–236.
76 J. Hetfleijs, G. Kuncova, S. Sabata, V. Blechta and J. Brus, *J. Sol-Gel Sci. Technol*., 2006, **38**, 121–131.
77 R. C. Pinheiro, C. M. F. Soares, O. A. A. dos Santos, H. F. de Castro, F. F. de Moraes and G. M. Zanin, *J. Mol. Catal. B*.: *Enzym*., 2008, **52**–**53**, 27–33.
78 P. Hara, U. Hanefeld and L. T. Kanerva, *J. Mol. Catal. B*.: *Enzym*., 2008, **50**, 80–86.
79 J. Brem, M. C. Turcu, C. Paizs, K. Lundell, M.-I. Toşa, F.-D. Irimie and L. T. Kanerva, *Process Biochem.*, 2012, **47**, 119–126.
80 Summary of lipase immobilization methods with emphasis on biodiesel production, showing the superiority of the sol-gel approach[71]: M. Stoytcheva, G. Montero, L. Toscano, V. Gochev and B. Valdez, *The Immobilized Lipases in Biodiesel Production*, in *Biodiesel–Feedstocks and Processing Technologies*, eds. M. Stoytcheva and G. Montero, InTech, Croatia, Rijeka, 2011, pp. 397–410.
81 Review of lipase immobilization techniques: P. Adlercreutz, *Chem. Soc. Rev*., 2013, **42**, 6406–6436.
82 General review of enzyme immobilization: R. A. Sheldon and S. van Pelt, *Chem. Soc. Rev*., 2013, **42**, 6223–6235.
83 S. Furukawa, T. Ono, H. Ijima and K. Kawakami, *J. Mol. Catal. B Enzym*., 2002, **17**, 23–28.
84 X. Cao, J. Yang, L. Shu, B. Yu and Y. Yan, *Process Biochem*., 2009, **44**, 177–182.
85 G. Kuncova, J. Szilva, J. Hetflejs and S. Sabata, *J. Sol-Gel Sci. Technol.*, 2003, **26**, 1183–1187.
86 A. Tomin, G. Hornyánszky, K. Kupai, Z. Dorkó, L. Ürge, F. Darvas and L. Poppe, *Process Biochem*., 2010, **45**, 859–865.
87 M. T. Reetz, W. Wiesenhöfer, G. Francio and W. Leitner, *Chem. Commun.,* 2002, 992–993.
88 M. T. Reetz, A. Zonta, V. Vijayakrishnan and K. Schimossek, *J. Catal. A: Chem.,* 1998, **134**, 251–258.
89 M. T. Reetz, R. Wenkel and D. Avnir, *Synthesis*, 2000, 781–783.
90 M. T. Reetz, *Angew. Chem., Int. Ed*., 2011, **50**, 138–174.
91 A. G. Sandström, Y. Wikmark, K. Engström, J. Nyhlen and J.-E. Bäckvall, *Proc. Natl. Acad. Sci. USA*, 2012, **109**, 78–83.
92 Q. Wu, P. Soni and M. T. Reetz, *J. Am. Chem. Soc*., 2013, **135**, 1872–1881.
93 A. Svendsen, *Biochim. Biophys. Acta,* 2000, **1543**, 223–238.
94 T. Ema, T. Fujii, M. Ozaki, T. Korenaga and T. Sakai, *Chem. Commun*., 2005, 4650–4651.

Nanoscale Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012