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Entrapment of *Pseudomonas cepacia* lipase with peracetylated β-cyclodextrin in sol–gel: application to the kinetic resolution of secondary alcohols

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Abstract—Co-lyophilized *Pseudomonas cepacia* lipase with peracetylated β-cyclodextrin was immobilized by the sol–gel process. The gel-entrapped lipase/cyclodextrin was prepared by the hydrolysis of methyltrimethoxysilane (MTMS) in the presence of the co-lyophilized lipase with peracetylated β-cyclodextrin prepared with different weight ratios (enzyme to CD). This type of enzyme preparation was subsequently used in the kinetic resolution of a set of secondary alcohols using isopropenyl acetate as an innocuous acyl donor in toluene as the organic medium. The resulting chiral alcohols (substrate) and the corresponding acetates (product) were baseline separated in one analysis without derivatization using gas chromatography on a new chiral stationary phase (CSP) Chirasil-β-Dex containing an undecamethylene spacer (C11-Chirasil-Dex).

1. Introduction

Lipases are well established as valuable catalysts for the enzymatic kinetic resolution (EKR) of racemates. They accept a broad range of substrates and are used as a tool for the synthesis of non-racemic chiral molecules under non-aqueous conditions.[1–6] However, some of their features limit their application in organic synthesis, especially the frequently lower enzyme activity under non-aqueous conditions, which constitutes a major drawback in the application of enzymes in organic solvents. To make enzymes more appealing to organic chemists, the use of cyclodextrins as regulators for the *Pseudomonas cepacia* lipase (PSL) and macrocyclic additives to enhance the reaction rate and enantioselectivity *E* in lipase-catalyzed enantioselective transesterifications of 1-(2-furyl)ethanol in organic solvents has been reported.[4,5] However, such processes are only economically and ecologically viable if lipases can be immobilized efficiently using cheap methods, and if such immobilizates can be separated from the reaction products and recycled without significant loss of activity. This renders the development of supports for immobilization of lipases an area of high interest.[7]

The immobilization of lipases allows for their recycling and reuse in a continuous fashion. Common immobilization techniques include physical adsorption onto a solid support, covalent binding to a solid support, and physical entrapment within a polymer matrix support (Fig. 1).

Entrapment of a lipase entails its capture within the matrix of cross-linkable resin. This method has several advantages over the other two methods. Unlike the covalent binding method, this method uses a relatively simple procedure that allows the immobilized lipase to maintain its activity and stability. A variety of methods have been used for entrapping lipases in a polymer matrix. In one method, the lipase is entrapped in a photo-cross-linkable or solution cross-linkable resin. This technique consists of mixing a liquid photo-cross-linkable resin such as polyethylenegylcol dimethacrylate, containing the photosensitive functional groups with an appropriate initiator such as benzoin ethyl ether, and an enzyme solution followed by irradiation with near-ultraviolet light for a few minutes or addition of a polymerization accelerator such as dimethylaniline. The immobilized lipase produced by this method has been successfully applied in the hydrolysis of triglycerides,[8,9] the esterification of fatty acids,[10] and the conversion of other water-soluble[11] and insoluble compounds.[12]

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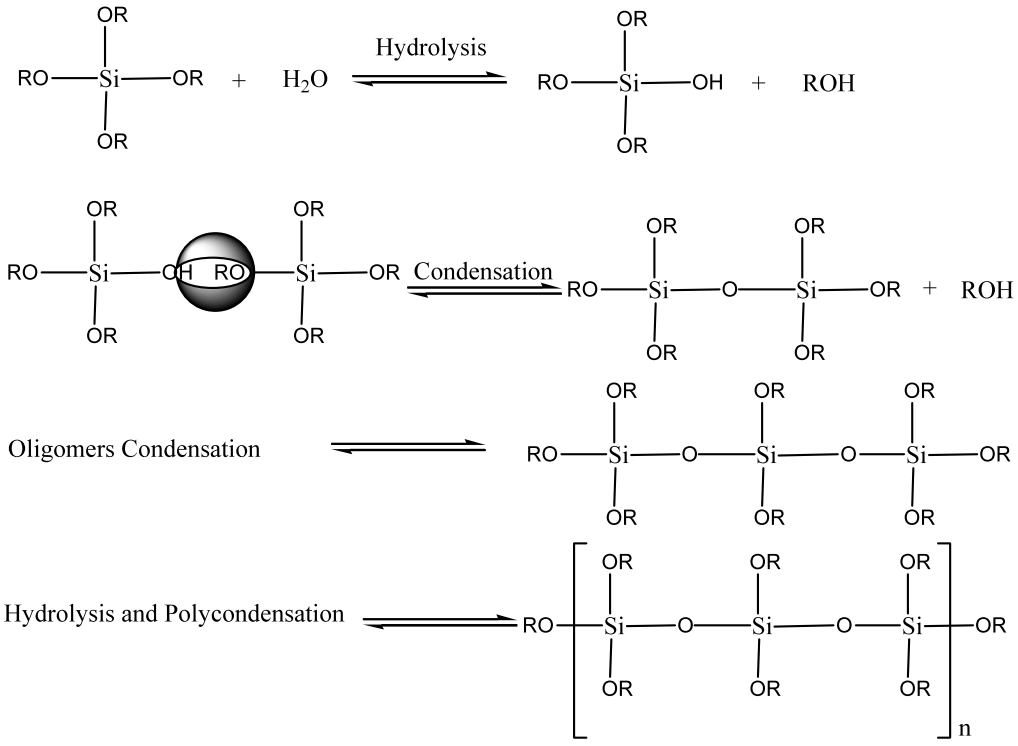


Figure 1. The sol–gel process.

The entrapment of enzymes in an inorganic polymer matrix has received substantial attention in recent years. This method pioneered by Avnir et al. is based on the sol–gel process,[13] which is well documented.[14] A typical immobilization procedure uses an aqueous solution of enzyme with sodium fluoride (NaF) acting as a catalyst, and alkoxysilane derivatives such as $RSi(OMe)_3$ where R = alkyl, aryl, or alkoxy as the gel precursors (Fig. 1).

Inorganic matrices offer a number of advantages: (1) They have controllable surface area, average pore size, narrow pore size distribution, and fractal dimension; (2) they are thermally stable; (3) leakage of the enzyme is prevented due to the rigidity of the caging; (4) they can be simply prepared from inexpensive chemicals; (5) they retain high enzyme activity; (6) they are easily obtained in a variety of forms, e.g. monoliths, thin films, fibers, powders, etc. and (7) they enhance the stability of the entrapped molecule and increase the activity of the enzyme.

Reetz et al. reported a significant increase in the activity of *Pseudomonas cepacia* lipase by entrapment in a sol–gel matrix using tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS), and other alkyl modified silane precursors.[15–18] The relative stability of the entrapped lipase increased with an increase in the pendant alkyl group of the polymerization precursors. The entrapped lipase also showed good stability when it was reused. Hsu et al. developed a procedure for the immobilization of lipase from *Pseudomonas cepacia* (PS-30) within a phyllosilicate sol–gel matrix. The procedure was based on a cross-linking polysilicate clay with a silicate polymer produced by the controlled hydrolysis of TMOS. The immobilized lipase showed more stability compared with free lipase in the esterification of lauric acid with octan-1-ol.[19] Other studies in this area have also indicated a higher activity and stability for the entrapped lipases within the inorganic matrix.[20,21]

In comparison with the immobilization of the lipase by the sol–gel method without additives, the activity of the enzyme can be enhanced by using macromolecular additives such as gelatin. Using cyclodextrins (CD) as macrocyclic additives rather than gelatin is expected to have another benefit. Peracetylated β-cyclodextrin has been used as an additive in enzymatic reactions in order to enhance the reaction rate and enantioselectivity *E* in transesterification reactions in organic solvents.[4] Herein, we benefit from the effect of CD on the lipase and entrap both components within the sol–gel matrix. The gel-entrapped lipase/cyclodextrin was prepared by the hydrolysis of methyltrimethoxysilane (MTMS) in the presence of the co-lyophilized lipase with peracetylated β-cyclodextrin. This type of enzyme preparation was subsequently used in the transesterification of a set of secondary alcohols in toluene as the organic solvent.

Figure 2. Preparation of lipase/CD containing gels.

2. Results and discussion

Three methods of enzyme preparation have been investigated. The first consists of a lyophilization of *Pseudomonas cepacia* lipase (PSL), the present model enzyme, from a phosphate buffer (pH 6) without the CD additive. The second enzyme preparation was a co-lyophilization of PSL with peracetylated β-cyclodextrin using the same phosphate buffer. Three different mixtures have been investigated, i.e. 1:1; 1:2; 1:6 (enzyme to cyclodextrin, w/w). The third enzyme preparation is the entrapment of the co-lyophilized lipase with peracetylated β-cyclodextrin (1:1; 1:2; 1:6) in the sol–gel matrix. The sol–gel process (initiated by the hydrolysis of MTMS) is performed in the presence of the co-lyophilized PSL with peracetylated β-cyclodextrin. Hydrolysis and condensation of the Si monomers, in the presence of an NaF catalyst trigger, cross-linking with the formation of SiO_2, a porous inorganic matrix which grows around the enzyme/CD in a three-dimensional manner (Fig. 2).

The effect of the entrapment of PSL/CD in the sol gel was first investigated for the model kinetic resolution of 1-(2-furyl)ethanol **1** using isopropenyl acetate as an acyl donor and toluene as the organic solvent. The new enzyme preparation was then investigated in the asymmetric acetylation of a set of secondary alcohols affording the non-racemic chiral alcohols and the corresponding acetates in high enantiomeric excess (up to 99%). The enantiomers of both substrate and product was separated in one analysis without derivatization using gas chromatography on a new chiral stationary phase (CSP) Chirasil-β-Dex containing an undecamethylene spacer (C11-Chirasil-Dex)[22] (Figs. 3–5 and Table 1).

2.1. Effect of cyclodextrin additives

Compared to the commercially available PSL (used as purchased), a slight decrease in the catalytic activity has been observed when the enzyme was lyophilized from the phosphate buffer (pH 6). This finding is accounted

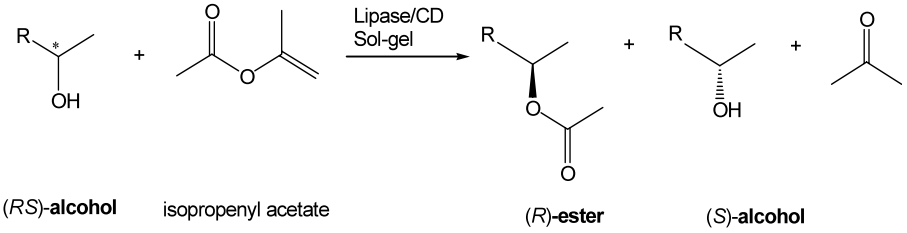


Figure 3. Lipase/CD entrapped in sol–gel-catalyzed transesterification of secondary alcohols using isopropenyl acetate as acyl donor in toluene.

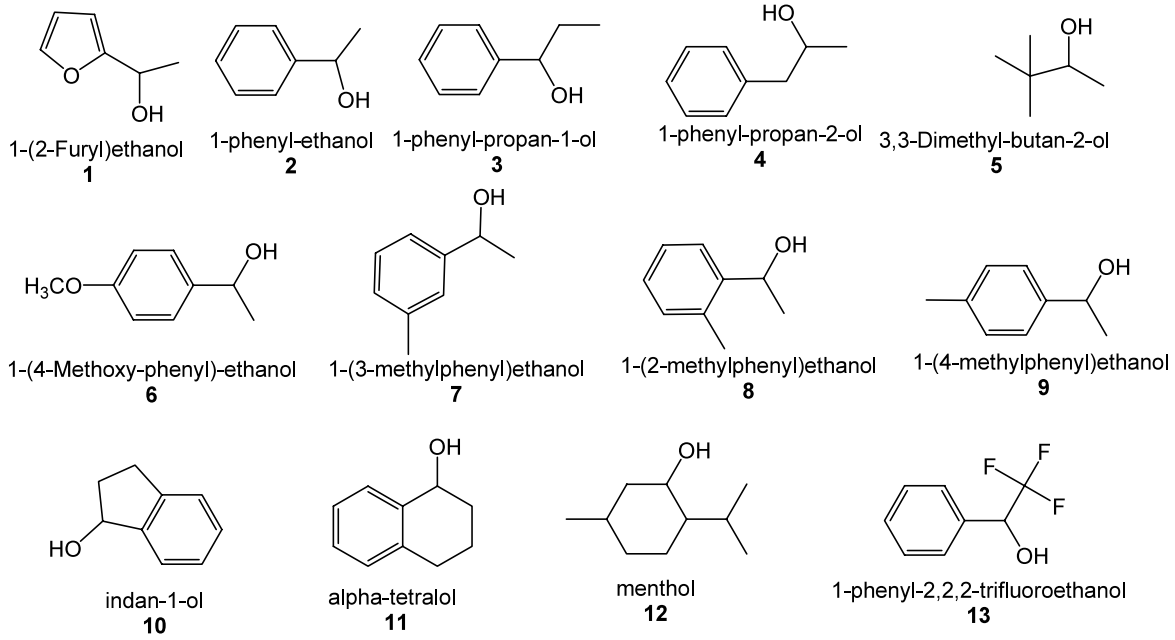


Figure 4. A set of secondary alcohols used in the lipase/CD entrapped in sol–gel-catalyzed kinetic resolution using isopropenyl acetate as acyl donor in toluene. All have been base-line separated on Chirasil-β-Dex with a new C11-spacer (C11-Chirasil-Dex).

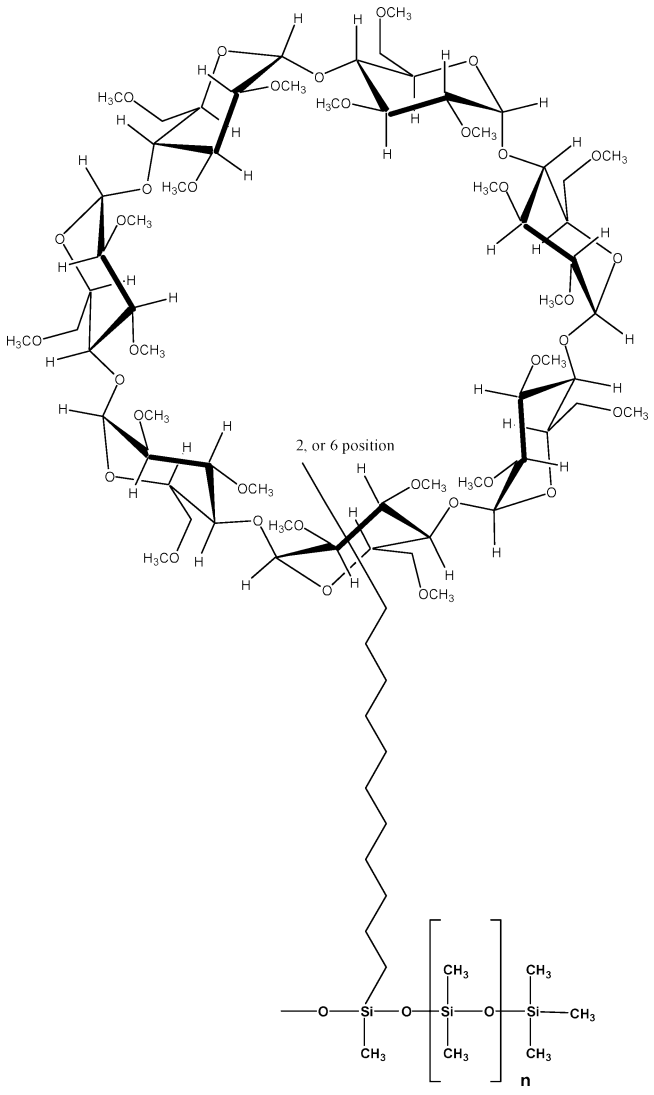


Figure 5. The structure of the chiral stationary phase consisting of permethylated β-cyclodextrin with a new C11-spacer (C11-Chirasil-Dex) bonded to a polysiloxane backbone.

for by partial denaturation of PSL during lyophilization. Interestingly, no such decrease in catalytic activity was observed when the enzyme was co-lyophilized with peracetylated β-cyclodextrin using the same phosphate buffer or when both were entrapped in the sol–gel matrix. This finding can be explained by the ability of the CD and sol–gel to protect the enzyme from aggregation or from denaturation effects.

Since the magnitude of the enzyme activation in organic solvents by macrocyclic compounds depends on the ratio of the enzyme and the additive,[23] three different mixtures have been investigated, i.e. 1:1; 1:2; 1:6 (enzyme to cyclodextrin, w/w), and added during the gel process. When the peracetylated β-cyclodextrin was employed at a 1:6 weight ratio of enzyme to cyclodextrin entrapped in sol–gel, a significant activation of PSL was detected when compared to the data of the lyophilized PSL from buffer alone.

In all of the cases of transesterification, the enhancement of the reaction rate and the enantioselectivity E of the PSL/CD in sol–gel-catalyzed transesterification of **1** in toluene was in the order 1:6>1:2>1:1 (enzyme to peracetylated β-cyclodextrin, w/w).

The enhancement effect observed in the co-lyophilized lipase with peracetylated β-cyclodextrin entrapped in sol–gel was almost the same as that observed before when using co-lyophilized lipase with peracetylated β-cyclodextrin (without entrapment in sol–gel). Therefore, it was concluded that the sol–gel was only working as an immobilizing agent for the co-lyophilized lipase with peracetylated β-cyclodextrin. Hence, the enhancement in the reaction rate and enantioselectivity E is believed to be based mainly on the beneficial effect of the CD additive on the enzyme. This arises from the direct interaction with the enzyme rather than the complexation with either substrate or product. Thus, in the co-lyophilization step, the CD may interact with the enzyme in a specific but as yet unknown way by changing its conformation and hence influencing its catalytic behavior as already inferred above. Since the CD is used in excess, host–guest complexation in solution with the substrate and product may not be excluded.

2.2. Stability of sol–gel entrapped PSL/CD

The sol–gel entrapped lipase from *Pseudomonas cepacia* is highly stable and can be stored at room temperature for months without significant loss of activity. The entrapped lipase in MTMS gels has been repeatedly used in the transesterification of 1-(2-furyl)ethanol in toluene. Adding to that, this kind of enzyme preparation was thermally stable. The lipase/CD entrapped in the sol–gel catalyzed transesterification of **1** using isopropenyl acetate in toluene was repeated at several temperatures from 30 to 70°C. A parallel experiment was also performed using free lipase. These results suggest that as the reaction temperature is increased the activity of lipase is also increased and the enhancement in activity was much stronger for the entrapped lipase/CD than for the free one.

2.3. Structural and morphological properties

2.3.1. Solid-state ^{29}Si NMR. Solid-state NMR and especially Si-NMR yields information about the chemical structure and degree of cross linking in the Si-containing gels, but tells us nothing about the actual enzymes, which are present in small amounts within the gel. In ^{29}Si NMR, it was interesting to determine the actual degree of cross-linking in the prepared gel. For this purpose only PSL in sol–gel only as well as PSL/CD 1:1, 1:2, 1:6 entrapped in sol–gel was investigated. The results suggest that a high degree of cross-linking was present and as much as the amount of CD (added to the lipase during lyophilization) was increased, the agglomeration of the spheres also increased (Fig. 6).

Table 1. Oven temperature (T), retention time (t_R), resolution (R_s) and the separation factor (α) of the simultaneous baseline separation of racemic secondary alcohols and their corresponding acetates[22]

Compounds[a]	Oven temperature[b]	t_R (R)	t_R (S)	R_s	α
1	85°C	4.6	4.8	1.88	1.05
1a		3.6	4.4	9.92	1.26
2	80°C	27.1	26.1	2.02	1.04
2a		30.2	24.9	11.34	1.22
3	95°C	10.1	11.2	5.83	1.12
3a		7.3	6.2	9.10	1.19
4	100°C	14.2	15.2	4.20	1.08
4a		8.0	7.4	4.74	1.09
5	50°C	5.4	5.8	1.78	1.07
5a		6.1	4.9	7.91	1.26
6	110°C	20.5	22.1	4.76	1.08
6a		19.0	16.7	8.45	1.14
7	105°C	10.6	11.5	4.68	1.09
7a		7.2	6.5	5.83	1.12
8	90°C for 13 min, then increased to 120°C at a rate of 10°C per min	18.2	20.0	10.66	1.10
8a		12.0	11.5	2.34	1.04
9	105°C	9.3	10.5	6.63	1.14
9a		8.4	7.4	7.44	1.14
10	100°C	22.1	21.4	1.79	1.03
10a		17.9	17.2	2.27	1.04
11	120°C	16.7	15.9	2.68	1.05
11a		12.8	13.3	2.64	1.04
12	100°C	10.6	10.3	1.64	1.04
12a		7.9	9.1	7.91	1.16
13	90°C for 4 min, then increased to 120°C at a rate of 10°C per min	10.9	11.3	3.47	1.04
13a		3.4	3.3	2.32	1.06

[a] **a** is designed for the acetate of the corresponding secondary alcohol.
[b] The head pressure is 50 kPa, the injector temperature is 200°C and the FID temperature is 250°C.

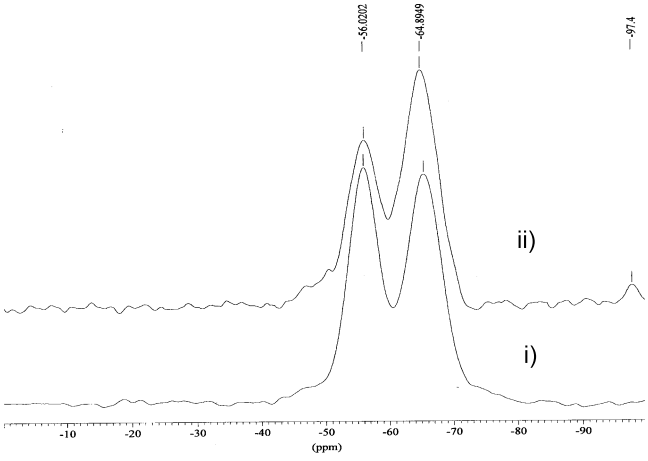


Figure 6. ^{29}Si NMR spectrum of (i) PSL-containing gel derived from MTMS (ii) co-lyophilized PSL with peracetylated β-cyclodextrin entrapped in sol gel, derived from MTMS.

2.3.2. Scanning electron microscopy (SEM) and energy dispersive X-ray studies (EDX). SEM studies were carried out (Figs. 7–10) to determine the morphology of lipase-containing gel derived from MTMS (control) and thus compare it with the co-lyophilized PSL with peracetylated β-cyclodextrin (1:1, 1:2, 1:6 w/w)-containing gel. They revealed large amorphous regions as well as spherical particles in the case of PSL (only) entrapped in sol–gel. As soon as the lipase was co-lyophilized with the CDs, the morphology of the particles changed to form agglomerates. When the peracetylated β-cyclodextrin increased (from 1:1 to 1:6 w/w lipase/CD ratio) the agglomeration is increased by the same amount.

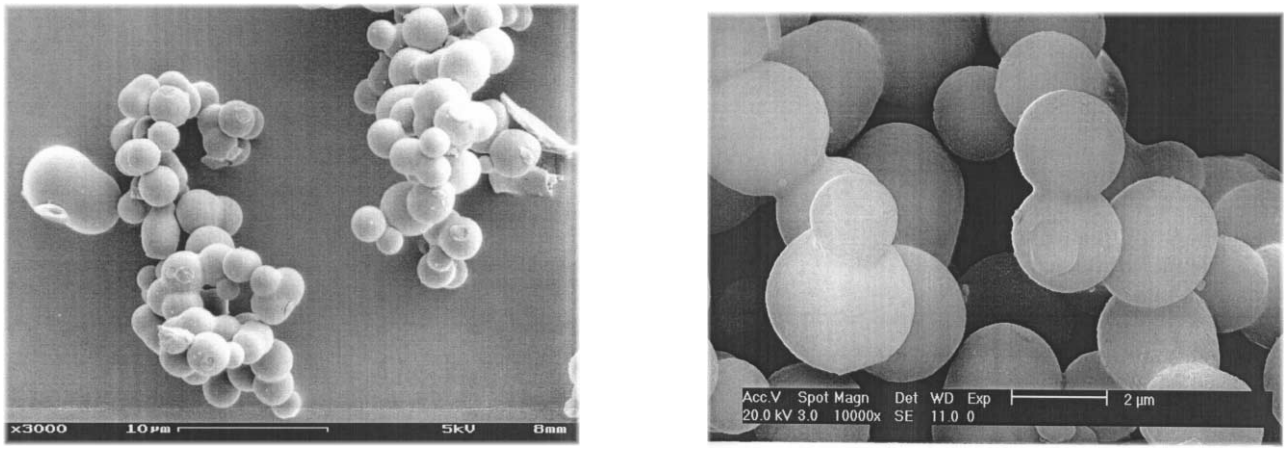
In order to define whether the lipase was simply physically entrapped or adsorbed on the surface of the particle, an EDX study was performed. This study revealed the existence of lipase/CDs inside the spherical particles as well as on the surface (Fig. 11).

2.4. Lipase-catalyzed enantioselective transesterification of secondary alcohols

The new type of enzyme preparation (lipase/CD 1:6 w/w entrapped in sol–gel) was subsequently used in the kinetic resolution of a set of secondary alcohols using isopropenyl acetate as an innocuous acyl donor in toluene, which was used as the organic medium. Results are summarized in Table 2.

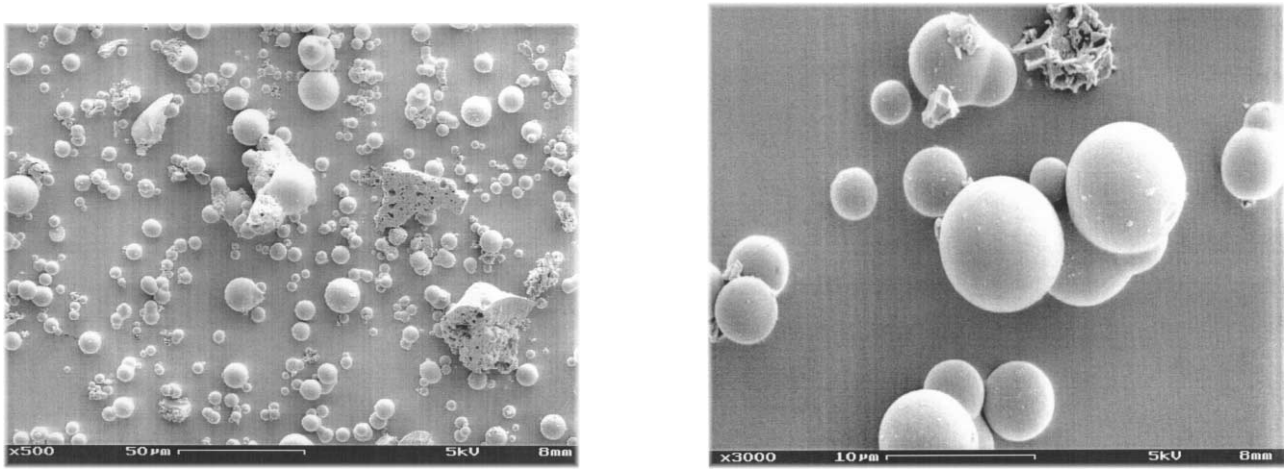
The transesterification was carried out at 40°C in toluene at a molar ratio of isopropenyl acetate to racemic alcohol of 2:1 to ensure the irreversibility of the reaction.

In all cases, the (R)-alcohol was the faster reacting enantiomer yielding the (R)-ester in high enantiomeric excess and leaving the (S)-alcohol in enantiomerically pure form. The absolute configuration of the resulting



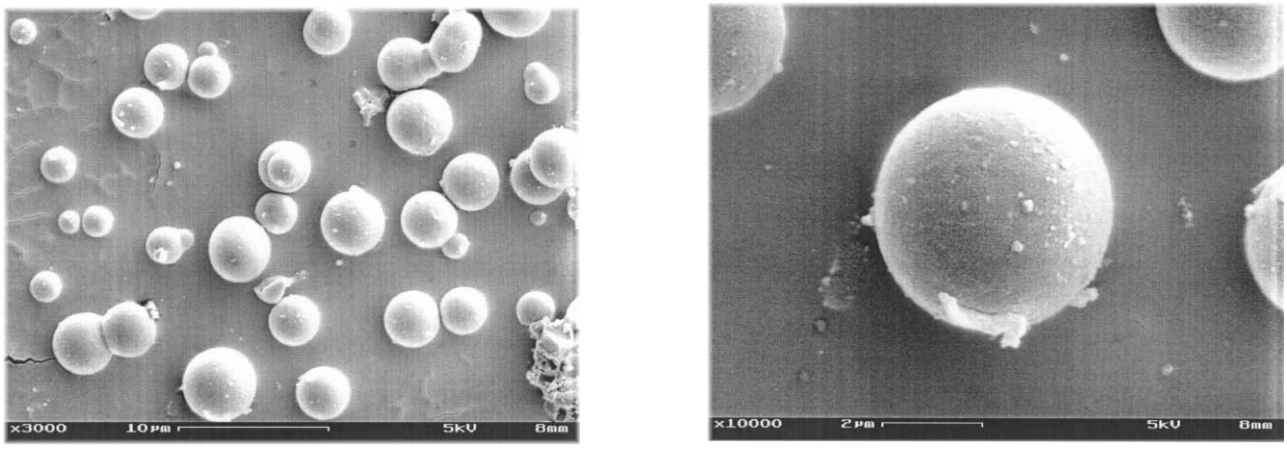
(a) (b)

Figure 7. A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated β-cyclodextrin (1:6 w/w) entrapped in sol–gel.



(a) (b)

Figure 8. A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated β-cyclodextrin (1:2 w/w) entrapped in sol–gel.



(a) (b)

Figure 9. A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated β-cyclodextrin (1:1 w/w) entrapped in sol–gel.

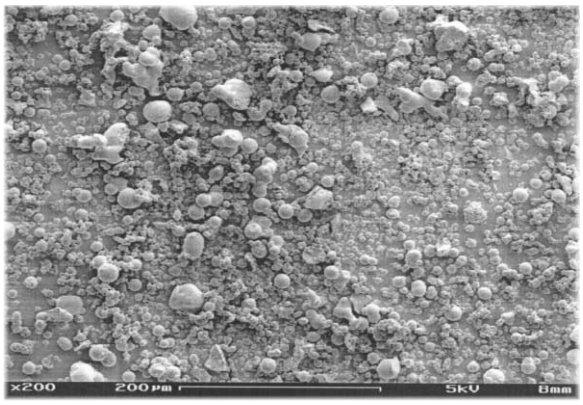


Figure 10. A typical SEM-micrograph of PSL entrapped in sol–gel.

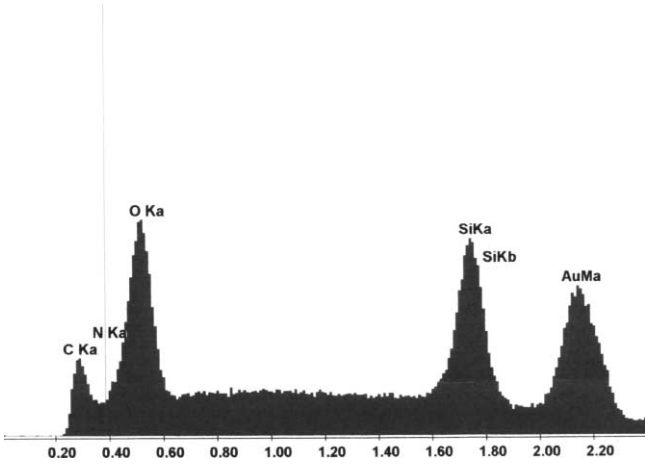


Figure 11. A typical EDX spectrum of the co-lyophilized lipase (PSL) with peracetylated β-cyclodextrin (1:6 w/w) entrapped in sol–gel.

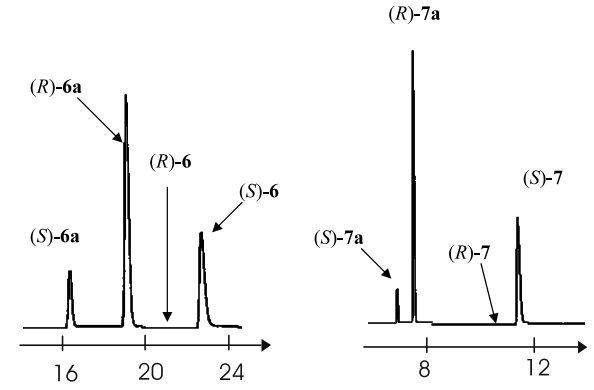


Figure 12. Lipase/CD (1:6 w/w) entrapped in sol–gel-catalyzed transesterification of **6** ($t=8$ h, $ee_s=>99\%$, $ee_p=65.2\%$, conv. $=60.5\%$ and $E=42$) and **7** ($t=2$ h, $ee_s=>99\%$, $ee_p=80\%$, conv. $=55\%$ and $E=93$) using isopropenyl acetate as acyl donor in toluene.

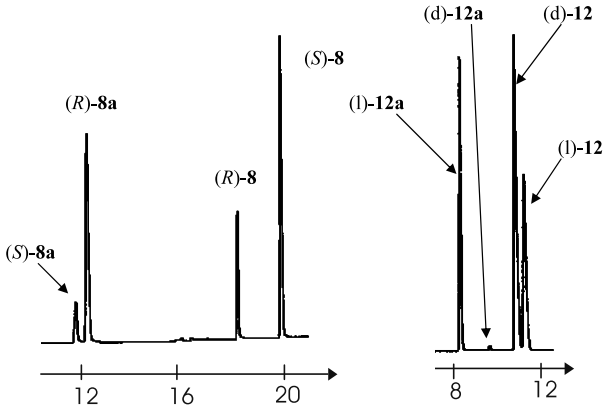


Figure 13. Lipase/CD (1:6 w/w) entrapped in sol–gel-catalyzed transesterification of **8** ($t=2$ h, $ee_s=48.3\%$, $ee_p=67.9\%$, conv. $=41.5\%$ and $E=9$) and **12** ($t=2$ h, $ee_s=27.4\%$, $ee_p=94.6\%$, conv. $=22.4\%$ and $E=47$) using isopropenyl acetate as acyl donor in toluene.

alcohols and esters were determined by comparaison with authentic samples. Molecular sieves (4 Å, 100 mg) were added prior to the enzymatic reaction in order to scavenge the liberated acetone (as a by product from the enzymatic reaction) thereby preventing any undesired reaction[2] (Figs. 12 and 13).

Table 2. Lipase/CD entrapped in sol–gel-catalyzed transesterification of secondary alcohols using isopropenyl acetate as acyl donor in toluene as organic solvent

Compounds	Time (h)	ee_s (*S*)-alcohol (%)[a]	ee_p (*R*)-ester (%)[a]	Conversion (%)[a]	*E*
1	8	>99	79	56	88
2	2	>99	91	52	>200
3	2	>99	78.6	55.9	93
4	2	>99	96	51	680
5	14	94.3	76.4	55.2	26
6	2	>99	87	53.4	141
7	2	>99	80	55	93
8	8	>99	36.1	73.4	16
9	12	>99	49	67	24
10	2	>99	18.3	84.4	9
11	2	>99	28.8	77.5	10
12	8	85.2	88	49	42
13	8	>99	53.8	65	29

[a] ee_s: enantiomeric excess of substrate (alcohol). ee_p: enantiomeric excess of product (ester). *E*: enantioselectivity factor.

3. Experimental

3.1. Chemicals and instrumentation

All alcohols were commercially available and dried over molecular sieves prior to use. The racemic esters were synthesized on an analytical scale to optimize a baseline separation of the enantiomers by gas chromatography. Scanning electron micrographs (SEM) and energy dispersive X-ray analysis (EDX) were performed on a DSM 962 scanning electron microscope (SEM) (Zeiss, LEO) equipped with a DX-4 X-ray detection system by EDAX. This consisted of an energy dispersive Si(Li)-detector with an active area of 10 mm^2 and the EDX software package. Micrographs were recorded detecting secondary electrons generated by a probe current of 168 pA, whereas a 623 pA probe current was applied for carrying out elemental analysis by EDX.

3.2. General procedure for the synthesis of esters (analytical scale)

Acetic anhydride (30 μl) was added to an alcohol (20 μl) in pyridine (200 μl). This mixture was then kept at 100°C for 1 h and injected without further clean-up.

3.3. Co-lyophilized lipase with cyclodextrin-containing gel

The *Pseudomonas cepacia* lipase (PSL) was dissolved (2 mg/1 μl) in a 20 mM phosphate buffer (pH 6) and lyophilized for 48 h (control). The co-lyophilization of the lipase with peracetylated β-cyclodextrin was performed by the same method, except that the CD was added prior to the lyophilization in the weight ratio 1:1; 1:2; 1:6 of lipase to cyclodextrin. The co-lyophilized lipase with peracetylated β-cyclodextrin was then added into a flask and stirred in 4 ml of phosphate buffer (pH 6) for 5 min to generate a homogeneous lipase/CD suspension. To this mixture 400 μl of 1 M NaF solution, 800 μl gelatin (4% w/w) and 628.2 μl (5 mmol) MTMS were added. The two-phase mixtures were vigorously shaken by hand until the evolution of heat began (1–3 min). The resulting milky emulsion cleared up to form a white solid material. This solid was then cooled down in an ice bath for 10 min and left in a closed vessel for 24 h at rt. The material was dried in an oven at 40°C for 3 days under atmospheric pressure. The resulting solid was grounded in a mortar and shaken with 16 ml of buffer (pH 6) for 2 h. The product was collected on a glass frit (D4), washed with buffer (20 ml) and *n*-pentane(20 ml). The resulting gel was then dried at 40°C for 24 h, grounded and kept for further use.

3.4. Lipase-catalyzed irreversible transesterification of secondary alcohols

All reactants (alcohol, ester) were stored over activated molecular sieves 4 Å.

Racemic alcohol (0.5 mmol) and isopropenyl acetate (1 mmol) were dissolved in toluene (3 ml) in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath to 40°C. 100 μl of the reaction mixture was then withdrawn for GC analysis ($t=0$ of sample). Afterward, 100 mg of lipase (PSL-C) colyophilized with either 100, 200 or 600 mg peracetylated β-cyclodextrin and entrapped in sol–gel was added followed by the addition of 100 mg molecular sieve (4 Å). Samples (100 μl) were taken after several time intervals and centrifuged to separate lipase. The organic layer was diluted by solvent (100 μl). The reaction progress was monitored qualitatively by thin layer chromatography. An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. The lipase powder was then dried in air for further use.

3.5. Enantioselective gas-chromatographic analysis

Enantioselective gas-chromatographic analysis was performed on a Hewlett Packard instrument (Waldbronn, Germany) equipped with a flame ionisation detector (FID). The chiral stationary C11-Chirasil-Dex was coated on a non-deactivated 19 m×0.25 mm fused silica capillary column (0.25 μm film thickness) according to Ref. 22. The analytical conditions were: injector temperature, 200°C; FID temperature, 200°C; oven temperature varying from 50 to 120°C depending on the analysed compound for the simultaneous separation of enantiomers of both substrate and product. Hydrogen was used as the carrier gas (50 kPa column head pressure). The enantiomeric excess of both the substrate and product as well as conversion and enantioselectivity E was determined as described previously.[24,25]

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