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Journal of Molecular Catalysis A: Chemical 134 (1998) 251–258

JOURNAL OF MOLECULAR CATALYSIS A: CHEMICAL

Entrapment of lipases in hydrophobic magnetite-containing sol-gel materials: magnetic separation of heterogeneous biocatalysts [1]

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Received 29 September 1997; accepted 5 December 1997

Abstract

The simultaneous entrapment of a lipase such as *Pseudomonas cepacia* or *Candida antarctica* and nanostructured superparamagnetic magnetite (Fe_3O_4) in hydrophobic sol–gel materials derived from $CH_3Si(OCH_3)_3$ (MTMOS) or other hydrophobic precursors leads to catalytically active, mechanically stable and magnetically separable heterogeneous biocatalysts. The relative enzyme activity in the test reaction involving the esterification of lauric acid by *n-octanol* in isooctane is typically 200–300%, with respect to the same reaction using a conventional suspension of the non-immobilized enzyme. Separation of the catalyst by applying a simple magnet poses no problems. In the kinetic resolution of 2-pentylamine, enantioselectivity is essentially complete (ee = 97–99%).

Keywords: Lipases; Immobilization; Esterification; Sol–gel; Magnetic separation; Enantioselectivity

1. Introduction

A wide variety of biocatalysts are currently available to the practicing organic chemist for many different kinds of synthetically important transformations [1–5]. In addition to enzymes, other types of biocatalysts have been introduced, including catalytic antibodies [6]. Nevertheless, enzymes remain the most important biocatalysts in synthetic organic chemistry [1–5]. A milestone in the area of enzymes as catalysts for organic chemistry was the discovery that some of them retain their catalytic activity in non-aqueous media [1–5,7–12]. An example of great importance pertains to the catalytic activity of lipases in organic solvents. Normally, these enzymes catalyze the hydrolysis of carboxylic acid esters with formation of carboxylic acids and alcohols. Since an excess of water is present, the reverse reaction, i.e., esterification, which is often the actual synthetic goal, cannot be achieved. However, use of a suspension of a lipase in an organic solvent does, in fact, make chemo-, regio- and stereoselective esterification or transesterification possible [1–5,7–10]. More recently, lipases have been used to catalyze the enantioselective acylation of amines [13–16]. Thus, they are the most frequently used enzymes in organic chemistry. Many different kinds of chiral esters, diesters,

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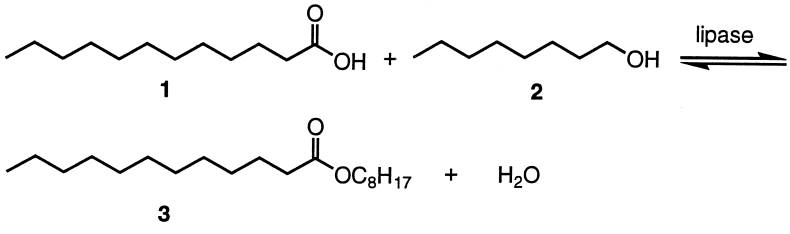
[1] Dedicated to Prof. Herman van Bekkum on the occasion of his 65th birthday.

1381-1169/98/$19.00
PII: S1381-1169(98)00043-0

lactones, alcohols, lactols, cyanohydrins, diols, amines, amino alcohols, and amino acids have been produced enantioselectively. However, the number of *industrial* lipase-based enantioselective syntheses of chiral organic compounds is limited, cheap and efficient recyclization being one of the problems. Immobilization of enzymes not only makes recyclization possible, it also offers the potential to increase catalytic activity [17–26] (for recent examples of lipase immobilization, see Refs. [27–32]. Since studies in these areas are on-going endeavors, it is currently difficult to assess the relative merits of the different approaches.

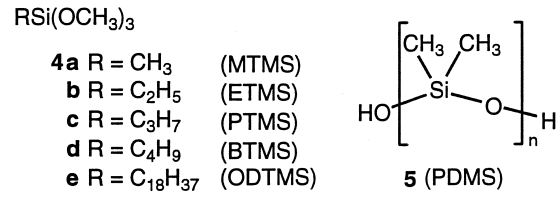
A unique strategy concerns entrapment of enzymes using inorganic matrices such as silica gel. Accordingly, the so-called sol–gel process [33,34], initiated by the hydrolysis of $Si(OR)_4$, is performed in the presence of the enzyme. Hydrolysis and condensation of the Si-monomers in the presence of an acid or base catalyst trigger cross-linking with formation of amorphous SiO_2, a porous inorganic matrix which grows around the enzyme in a three-dimensional manner. Based on previous work by Dickey [35], Johnson [36] and Mosbach [37], the group of Avnir [38–43] generalized the sol–gel entrapment to include a fairly wide range of enzymes such as phosphatase, trypsin, aspartase, glucose oxidase, carbonic anhydrase, chinitase and monoamine oxidase. Gel-like materials were obtained which in optimal cases have enzyme activities approaching 100% relative to those of the natural non-encapsulated forms [38–41].

In the early 1990s we became interested in immobilizing lipases and catalytic antibodies in sol–gel materials [44,45]. Upon employing $Si(OR)_4$ as the gel-precursor in the presence of lipases, we obtained heterogeneous biocatalysts displaying disappointingly low enzyme activities [44–48]. Using the esterification of lauric acid **1** by *n-octanol* **2** in isooctane with formation of octyl laurate **3** as the model reaction, relative activities of 1–20% were observed. In this test reaction, which was used in all of the following studies, the relative activity x is defined as v (immobilized enzyme)$/v$ (commercial enzyme), where v is the initial rate of the reaction (in μmol h^{-1} mg $lipase^{-1}$). Similar results were reported by other groups [49–51].

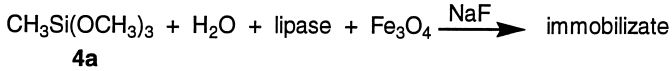


We then took a different approach to the problem by considering the structure and mode of catalytic action of lipases. It is well known that lipases are interfacial-active enzymes with hydrophobic domains [52–62] [63]. Lipophilic interactions with substrate molecules induce conformational changes in the lipase. In certain types of lipases it is the movement of a short α-helical loop which uncovers the active site (lid mechanism). We speculated that pure SiO_2 is not the ideal matrix for lipases, and that hydrophobic matrices could provide a better microenvironment [44–48]. Thus, monomers of the type $RSi(OCH_3)_3$ or mixtures of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$ were used in the sol–gel process. This strategy turned out to be successful. The following list includes the most important sol–gel precursors which we used. Hydrophobic lipase-containing gels were obtained which display relative enzyme activities of 400–8800% in the test reaction. They can be re-used many times without significant loss of enzyme activity. These and other developments [46–48], including 'double immobilization' in hydrophobic sol–gel materials on glass beads such as SIRAN® [64], are described

in a recent review article [65]. These heterogeneous biocatalysts are now commercially available [66] and can be employed in regio- and enantioselective acylations [65] and even in hydrolysis reactions [65]. Several groups have used these catalysts in related applications [67–71].



In the present paper we describe yet another facet of these lipase-immobilizates, namely the use of magnetite-containing analogs [44,45]. Although we generally separate the catalyst from the reaction mixture by simple filtration [44–48,64,65], we envisioned an alternative method, specifically magnetic separation known to be efficient in other applications [72–76]. Following our initial report on the utilization of this method concerning lipase-containing gels [44,45], a related publication recently appeared in which a lipase was immobilized on ferrite powder followed by a sol–gel process [77]. Our own strategy is somewhat different, namely to carry out the usual immobilization of a lipase [65] in the presence of nanostructured super-paramagnetic magnetite (Fe_3O_4). We speculated that both the lipase and the Fe_3O_4 nanoparticles would be entrapped in the hydrophobic sol–gel material derived from such precursors as **4a**.



2. Immobilization

2.1. Preparation and characterization of Fe_3O_4-nanoparticles

It is well known that the optical and magnetic properties of nanoparticles are size-dependent [78–81]. In the case of magnetite, particles larger than about 30 nm display ferromagnetic properties, whereas those smaller than 30 nm are superparamagnetic. Therefore, we had to prepare magnetite particles smaller than 30 nm. In doing so, we utilized the procedure of Kobayashi based on co-precipitation from a solution of $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$ [82]. Following isolation of the colloidal magnetite, a sample was characterized by transmission electron microscopy (TEM). Fig. 1 shows the presence of crystallites appearing to have quadratic and rhombic shapes. Although this needs further study, the conclusion important for this study concerns the size of the particles. They are in the range of 10–20 nm and are therefore expected to have superparamagnetic properties.

2.2. Simultaneous entrapment of lipases and magnetite

In an initial study, magnetite and lipase Amano PS (from *Pseudomonas cepacia*) were entrapped in a MTMS-gel by mixing an aqueous suspension of Fe_3O_4 (Section 2.1) with the mixture of the enzyme and gelatin as an additive, followed by the addition of the sol–gel precursor $CH_3Si(OCH_3)_3$. NaF served as the catalyst. This process turned out to be successful, affording mechanically stable, paramagnetic material which could easily be separated by applying a magnetic field (simple magnet). The degree of enzyme immobilization was measured to be 88%.

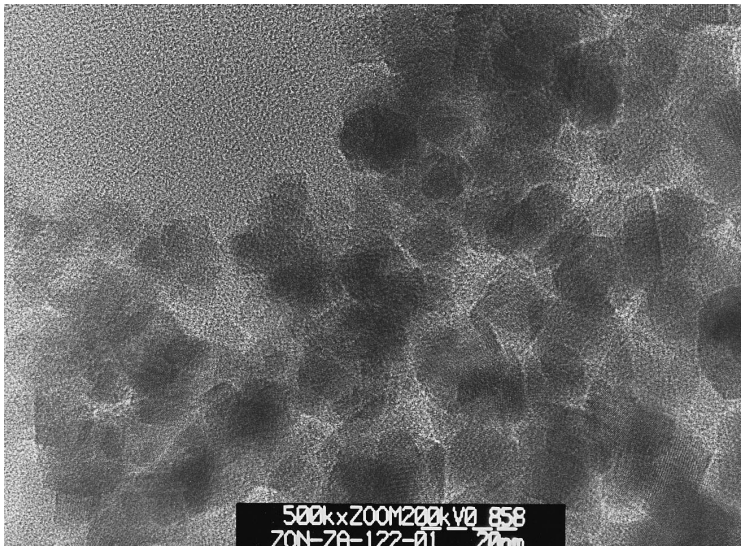


Fig. 1. TEM image of colloidal magnetite.

The gel was characterized by TEM analysis (Fig. 2). Since the iron oxide particles have a higher contrast than the surrounding silicon-matrix, a sufficient degree of visualization is possible. Indeed, numerous entrapped magnetite crystallites are visible (~20 nm in size). An EDX spot analysis clearly showed the presence of iron in the matrix.

The same lipase was then simultaneously entrapped with magnetite in gels originating from MTMS/PDMS, BTMS/TMOS and PTMS/TMOS using similar procedures except that poly(vinyl alcohol) (PVA) was used as an additive in place of gelatin. Finally, three additional gels containing the lipase from *C. antarctica*, PVA and magnetite were prepared using the gel precursors MTMS/PDMS, BTMS/TMOS and PTMS/TMOS.

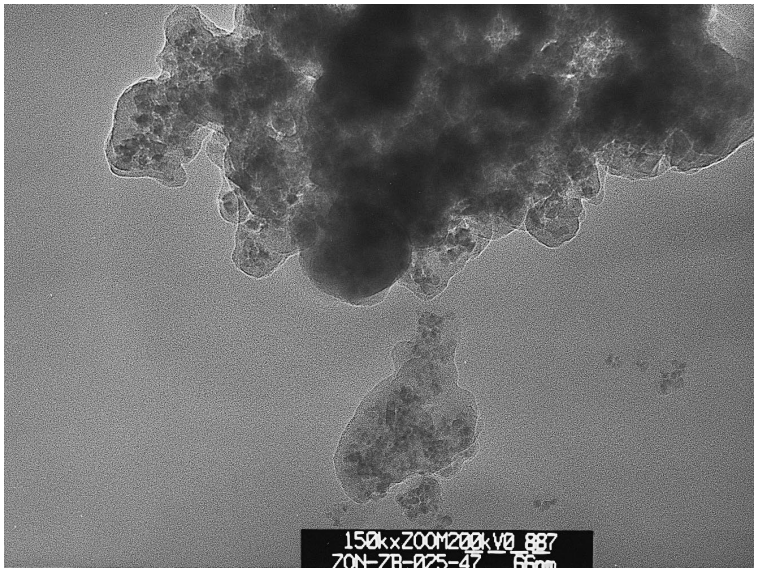
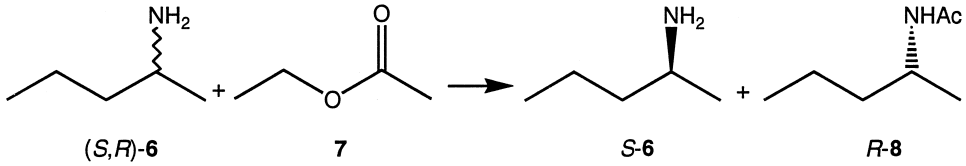


Fig. 2. TEM image of magnetite-containing lipase (Amano PS) immobilizate.

3. Enzyme activity and stereoselectivity

In order to determine the enzyme activity of the magnetite-containing gels, catalysis of the test reaction involving the usual esterification of lauric acid by *n-octanol* was studied. It turned out that the relative catalytic activity of our initial gel amounts to 330%, i.e., the material is 3.3 times as active as a suspension of the non-immobilized enzyme under otherwise identical conditions. Although an analogous MTMS-gel in the absence of magnetite was previously shown to display higher activity [46–48,65], the present results are very encouraging. Indeed, the heterogeneous biocatalyst was easily separated from the reaction mixture simply by using a magnet. The material showed similar enzyme activity upon re-use. The other lipase/magnetite containing gels also showed pronounced degrees of an increase in enzyme activity (Table 1).

Finally, one of magnetite containing gels (*C. antarctica*; MTMS/PDMS; PVA) was tested as an enantioselective catalyst in the kinetic resolution of racemic 2-pentylamine **6** using ethyl acetate **7** as the acylating agent and *t*-butylmethyl ether as the solvent (Fig. 3). As the reaction proceeded up to the theoretically maximum value of 50% conversion, samples were taken to monitor the degree of enantioselectivity (ee). Gratifyingly, it remained approximately constant in the range of 97–99% ee. This is identical to the behavior of a similar gel not containing magnetite [83,84].



4. Conclusions

We have demonstrated for the first time the feasibility of incorporating nanostructured magnetite in lipase-containing hydrophobic sol–gel materials for the purpose of simple magnetically induced separation. Although optimization was not strived for, the principles are clear. Lipases tolerate the presence of magnetite in the entrapment procedure, affording catalytically active mechanically stable, and magnetically separable heterogeneous biocatalysts. The presence of magnetite does not adversely affect enantioselectivity in such reactions as the acylation of chiral amines. Generalization to include other enzymes should be straightforward.

Table 1
Relative activity of magnetite-containing lipase immobilizates (test reaction: esterification of lauric acid (**1**) by *n*-octanol (**2**))

Lipase	Gel-precursor	Additive	Relative activity (%)
Pseudomonas cepacia	**4a**	gelatin	330
	4a/**5** (1: 1)	gelatin	210
	4a/**5** (1: 1)	PVA	275
	TMOS/**4d** (1: 1)	PVA	202
	TMOS/**5** (1: 1)	PVA	200
Candida antarctica	**4a**/**5** (1: 1)	PVA	210
	TMOS/**4d** (1: 1)	PVA	160

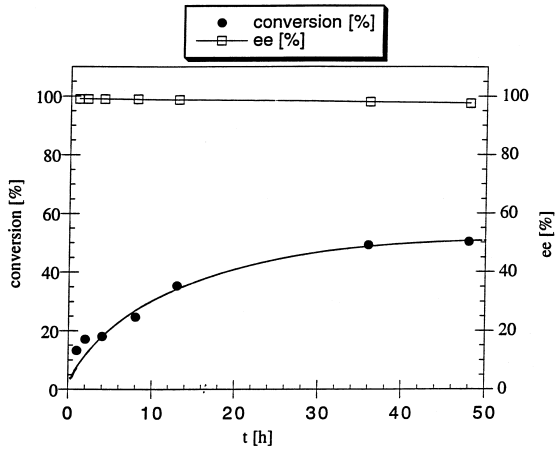


Fig. 3. Kinetic resolution of racemic 2-pentylamine (**6**).

4.1. Preparation of colloidal magnetite

Basically, the method of Kobayashi was used [82]. The solution of $FeSO_4 \cdot 7H_2O$ (41.7 g 0.15 mol) in 180 ml of de-ionized water is added to a solution of $FeCl_3 \cdot 6H_2O$ (80.7 g 0.3 mol) in 480 ml of water. Within 45 min a 20% (by w/v) solution of NaOH is added dropwise with rapid stirring until the pH reaches 11.5. During this time dark-brown magnetite precipitates out. The mixture is stirred for an additional hour at 60°C. Upon cooling to 0°C, the whole mixture is poured onto 4 l of ice-cold de-ionized water. Following deposition of the magnetite, the solution is carefully decanted, and the solid material is washed three times with 3 l of de-ionized H_2O. The moist colloid (196 g 77% H_2O content) is kept in a closed vessel in the refrigerator. The dried material was characterized by TEM analysis using a Hitachi HF 2000 FE-TEM instrument (200 kV).

4.2. Typical entrapment procedure

Essentially the same method for entrapment of lipases in hydrophobic sol–gel materials was employed as previously described [44–48], except that magnetite was used as an additive. For example, using a polypropylene vessel, the mixture of lipase Amano PS (commercially available from Amano) (25 mg in 1 ml de-ionized H_2O), 200 μl of an aqueous gelatin solution (4% w/v), 100 μm of an aqueous 1 M NaF-solution and 500 mg of the above colloidal magnetite is mixed intensively for 10 s by applying a Vortex-mixer. Then $CH_3Si(OCH_3)_3$ (MTMS) (857 μl 6 mmol) is added, and the two-phase mixture is again dispersed using a Vortex-mixer (3 s). The vessel is shaken by hand for about 2 min and then cooled in an ice bath for 5 min. The precipitate is left to stand 24 h at room temperature in the closed vessel, after which it is dried at 35°C for 3 d at atmospheric pressure. The solid material is ground in a mortar and shaken with 4 ml of de-ionized water for 2 h at room temperature. The product is filtered over a glass-frit (D4) and washed with 20 ml of de-ionized H_2O, twice with 20 ml of acetone and 20 ml of pentane. The immobilizate is stored at 35°C for 24 h and is then ground in a grinder. Characterization by TEM analysis and energy dispersive X-ray analysis (EDX) was performed using the Hitachi instrument (see above).

4.3. Enzyme activity

The test reaction was performed as described previously [46–48,65]. In the case of lipase Amano PS in the gel from MTMS (sel above), an activity of 106.4 μmol h^{-1} mg^{-1} protein was observed,

V_{free} being 32.5 μmol h^{-1} mg^{-1} enzyme. This amounts to a relative activity of 330%. The enzyme activities of the other gels were determined analogously (Table 1).

*4.4. Kinetic resolution of racemic 2-pentylamine (**6**)*

Magnetite-sol–gel immobilizate (360 mg containing less than 58 mg of lipase *C. antarctica* SP 525) was suspended in 10 ml of *t*-butyl methyl ether; 2 mmol of (*R*,*S*)-2-pentylamine and 8 mmol of ethyl acetate were added and the mixture was shaken at room temperature at 300 rpm. The reaction was monitored by taking samples (0.15 ml) at regular intervals, removing the immobilizate using a magnet and assaying the solution for the acylated amine. The enantiomeric excess of the acylated amine **8** was measured by GC with a Becker-Packard 427 instrument (column: 0.25 mm fused-silica capillary, 25 m column length, stationary phase: 20% 2,6-dimethyl-3-pentyl-β-cyclodextrin in SE 54; flame ionization detector, operating temperature: 60–180°C/2°C min^{-1}; carrier gas: H_2 at 0.12 MPa). The GC-values obtained as moles/100 moles were corrected by experimentally determined response factors.

References

[1] K. Faber, Biotransformations in Organic Chemistry, 3rd edn., Springer, Berlin, 1997.
[2] W. Boland, C. Frößl, M. Lorenz, Synthesis (1991) 1049.
[3] C.H. Wong, G.M. Whitesides, Enzymes in Synthetic Organic Chemistry, Pergamon, Oxford, 1994.
[4] H.G. Davies, R.H. Green, D.R. Kelly, S.M. Roberts, Biotransformations in Preparative Organic Chemistry: The Use of Isolated Enzymes and Whole Cell Systems in Synthesis, Academic Press, London, 1989.
[5] F. Theil, Chem. Rev. 95 (1995) 2203.
[6] R.A. Lerner, S.J. Benkovic, P.G. Schultz, Science 252 (1995) 659.
[7] A.M. Klibanov, Acc. Chem. Res. 23 (1990) 114.
[8] I. Mingarro, C. Abad, L. Braco, Proc. Natl. Acad. Sci. USA 92 (1995) 3308.
[9] G. Carrea, G. Ottolina, S. Riva, Trends Biotechnol. 13 (1995) 63.
[10] J. Tramper, M.H. Vermue, Chimia 47 (1993) 110.
[11] C.-S. Chen, C.J. Sih, Angew. Chem. 101 (1989) 711.
[12] C.-S. Chen, C.J. Sih, Angew. Chem. Int. Ed. Engl. 28 (1989) 695.
[13] V. Gotor, R. Brieva, F. Rebolledo, J. Chem. Soc., Chem. Commun. (1988) 957.
[14] M.T. Reetz, C. Dreisbach, Chimia 48 (1994) 570.
[15] A. Mattson, C. Orrenius, N. Öhrner, C.R. Unelius, K. Hult, T. Norin, Acta Chem. Scand. 50 (1996) 918.
[16] V.M. Sanchez, F. Rebolledo, V. Gotor, Tetrahedron Asymmetry 8 (1997) 37.
[17] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill Jr., C.H. Amundson, Enzyme Microb. Technol. 14 (1992) 426.
[18] M. Norin, J. Boutelje, E. Holmberg, K. Hult, Appl. Microbiol. Biotechnol. 28 (1988) 527.
[19] S.-W. Cho, J.S. Rhee, Biotechnol. Bioeng. 41 (1993) 204.
[20] M. Arroyo, J.M. Moreno, J.V. Sinisterra, J. Mol. Catal. 83 (1993) 261.
[21] Y. Kimura, A. Tanaka, K. Sonomoto, T. Nihira, S. Fukui, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 107.
[22] P.C. de Jesus, M.C. Rezende, M. da Graca Nascimento, Tetrahedron Asymmetry 6 (1995) 63.
[23] I. Uemasu, W.L. Hinze, Chirality 6 (1994) 649.
[24] K. Wannerberger, S. Welin-Klintström, T. Arnebrant, Langmuir 13 (1997) 784.
[25] D. Cowan, Trends Biotechnol. 14 (1996) 177.
[26] G. Pencreac'h, M. Leullier, J.C. Baratti, Biotechnol. Bioeng. 56 (1997) 181.
[27] K. Mosbach (Ed.), Immobilized Enzymes and Cells, Part B, C, D, Methods in Enzymology, Vol. 135–137, Academic Press, Orlando, San Diego, (1987–1988).
[28] A. Tanaka, T. Tosa, T. Kobayashi (Eds.), Industrial Application of Immobilized Biocatalysts, Bioprocess Technol., Vol. 16, Dekker, New York, 1993.
[29] J.F. Kennedy, E.H.M. Melo, K. Jumel, Chem. Eng. Prog. 7 (1990) 81.
[30] Y. Okahata, T. Mori, Trends Biotechnol. 15 (1997) 50.
[31] R.V. Parthasarathy, C.R. Martin, Nature (London) 369 (1994) 298.
[32] J. Lalonde, CHEMTECH 27 (2) (1997) 38.
[33] L.L. Hench, J.K. West, Chem. Rev. 90 (1990) 33.
[34] C.J. Brinker, G.W. Scherer, Sol–gel Science: The Physics and Chemistry of Sol–gel Processing, Academic Press, Boston, 1990.

[35] F.H. Dickey, J. Phys. Chem. 58 (1955) 695.
[36] P. Johnson, T.L. Whateley, J. Colloid Interface Sci. (1971) 557.
[37] M. Glad, O. Norrlöw, B. Sellergren, N. Siegbahn, K. Mosbach, J. Chromatogr. 347 (1985) 11.
[38] D. Avnir, S. Braun, O. Lev, M. Ottolenghi, Chem. Mater. 6 (1994) 1605.
[39] D. Avnir, Acc. Chem. Res. 28 (1995) 328.
[40] J. Livage, C.R. l'Academie Sci. Ser. IIb: Mec. Phys. Chim. Astron. 322 (1996) 417.
[41] S. Braun, S. Rappoport, R. Zusman, D. Avnir, M. Ottolenghi, Mater. Lett. 10 (1990) 1.
[42] S. Shtelzer, S. Rappoport, D. Avnir, M. Ottolenghi, S. Braun, Biotechnol. Appl. Biochem. 15 (1992) 227.
[43] S. Shtelzer, S. Braun, Biotechnol. Appl. Biochem. 19 (1994) 293.
[44] M.T. Reetz, J. Simpelkamp, A. Zonta, patent DE 44 08 152 A1, Germany, 1994.
[45] A. Zonta, Dissertation, Universität Bochum 1995.
[46] M.T. Reetz, A. Zonta, J. Simpelkamp, Angew. Chem. 107 (1995) 373.
[47] M.T. Reetz, A. Zonta, J. Simpelkamp, Angew. Chem. Int. Ed. Engl. 34 (1995) 301.
[48] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527.
[49] K. Kawakami, S. Yoshida, Biotechnol. Tech. 8 (1994) 441.
[50] S. Sato, T. Murakata, M. Ochifuji, M. Fukushima, T. Suzuki, J. Chem. Eng. Jpn. 27 (1994) 732.
[51] T. Antczak, J. Mrowiec-Bialon, S. Bielecki, A.B. Jarzebski, J.J. Malinowski, A.I. Lachowski, E. Galas, Biotechnol. Tech. 11 (1997) 9.
[52] L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, U. Menge, Nature (London) 343 (1990) 767.
[53] 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, L. Thim, Nature (London) 351 (1991) 491.
[54] F.K. Winkler, A. d'Arcy, W. Hunziker, Nature (London) 343 (1990) 771.
[55] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, K.H.G. Verschueren, A. Goldman, Protein Eng. 5 (1992) 197.
[56] H. van Tilbeurgh, L. Sarda, R. Verger, C. Cambillau, Nature (London) 359 (1992) 159.
[57] K.-E. Jaeger, S. Ransac, H.B. Koch, F. Ferrato, B.W. Dijkstra, FEBS Lett. 332 (1993) 143.
[58] M. Cygler, P. Grochulski, R.J. Kazlauskas, J.D. Schrag, F. Bouthillier, B. Rubin, A.N. Serreqi, A.K. Gupta, J. Am. Chem. Soc. 116 (1994) 3180.
[59] R.J. Kazlauskas, Trends Biotechnol. 12 (1994) 464.
[60] M.E.M. Noble, A. Claesby, L.N. Johnson, M.R. Egmont, L.G.J. Frenken, FEBS Lett. 331 (1993) 123.
[61] R. Verger, Trends Biotechnol. 15 (1997) 32.
[62] J.D. Schrag, Y. Li, M. Cygler, D. Lang, T. Burgdorf, H.-J. Hecht, R. Schmid, D. Schomburg, T.J. Rydel, J.D. Oliver, L.C. Strickland, C.M. Dunaway, S.B. Larson, J. Day, A. McPherson, Structure (London) 5 (1997) 187.
[63] K.K. Kim, H.K. Song, D.H. Shin, K.Y. Hwang, S.W. Suh, Structure (London) 5 (1997) 173.
[64] M.T. Reetz, A. Zonta, J. Simpelkamp, W. Könen, Chem. Commun., Cambridge, (1996) 1397.
[65] M.T. Reetz, Adv. Mater. 9 (1997) 943.
[66] Information sheet from Fluka, Switzerland, Reagent of the Year 1997.
[67] G. Kuncova, M. Guglielmi, P. Dubina, B. Safar, Collect Czech. Chem. Commun. 60 (1995) 1573.
[68] M. Heidt, U. Bornscheuer, R.D. Schmid, Biotechnol. Tech. 10 (1996) 25.
[69] K. Kawakami, S. Yoshida, J. Ferment. Bioeng. 82 (1996) 239.
[70] K. Kawakami, S. Yoshida, Biotechnol. Tech. 9 (1995) 701.
[71] K. Kawakami, S. Yoshida, J. Ferment. Bioeng. 82 (1996) 239.
[72] P.J. Robinson, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 15 (1973) 603.
[73] K. Takahashi, Y. Tamaura, Y. Kodera, T. Mihama, Y. Saito, Y. Inada, Biochem. Biophys. Res. Commun. 142 (1987) 291.
[74] M.D. Butterworth, S.P. Armes, A.W. Simpson, J. Chem. Soc., Chem. Commun. (1994) 2129.
[75] G.M. Whitesides, R.J. Kazlauskas, L. Josephson, Trends Biotechnol. 1 (1983) 144.
[76] P.J. Halling, P. Dunnill, Enzyme Microb. Technol. 2 (1980) 2.
[77] G. Kuncova, M. Sivel, J. Sol–gel Sci. Technol. 8 (1997) 667.
[78] H. Weller, Angew. Chem. 105 (1993) 43.
[79] H. Weller, Angew. Chem. Int. Ed. Engl. 38 (1993) 41.
[80] A. Henglein, J. Phys. Chem. 97 (1993) 5457.
[81] G. Nimtz, P. Marquard, H. Gleiter, J. Cryst. Growth 86 (1988) 66.
[82] H. Kobayashi, T. Matsunaga, J. Colloid Interface Sci. 141 (1991) 505.
[83] K.-E. Jaeger, K. Liebeton, A. Zonta, K. Schimossek, M.T. Reetz, Appl. Microbiol. Biotechnol. 46 (1996) 99.
[84] K.-E. Jaeger, B. Schneidinger, F. Rosenau, M. Werner, D. Lang, B.W. Dijkstra, K. Schimossek, A. Zonta, M.T. Reetz, J. Mol. Catal. B: Enzyme 3 (1997) 3.