POLYMERIC MATERIALS

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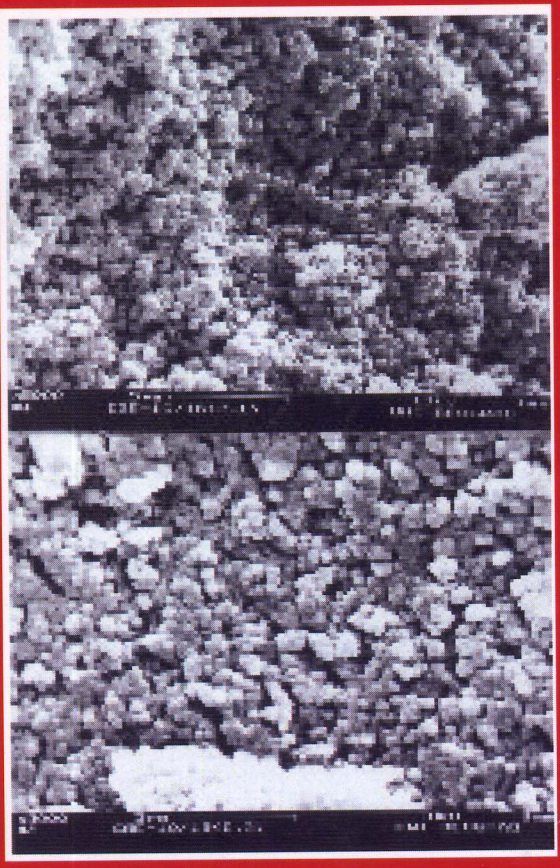

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6 Immobilization – A route for improving enzyme performance

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Abstract

Lipases are, due to their availability and promising characteristics, widely used in academic research and industry.

In nature lipases catalyze fat hydrolysis and show high catalytic activity, specificity and selectivity in their biological environment. However, under industrial conditions lipases often denature, because of the high temperatures, mechanical shear or presence of organic solvents used. Separation of enzymes from reaction solutions and their reusability are generally difficult as well.

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An important route to improve enzyme performance in non-natural environments is to immobilize them by either adsorption, covalent attachment or by incorporation in hydrophobic organic-inorganic hybrid materials with the help of a sol-gel process. These immobilization procedures have resulted in remarkable improvements in performance:

- increased enzyme activity (up to a factor of 100) in organic solvents.
- increased enantioselectivity - remarkable long-term stability.
- increased temperature stability.
- convenient recovery by filtration or centrifugation.

This review will describe the immobilization techniques used for the immobilization of lipases and will compare their results, advantages and disadvantages in general. Comparative studies between adsorption and covalent attachment will be discussed in more details.

1. Introduction

Enzymes are promising biocatalysts for many organic reactions. They have excellent features like high activity, specificity and selectivity, and can catalyze under mild and environmental friendly conditions. However, enzymes have been optimized, via natural evolution, to fulfill their biological function: to catalyze reactions in complex metabolic pathways exposed to many levels of regulation. Therefore they can catalyze reactions with an accuracy that cannot be obtained via conventional methods. However, most of the enzymes show none of their profound characteristics in organic solvents and can easily denature under industrial conditions. Recovery of enzymes from reaction solutions and separation of the enzymes from substrates and products are generally difficult. This all together causes the little use of biocatalysts in industry at that point.

An important route in improving enzyme performances in non-natural environments is to immobilize them. Immobilization is the localization of an enzyme and entails attaching or incorporating enzyme onto or into larger structures, including immobilization on surfaces, attachment within porous solids, or encapsulation in polymers, sol-gels, or composite materials or by crosslinking enzymes to crystals or aggregates. These procedures have resulted in considerable improvements in the activity of the enzymes, the stability towards high temperatures and organic solvents, long term stability, increased enantioselectivity, easy recovery and possibilities for reuse.

The success of the immobilized enzymes is closely related to the properties of the carrier material and the characteristics of the enzyme. The interactions between support and enzyme are critical for the activity of the enzyme. These interactions can change the enzyme three-dimensional

structure, which can either lead to inactivation or to hyper activation. Due to the growing improvements in the characterization and the elucidation of the reaction mechanism of enzymes, these interactions can become clearer and better understood. This can eventually help in the search for good supports for enzyme immobilization.

One class of enzymes that shows great improvements through immobilization and already have several industrial applications are lipases. Lipases are used by nature for fat hydrolysis and have therefore proven to be useful in laundry detergents and cleaning agents. Lipases are commercially available or can be isolated from lots of microorganisms. Due to the improvements in recombinant DNA technology completely pure enzyme, ready for use in organic chemistry can be obtained.

A lot of research has been already conducted on the immobilization of enzymes [1-3]. This review focuses on the immobilization of lipases, shows the results of different supports and outlines the promising features of immobilized lipases.

2. Lipases

Lipases are water-soluble enzymes, produced in nature by many microorganisms and higher eukaryotes (Table 1).

Table 1. Some examples of the lipases produced by microorganisms [4].

Source	Genus	Species
Bacteria	Bacillus	*B. megaterium*
		B. cereus
	Staphylococcus	*S. canosus*
		S. aereus
	Micrococcus	*M. luteus*
		M. fredenreichii
	Pseudomonas	*P. glumae*
		P. fluorescens
Fungi	Rhizopus	*Rhizop. Delemar*
		Rhizop. Oryzae
	Aspergillus	*A. flavus*
		A. niger
	Penicillium	*Pe. cyclopium*
		Pe. Camambertii
	Mucor	*Mucor miehei*
		Mu. Javanicus
Yeasts	Candida	*C. rugosa*
		C. antartica
	Pichia	*Pi. Bispora*
		Pi. Sivicola
Actinomycetes	Streptomyces	*Str. Cinnamomeus*
		Str. Coelicolor

Lipases catalyze the hydrolysis of triglycerides to the corresponding fatty acids and glycerol (Figure 1). Triglycerides are natural fats that contribute to the metabolic energy storage in humans. Since the water insoluble lipid interferes with the water soluble lipase, digestion of these triglycerides takes place at the water-oil interface. The surface area of the interface therefore determines the rate of the hydrolysis; more interface results in more interacting space for enzyme and triglycerides.

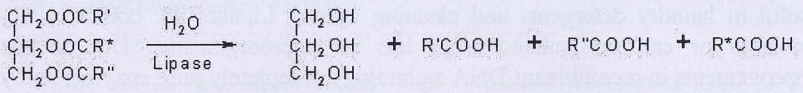

Figure 1. Lipase reaction.

For usage in chemistry lipases from microbial origin are commonly used and commercially available.

Applications of lipases

Biocatalysts, especially lipases, are increasingly used in organic chemistry due to their excellent properties. First of all, the availability of lipases makes them very attractive. Lipases can be obtained by isolation from several microorganisms (Table 2) and can nowadays be bought from several commercial suppliers. With recombinant DNA techniques lipases can even be obtained in very high purities, which make them ready for use in organic chemistry.

A second advantage of lipases is their high stability. Lots of lipases tolerate elevated temperatures and several organic solvents. For example high activities for the lipase from *Candida antarctica* at high temperature and in hydrophobic solvents have been reported [5-8].

Another very important property of lipases is the broad range of unnatural substrates that are accepted. Substrates different from the natural ones can be used, which increases the applicability of lipases [9].

Furthermore lipases catalyze in a regio- and stereoselective fashion. Lipases from different sources have different enantioselective properties and different substrate specificities. Therefore it is most of the time possible to choose the right enzyme for a specific synthesis from a large database of lipases.

A final advantage of lipases is the ability to catalyze several different reaction types. Ester hydrolysis, esterification, interesterification, polymerization and amidation reactions are reported in literature [2]. All these advantages together, the great variety of unnatural substrates, the different reaction types

that can be catalyzed, the stability at elevated temperatures and in organic solvents, their enantioselectivity and their availability make lipases very promising for use in organic chemistry.

Lipases find a lot of applications already for example as detergents, in food processing or in the synthesis of cosmetics, pharmaceuticals and chemicals [4]. However, to be added to a laundry detergent the lipase has to tolerate washing conditions (30-60°C), proteases and surfactants (which are ingredients of many detergents) and they need to have a low substrate specificity in order to remove fats of different compositions [4]. In food processing among other things lipases have been used to replace undesirable fat with a high-value fat. For example, Undurraga *et al.* have used Novo-Nordisks lipozyme™ for the transesterification reaction which replaces the palmitic acid in palm oil with stearic acid to produce a cocao butter substitute [10]. The cosmetic, pharmaceutical and chemical industry use lipases among other things as catalysts to make enantioselective products.

Structure and the function of lipases

Lipases, like almost all enzymes, are generally globular proteins. Most lipases are usually very large in comparison with substrate they act on, and only a small portion of the enzyme (around 3-4 amino acids) is near or in direct contact with substrate and directly involved in the catalysis. The part of enzyme that contains these catalytics residues, on which the substrate attaches, and then chemical change occurs is known as the active site (in case of 3 amino acids involved, the active site is called catalytic triad). Like all proteins and enzymes, lipases are long, linear chains of amino acids that fold to produce a three-dimensional product. Most of the lipases have the lid, i.e. a surface helix covering the active site of the lipase. In order to perform full catalytic activity, lipases also need additional non-protein components called procolipases.

The structure and function of lipases have been thoroughly studied. Already in 1990 Winkler *et al.* [11] elucidated the structure of the human *pancreatic* lipase. Three years later van Tilbeurgh *et al.* [12] revealed the mechanism involved in the interfacial activation (Figure 2).

Namely, during the catalysis the lid adopts a totally different conformation which allows the substrate to bind to the enzyme's active site.

First of all, Trp 252 (tryptophan) moves from the active site pocket to the surface of the molecule, which clears the active site pocket, allowing the substrate to enter.

Secondly, a second loop (β5-loop, red in Figure 2), which makes van der Waals contacts with the closed lid, folds back upon the core of the protein when the lid is opened.

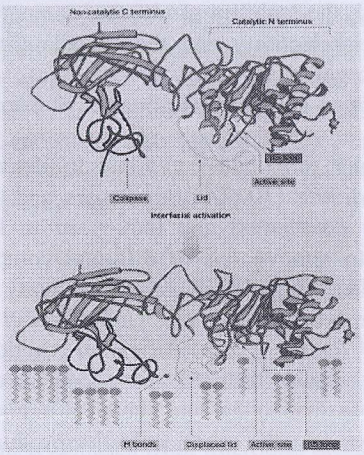

Figure 2. Mechanism of interfacial activation of triacylglyecrol lipase [6].

Another consequence of the structural rearrangements is the change in hydrophobicity around the active site. The lid and the β5-loop both fold away from the active site, which is stabilized by many hydrogen bonds with the exterior of the molecule. As a result, hydrophobic side chains become exposed, whereas hydrophilic ones are buried, and therefore the hydrophobicity around the active site is considerably increased. A major contributor to this increase is the hydrophobic site of the amphiphatic main helix of the lid.

As a further consequence, interaction of the catalytic N-terminal domain with procolipase via the open lid occurs and is stabilized by hydrogen bonds and salt bridges. Through this binding three isoleucines are exposed, which is important for the function of the procolipase.

3. Immobilization

Enzyme immobilization is the localization of an enzyme, i.e. restricting the enzyme mobility. The first intentionally prepared immobilized enzymes were already reported in the 1950s [13] and since then a lot of research has been conducted.

The main advantage of immobilization is the ease with which the enzyme can be recovered after the reaction. This also enables reuse of the enzyme afterwards in another reaction. Another advantage can be the increased stability of the enzyme. After immobilization some enzymes show an increased temperature resistance and an increased tolerance towards organic solvents [14, 15].

There are different ways to establish immobilization:

i. Physical adsorption: the enzyme is bound to a support by hydrophobic, van der Waals or ionic interactions.
ii. Covalent binding: the enzyme is covalently bound to a support.
iii. Entrapment via inclusion: the enzyme is trapped in a polymer network or a membrane device, like for instance a microcapsule.
iv. Crosslinking of enzyme aggregates: the enzyme molecules are cross-linked by a bifunctional reactant to create carrierless macroparticles.

3.1. Properties of immobilized enzymes

The properties of immobilized enzymes are determined by the properties of the enzyme and of the support. This is schematically represented by figure 3.

The biochemical properties of the enzyme, like molecular mass, functional groups on the surface and the purity of the enzyme are important factors for the immobilization. The functional groups on the surface of the enzyme for instance give information about which kind of interactions between the support and the enzyme can take place. Also the purity of the enzyme is important, as if for instance an impure enzyme is used the impurities can interfere with the substrates.

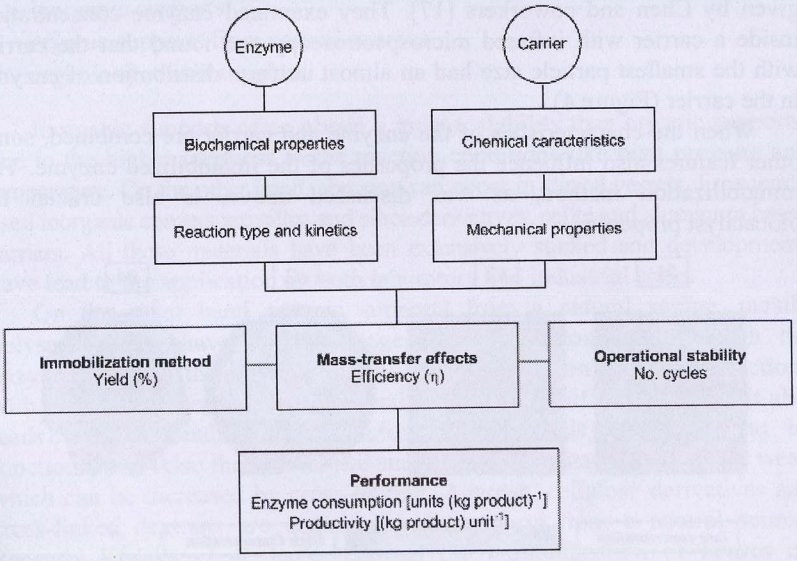

Figure 3. Properties of enzyme and carrier determine the properties of the immobilized enzyme [16].

Other features of the enzyme that determine the parameters of the immobilized enzyme are the reaction type and the kinetics of the reaction that is catalyzed by the enzyme. Specific activities, kinetic parameters for activation and inhibition and stability against pH, temperature, solvents and impurities have impact on the immobilized enzyme as well.

The characteristics of the carrier material also influence the properties of the immobilized enzyme. One of the most important features of the carrier is the chemical structure that will determine interaction with enzyme. If the support material is highly porous, pore size and pore size distribution will play an important role in determining the immobilized enzyme properties. A small pore sizes can cause diffusion limitation and thereupon enzymes get structurally rearranged and becomes inactive. However, in a very large pore sizes enzymes can cluster together and as well lose some of their activity.

The mechanical properties of the carrier material are crucial in the application of the immobilized enzyme. When an immobilized enzyme is applied in a stirred tank, it has to have other properties than when it is applied in a column. In a stirred tank the carrier material must be resistant to abrasion, while in a column it has to have some flow resistance.

Carrier particle size is momentous as well. An example of the influence of particle size on the distribution of enzyme throughout the carrier bead is given by Chen and coworkers [17]. They examined enzyme concentration inside a carrier with infrared microspectroscopy and found that the carrier with the smallest particle size had an almost uniform distribution of enzyme in the carrier (Figure 4).

When the characteristics of the enzyme and carrier are combined, some other features also influence the properties of the immobilized enzyme. The immobilization method, as was discussed above, is also crucial for biocatalyst properties.

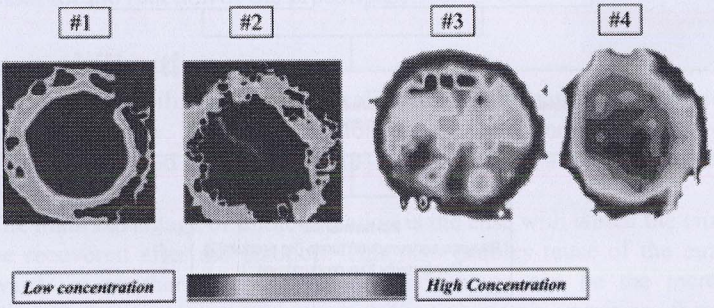

Figure 4. Infrared microspectroscopy images of a series of MMA resins with different particle sizes (diameters: #1=600μm, #2=120μm, #3=75μm, #4=35μm) [17].

The rate of the enzyme-catalyzed reaction is greatly influenced by mass-transfer effects. Due to mass transfer to, from an inside the immobilized enzyme, micro and nano environments occur with different pH, concentration etc. This effect arises from the fact that an immobilized enzyme is bound to the support and has a deliberate restricted mobility. It can affect the mobility of the solutes as well. The solutes can be adsorbed to the support, resulting in a reduced mobility which causes a decreased reaction rate compared with soluble enzymes [13]. Not only this reduced mobility of the solutes cause a reduced reaction rate, but also diffusion restrictions on the surface carrier or effects from internal or porous diffusion can lower the reaction rate [16].

3.2. Supports

Two of the above mentioned immobilization techniques require a support, physical adsorption and covalent binding. These two are also the most commonly used immobilization techniques. As can be seen from figure 4 the properties of an enzyme immobilized on a carrier greatly depend on the properties of the support. A support is therefore important and should be chosen with care.

Frequently used carriers are categorized in:

- inorganic supports
- organic supports from natural sources
- organic synthetic supports

Inorganic supports often obtain a greater stability than organic supports, due to the higher inertness to the reaction conditions like high pressure and temperature. On the other hand, abrasion can occur in stirred vessels. Frequently used inorganic carriers are silica and silica derivatives, celite and aluminum based carriers. All these materials have been extensively studied and developments have lead to the application on both laboratory and industrial scale.

On the other hand organic supports from a natural source, mostly polysaccharides, have the advantage of a great compatibility with the enzymes. Due to their hydrophilicity, they undergo only weak interactions with the enzyme, leading to a minimal inactivation, but unfortunately it also leads to poor binding and therefore the materials often have to be functionalized. Also the mechanical stability of these materials is rather weak which can be increased by cross-linking. Agarose, cellulose derivatives and cross-linked dextrans are common used supports from a natural source. Recently Kosaka *et al.* have reported the immobilization of lipases on ultrathin films of cellulose esters. They claim that the immobilized lipases show a higher activity than the free lipases [18].

Organic synthetic supports, as for instance synthetic polymers, are widely used as immobilization carriers. Ion exchange materials, frequently based on polystyrene that can be cross-linked with divinylbenzene, have been used for enzyme immobilization. By adding various amounts of divinylbenzene different network densities can be obtained.

Furthermore, several copolymers are used in enzyme immobilization. Changing the ratio between the two polymers, the properties of the copolymer (hydrophilicity/hydrophobicity, amount of functional groups, mechanical properties, porosity etc.) can be desirable changed. Therefore custom-made carriers for enzyme immobilization for all kinds of different purposes can be produced.

3.3. Kinetics of immobilized enzymes

Most of the enzyme mediated reactions follow a kinetic model called Michalies-Menten kinetics. This kinetic model is relevant to situations where the concentration of the enzyme is much lower than the concentration of the substrate (i.e. where enzyme concentration is the limiting factor). It is established that enzymes form a bound complex to their reactants (i.e. substrates) during the course of their catalysis and prior to the release of products. The model describes enzyme reactions with one or more substrates. For most of the enzymes, two-substrates reactions can be applied (Figure 5).

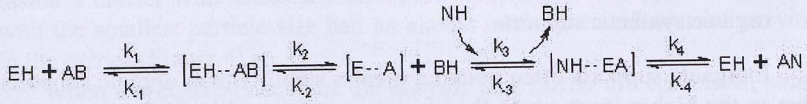

Figure 5. Enzymatic two substrate reaction. In this reaction EH is the enzyme (H is depicted because proton transfer often occurs), AB and NH are substrates.

The rate of product formation is given by equation 2.1.

$$V = \frac{d[AN]}{dt} = k_4[EA\ NH] \tag{2.1}$$

The concentration of [NH--EA] can be expressed in the known concentrations of $[EH]_0$, $[AB]_0$ and $[NH]_0$, when a turnover number and Michaelis-Menten constant are defined as in equation 2.2 and 2.3.

$$turnover\ number = k_{cat,AB} = \frac{k_2 k_3 k_4 [NH]_0}{k_3(k_4 + k_2)[NH]_0 + k_2 k_4} \tag{2.2}$$

$$Michaelis-Menten\ const. = K_m = \frac{(k_{-1}+k_2)k_3k_4[NH]_0}{(k_3(k_4+k_2)[NH]_0+k_2k_4)k_1} \quad (2.3)$$

Then equation 2.4 can give an expression for the rate of product formation in the form of a Michaelis-Menten equation for two-substrate reactions.

$$V = \frac{k_{cat,AB}[AB]_0[EH]_0}{(K_{m,AB}+[AB]_0)} \quad (2.4)$$

Equation 2.4 was first derived in 1913 by Leonor Michaelis and Maud Menten in order to determine the general rate of an enzyme catalyzed reaction. The Michaelis-Menten constant is the substrate concentration at which 50% of the maximal velocity, V_{max}, is reached. The turnover number is the number of moles converted per second by one enzyme molecule under substrate saturation conditions [13]. Those two parameters describe the entire system and can be determined from the enzyme saturation curve (Figure 6).

Kinetics change for immobilized enzymes because the rate of product formation is now limited by the maximum rate of substrate transfer to the particles and the biocatalyst content in the support. To account for this an efficiency coefficient or effectiveness factor, η, is defined (equation 2.5).

$$\eta = \frac{v_{IMM.}}{v_f} \quad (2.5)$$

In this equations, v_{IMM} is the rate of product formation in the immobilized enzyme catalyzed reaction and v_f that of the free enzyme reaction.

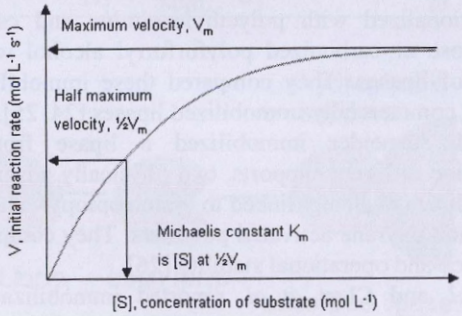

Figure 6. Saturation curve for an enzyme showing the relation between the concentration of substrate and rate.

4. Methods of immobilization

4.1. Physical adsorption

Physical adsorption is a simple and straightforward route for enzyme immobilization, in which the enzyme is bound to a support by hydrophobic, van der Waals or ionic interactions. It is often used because of the ease and low cost of the procedure. A support is added to an enzyme solution and after a few hours of mixing the enzyme-support complex is ready. Secondly, physical adsorption is reversible that enables the reuse of the support. Denatured enzymes can be removed from the support by changes in pH or ionic strength of the reaction medium and it can be replaced with fresh enzyme. However, a drawback is the leaking of enzyme upon use, so not all of the enzyme can be reused and the product might have to be purified.

Several different supports were used for the physical adsorption of lipases. Polymers [17, 19, 20], carbon nanofibers [24, 25], celite and silica [30] have been reported and are compared in this study. Due to the interfacial activation, that most lipases show, caused by hydrophobic interactions of the lid (exceptions are lipases form *Pseudomonas glumae*, *Psuedomonas aeruginosa* and *Candida antartica* lipase B, because of the smaller or missing lid [21]) physical adsorption to hydrophobic surfaces is promising.

Guisan and Fernandez-Lafuente *et al.* reported several comparison studies in which they compare interfacial adsorbed lipases with covalently immobilized lipases, multipoint covalently linked lipases, soluble lipases and soluble lipases with droplets of insoluble substrates. They show that interfacial adsorption is a one step route for obtaining, purification, stabilization and hyperactivation of lipases [14, 15, 19, 22, 23].

Lathouder *et al.* reported a whole different strategy. They immobilize lipases on carbon nanofibers amongst different types of carbon. Cordierite monoliths functionalized with polyethyleneimine and carbon nanofibers, carbonized sucrose or carbonized polyfurfurryl alcohol were used for the immobilization of lipases. They compared these immobilized lipases with free enzyme and commercially immobilized lipases [24, 25].

Ivanov and Schneider immobilized a lipase from *Pseudomonas fluorescens* on five different supports, two physically adsorbed to celite and alkyl-silica and three covalently linked to γ-aminopropyl- and gluteraldehyde-activated silica and oxyrane activated polymers. They compare the results on activity, efficiency and operational stability [26].

Santos *et al.* and Chen *et al.* reported immobilization by physical adsorption on different polymers. Poly(N-methylolacrylamide) was investigated by Santos *et al.* [20]. They compared the free enzyme with immobilized derivatives in terms of pH, temperature and thermal stability.

Furthermore they studied the effects of temperature, biocatalyst concentration and the ratio between the two substrates to obtain the optimal reaction conditions. Chen *et al.* studied the influence of particle size of a poly(methylmethacrylate) resin on the enzyme loading and catalytic properties [17].

Different supports have been used for the immobilization of lipases via physical adsorption: celite, octyl-silica, carbonized sucrose, poly(furyl alcohol), carbon nano fibers, methyl methacrylate (Amberchrom CG 71S) and octadecyl sepabeads. All of these materials have different interactions with lipases. The results of these materials as immobilization supports are shown in Table 2.

Although it is difficult to compare these results, because of the differences in reactions, reaction conditions, enzymes used etc., one can conclude that the octadecyl sepabeads show an extremely high catalytic activity, with a low enzyme loading. The authors show that such high activity is caused by the hyperactivation of the lipase due to interfacial adsorption to hydrophobic surfaces (Figure 7).

Table 2. Properties of physically adsorbed enzymes.

Support	Enzyme	Enzyme Loading (μg/mg)	Relative Activity	Reference
Celite	PFL		7.58	[26]
Octyl-silica	PFL		1.30	[26]
Carbonized sucrose	CAL	180.0	0.27	[24]
Carbonized poly(furryl alcohol)	CAL	50.0	0.24	[24]
Carbon nano fiber	CAL	10.0	0.27	[24]
Amberchrom CG 71S	CAL B	10.0		[17]
Octadecyl sepabeads	CAL B		2	[22]
Octadecyl sepabeads	CRL		4	[22]
Octadecyl sepabeads	MML		20	[22]

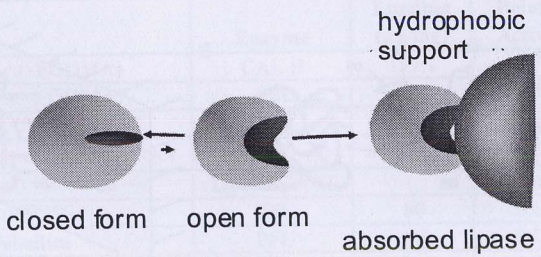

Figure 7. Interfacial adsorption [14].

According to the authors the closed form of the lipase is favored in solution, but when the lipase comes near a hydrophobic surface the lid opens because of favorable interactions and the enzyme remains in this open formation (due to the equilibrium) that is responsible for an increase in enzyme activity.

4.2. Covalent attachment

Covalent attachment is the second most widely used method of enzyme immobilization, in which the enzyme is covalently bound to a support. Covalent binding does not suffer from desorption or leaching of enzyme during catalytic reactions, due to the firm bondages between enzyme and support. On the other hand, enhancement of the bond strength between the enzyme and the support can cause changes in the enzyme conformation, often into a less favorable one, which can result in the deactivation of the enzyme [27]. In immobilization through covalent attachment two different global methods can be outlined: covalent attachment via long spacer arms or covalent attachment via multipoint attachments (Figure 8). The spacer arm has the advantage of only moderately restricting the enzymes configuration, while the multipoint attachment is supposed to have a higher stability. The multipoint attachment method is often created by crosslinking with gluteraldehyde [28].

Covalent immobilization was reported by several groups [26, 27, 28, 29, 30, 31, 32]. Different supports are used for immobilization and most of them are activated carriers with a high content of reactive groups, like epoxy rings or activated carbonyl groups, ready to bind mainly to amine groups of the enzyme.

Charusheela and Arvind [31] used reversibly soluble polymers for the immobilization. Lipase was immobilized on two polymers Eudragit S100, a copolymer of methylmethacrylate and methacrylic acid, and carboxymethyl cellulose using the carbodiimide coupling method respectively. They show that

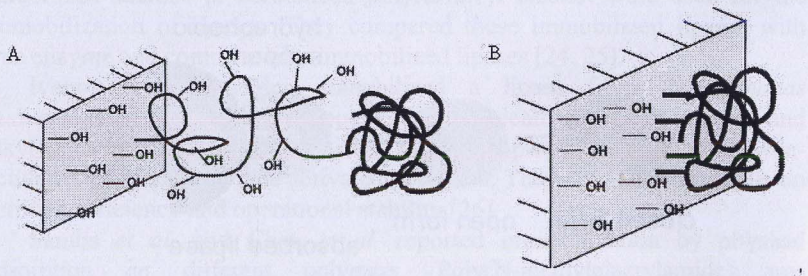

Figure 8. Immobilization through covalent attachment: (A) via long spacer arm, (B) via multipoint attachment.

reversibly soluble polymers keep the advantage of immobilization, namely the recovery and reuse of the enzyme, but it also opens up the possibility of performing the reaction in solution, thereby putting aside the problems from solid-liquid mass transfer and pore diffusion.

Dyal *et al.* [30] used γ-Fe_2O_3 magnetic nanoparticles as a support for the covalent immobilization of *Candida rugosa* lipase. The advantage of magnetic particles is the easy separation, simply by applying a magnetic field and furthermore the small size of the particles enables application in biological systems.

Warmuth *et al.* [28] used a combination of physical adsorption and multipoint covalent linking. They first physically adsorbed the lipase on a hydrophobic support by interfacial separation, followed by crosslinking with gluteraldehyde to obtain multipoint covalent attachment. Bertucci and coworkers [32] used a similar technique to immobilize lipases on a epoxy silica HPLC column. They compared their results with results obtained for the free enzyme under the same reaction conditions.

Most of the supports used for covalent attachment are activated supports, with functional groups on the surface suitable to bind the enzyme covalently. The supports reviewed here are presented in Table 3.

As can be seen, the relative activities with covalent attachment are quite low. This can be explained by the fact that covalent bondages between enzyme and support cause restricting chain mobility within protein molecules and therefore conformational changes that are essential during catalysis are disabled.

However, despite this rather low relative activity, immobilized enzymes with an activity approximately the same as the free enzyme have the potential to be used in industry. The costs saved by reusing and recovery of the enzymes can compensate for the loss of activity.

Table 3. Activity of enzymes immobilized via covalent attachment.

Support	Enzyme	Loading (μg/mg)	Relative Activity	Reference
Poly(GMA-*co*-EGDMA)	CAL B	157.1	2.10	[29]
Poly(GMA-*co*-EGDMA)	ABL		1.32	[33]
Eupergit 250L	SCL		0.08	[28]
γ-Fe_2O_3 magnetic nanoparticles	CRL	55.6	0.01	[30]
Eudragit S100	Lipolase 100T		4.46	[31]
Eupergit 250L	PFL	80	0.58	[26]
Aminopropyl-silica	PFL	80	0.03	[26]
Glutaraldehyde-activated silica	PFL	80	0.02	[26]
Glyoxyl-agarose	CAL B		0.90	[22]
Glutaraldehyde-agarose	CAL B		0.85	[22]

An improved stereospecificity can be the reason for using covalently immobilized enzymes as well. Fernandez-Lorente and coworkers reported that the lipase from *Pseudomonas fluorescens* shows an increased enantioselectivity upon multipoint covalent immobilization on glyoxyl-agarose. They reported a three-fold improvement in the enantioselectivity. According to the authors this increase is probably due to a distortion of the enzyme structure, since the activity was decreased, or/and due to an increase in rigidity of the enzyme, since the enzyme showed an increased stability [34].

The influence of the resin properties on the loading of *Candida antarctica* lipase B (Cal-B) during immobilization and on the hydrolytic (hydrolysis of para-nitrophenyl acetate) and synthetic (ring-opening polymerization of ε-caprolactone) activity of the immobilized Cal-B were studied by Miletić *et al* [29]. Crosslinked macroporous hydrophilic poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate)s [abbreviated poly(GMA-*co*-EGDMA)] with identical chemical structure (60 % of glycidyl methacrylate) but with varied average pore sizes and particle sizes were synthesized via suspension polymerization. The authors showed that about 80% of immobilized Cal-B was covalently attached to the macroporous resins via reaction between amino group of enzyme and epoxy group of copolymer. Table 4 shows the effect of these parameters on the enzyme loading and activity.

Increasing hydrolytic activity can be observed when resins of smaller particle sizes are used for catalysis which is due to the reduced hindrance of substrate and product diffusion in the matrix. On the other hand, increasing the pore size of the beads resulted in a large increase in the hydrolytic and synthetic activity. According to the authors this can be explained with the facilitated substrate and product diffusion to enzyme regions.

Another feature that is well illustrated by the results from Ivanov *et al.* is the influence of the carrier. Three chemically different supports were used for

Table 4. Effect of particle size and pore size on enzyme loading and activity [29].

Resin	Particle Size (um)	Average Pore Diameter (nm)	Enzyme Loading (ug/mg)	Activity (nmol/min* mg)
Poly(GMA-*co*-EGDMA)	630-300	30	222.7	1065.4
Poly(GMA-*co*-EGDMA)	630-300	92	178.2	1928.4
Poly(GMA-*co*-EGDMA)	630-300	30	184.8	1801.8
Poly(GMA-*co*-EGDMA)	300-150	87	220.8	2125.0
Poly(GMA-*co*-EGDMA)	300-150	270	207.8	2775.0
Poly(GMA-*co*-EGDMA)	300-150	560	172.1	2875.6
Poly(GMA-*co*-EGDMA)	150-100	59	166.9	4534.9
Poly(GMA-*co*-EGDMA)	<100	48	157.1	5027.2

the immobilization of the same enzyme. Immobilized biocatalysts had the same crude enzyme loading and were used under the same reaction conditions, but showed different relative activity. A 19-fold increase in activity of Eupergit® C250L, a copolymer of methacrylamide, N,N'-methylen-bis(acrylamide) and a monomer carrying oxirane groups, compared to aminopropyl-silica is reported [26]. The enzyme is coupled to the Eupergit® 250L through the free oxirane groups. The increase in activity is probably caused by differences in interactions between support and enzyme, but can also be the result of different particle or pore sizes.

4.3. Entrapment via inclusion

Another possibility of enzyme immobilization is entrapment via inclusion, meaning that the enzyme is entrapped in a polymer network or a membrane device. Many researchers started with inclusion in polymeric matrices [35], but more recently inclusion in hydrophobic sol-gel materials [36-39] and entrapment in hollow fibers [41] has been reported. In this immobilization method the enzyme is retained within a device or material. It does not completely prevent leaking, but it considerably decreases it with regard to physical adsorption.

Most literature found on immobilization by inclusion is on sol-gel materials. This technique is based on the production of silica matrices by acid or base catalyzed hydrolysis of silane compounds like tetraethoxysilane (TEOS).

Vidinha and coworkers used sol-gel matrices, prepared with a combination of alkyl-alkoxysilane precursors of different chain-lengths, for the immobilization of cutinase from *Fusarium solani pisi*. They reported several additives that improved the catalytic activity of cutinase as well as of lipase from *Pseudomonas cepacia* [39].

In 2003 Kuncova *et al.* reported a series of immobilized lipases in sol-gel materials, using silica prepolymers prepared form tetramethoxysilane, methyltrimethoxysilane, propyltrimethoxysilane and (3-aminopropyl) triethoxysilane. They measured the activities and compared them with lipases adsorbed on a carrier, lipases encapsulated in silicone rubber, lipases entrapped in nanoporous silica matrix and commercial sol-gel lipases. Influence of the precursor composition on the conversion of the substrate was also examined [40].

Reetz *et al.* have established a method that enables the immobilization of lipases via inclusion in sol-gel materials (Table 5). They studied the effects that several parameters (water content, type and amount of catalyst, variation of the precursor etc.) have on the stability and enzyme activity. They have examined several lipases and several sol-gel materials with and without additives [36, 38].

Table 5. Activity of sol-gel encapsulated enzymes.

Support	Enzyme	Enzyme Loading	Additive	Relative Activity	Reference
TMOS/PTMS (1:5)	ANL	91%	none	16.4	[36]
TMOS/PTMS (1:5)	PRL	28%	none	8.8	[36]
TMOS/PTMS (1:5)	SP 523 (novo)	76%	none	87.6	[36]
BTMS	BcL		none	55.7	[38]
BTMS	BcL		Celite ® / methyl-β-CD	140	[38]
BTMS	TIL		none	617	[38]
BTMS	TIL		Celite ® / Tween 80 ®	1391	[38]

As can be seen from these results, the additives Celite®, methyl-β-CD and Tween 80® have a significant effect on the activity of the enzyme. Possible explanations for this increase could be improved interactions between the enzyme and the matrix.

Enantioselectivity studies were also performed and are shown in Table 6. It becomes obvious that the enantioselectivity of *Candida antarctica* lipase B showed a significant increase upon immobilization in sol-gel materials. The enantioselectivity of Bc lipase remains unchanged upon immobilization and additives and the enantioselectivity of Pf lipase shows a slight increase.

Table 6. Enantioselectivity properties of entrapped enzymes in a polymer network [38].

Support	Enzyme	Additive	Enantioselectivity
	CAL B	-	70.1
TMOS/BTMS	CAL B	-	> 100
TMOS/BTMS	CAL B	18-crown-6	> 100
	B cL	-	4.4
TMOS/BTMS	B cL	-	4.1
TMOS/BTMS	B cL	18-crown-6	4.0
TMOS/BTMS	B cL	Tween 80	4.5
	P fL	-	2.2
TMOS/BTMS	P fL	-	3.3
	P fL	18-crown-6	6.0

4.4. Crosslinking of enzyme aggregates

Immobilization by cross-linked enzyme crystals (CLECs) or cross-linked enzyme aggregates (CLEAs) is a final method of immobilization. CLECs are microcrystals that can be grown from an aqueous solution and can be cross-linked with a bifunctional agent (Figure 9). CLECs are macroporous and contain about 50% solvent by volume. The channels allow diffusion of product, substrate and solvent in, through and from the crystals [42].

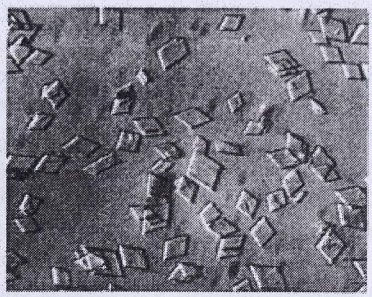

Figure 9. Optical microscope photograph cross-linked *C. rugosa* lipase crystals (CRL-CLEC's) (250x magnification (reproduced at 50% of original size)) [42].

CLEAs can be synthesized more easily than CLECs. Therefore CLEAs are possible alternatives to CLECs, since both can be prepared by precipitating the enzymes and subsequent cross-linking with for instance gluteraldehyde. CLEAs of *penicillin acylase* were reported by Cao *et al.* to give comparable activities as CLECs and showed furthermore a greater tolerance for organic solvents [43].

CLECs of lipases are reported by Lalonde and coworkers. Enantioselectivity of a crude, commercial available lipase with a highly purified enzyme crystal was compared. They attribute the increased enantioselectivity of the CLEC to the purity of the enzyme crystals in comparison to the crude enzyme preparation. The effect of substrate concentration on the hydrolysis rate was also tested [42].

Overbeeke *et al.* used the lipase from *Candida rugosa* for their experiments to see the influence of CLEC's purity and open and closed conformation of the lid on the enantioselectivity. According to the authors, changes in the active site are coupled to changes in the opening and closing of the lid [44].

Cross-linked enzyme aggregates have been reported by Schoevaart and coworkers. They prepared CLEAs of several different enzymes, like laccases, phytases, oxidases and also lipases (*Candida antartica* lipase A and B, *Thermomyces lanuginosus* and *Rhizomucor miehei*). Activity of the enzyme preparations, precipated in different non-solvents, with the activity of the enzyme preparations after cross-linking was compared [45].

Lopez-Serrano *et al.* have examined the catalytic activity of CLEAs of different lipases (*Candida antarctica* lipase A and B, *Thermomyces lanuginosus* lipase, *Rhizomucor miehei* lipase and *Aspergillus niger* lipase). They reported the effect that additives, like sodium dodecyl sulfate, Triton X-100 or dibenzo-18-

crown-6, have on the catalytic activity of the CLEA. The authors have also observed a higher activity of the CLEA than the free enzyme [46].

Lalonde *et al.* have examined cross-linked enzyme crystals with different substrates. A part of the results, including the conversion and the enantioselectivity of the CLECs, are listed in Table 7.

From this table it can be concluded that the activity of the crude enzyme preparation and the CLEC both depend on the substrate. The conversion of both enzyme preparations are comparable. The major difference can be found in the enantioselectivity as all CLECs show an improved (or the same) enantioselectivity. In some cases this increase is enormous. The authors attribute this improvement to the increase in purity between the crude enzyme preparation and the CLEC.

Lalonde *et al.* have also examined the thermostability in water-miscible organic solvents of crude and pure enzyme and CRL-CLECs at 40 °C (Figure 10). The catalytic activity of the CLEC shows an enormous increase in thermostability in comparison with the crude and free enzyme preparations. The stability of CLECs in 50% organic solvent solutions displays an amazing increase with regard to the crude enzyme preparation. The authors attribute these effects to a combination of crystallinity in the material and covalent cross-linking between the molecules. Due to the crystallinity the enzymes are closely packed, resulting in many protein-protein interactions, which can stabilize the enzyme crystals against unfolding, aggregation or dissociation.

Table 7. Conversion and enantioselectivity obtained by Lalonde *et al.* [42].

Species	Enzyme	Substrate	Conversion	Eapp
crude enzyme	CRL	chloroethylester of Ketoprofen	36.7	5.2
CLEC	CRL	chloroethylester of Ketoprofen	49.3	64
crude enzyme	CRL	N aproxen	46.8	12
CLEC	CRL	N aproxen	39	>100
crude enzyme	CRL	chloroethylester of methylphenylacetic acid	70.4	3.7
CLEC	CRL	chloroethylester of methylphenylacetic acid	48	>100
crude enzyme	CRL	ethylester of hydroxyhexanoic acid	63.2	10.4
CLEC	CRL	ethylester of hydroxyhexanoic acid	60	15.2
crude enzyme	CRL	acetoxycyclopentenol	84.3	1
CLEC	CRL	acetoxycyclopentenol	90.5	1

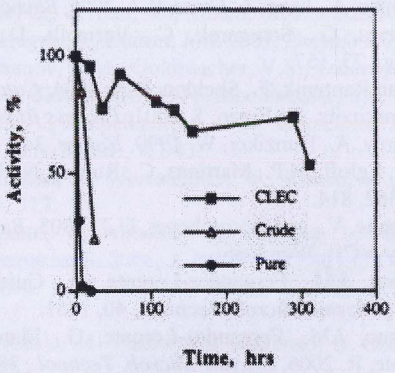

Figure 10. Thermostability of crude and pure enzyme preparations and CRL-CLECs at 40°C [42].

5. Summary

Immobilization of enzymes by adsorption, covalent attachment, entrapment or crosslinking, can significantly improve the enzymes performance. Immobilized enzymes show much higher activity and higher stability than crude enzymes.

Immobilizing enzymes by physical adsorption results in enhanced activity and stability. On the other hand, biocatalysts will suffer from leaching and therefore reusability is limited.

Covalently attached enzymes are much more stable, as the covalent binding prevents leaching and enables reusability. However a quite low activity can be observed due to changes in the enzymes tertiary structure and/or mobility.

From the reported work it becomes obvious that enzyme immobilization is an excellent tool to enhance the activity, reusability and even the enantioselectivity of biocatalysts. However it is also clear at this point that there are no general immobilization techniques and/or supports which work for a wide range of enzymes.

References

1. Krajewska, B. 2004, *Enzyme Microb. Technol.*, 35, 126.
2. Gross, R.A., Kumar, A., Kalra B. 2001, *Chem. Rev.*, 101, 2097.
3. Castro, G.R., Knubovets, T. 2003, *Crit. Rev. Biotechnol.*, 23, 195-231.
4. Sharma, R., Chisti, Y., Banerjee, U.C. 2001, *Biotechnol. Adv.*, 19, 627.
5. Rejasse, B., Lamare, S., Legoy, M.D., Besso, T. 2004, *Org. Biomol. Chem.*, 2, 1086.
6. Ong, A.L., Kamaruddin, A.H., Bhatia, S., Long, W.S., Lim, S.T., Kumari, R. 2006, *Enzyme Microb. Technol.*, 39, 924.

7. Azim, H., Dekhterman, A., Jiang, Z., Gross, R.A. 2006, *Biomacromolecules*, 7, 3093.
8. Secudno, F., Carrea, G., Soregaroli, C., Varinelli, D., Morrone, R. 2001, *Biotechnol. Bioeng.*, 73, 157.
9. de Zoete, M.C., van Rantwijk, F., Sheldon, R.A. 1994, *Catal. Today*, 22, 563.
10. Undurraga, D., Markovits, A., Erazo, S. 2001, *Process Biochem.*, 36, 933.
11. Winkler, F.K., Darcy, A., Hunziker, W. 1990, *Nature*, 343, 771.
12. van Tilbeurgh, H., Egloff, M.P., Martinez, C., Rugani, N., Verger, R., Cambillau, C. 1993, *Nature*, 362, 814.
13. Buchholz, K., Kasche, V., and Bornscheuer, U.T. 2005, *Biocatalysts and Enzyme Technology*, Wiley-VCH, Weinheim.
14. Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R. 2007, *Enzyme Microb. Technol.*, 40, 1451.
15. Wilson, L., Palomo, J.M., Fernandez-Lorente, G., Illanes, A., Guisan, J.M. Fernandez-Lafuente, R. 2006, *Enzyme Microb. Technol.*, 38, 975.
16. Tischer, W., Kasche, V. 1999, *Trends Biotechnol.*, 17, 326.
17. Chen, B., Miller, E.M., Miller, L., Maikner, J.J., Gross, R.A. 2007, *Langmuir*, 23, 1381.
18. Kosaka, P.M., Kawano, Y., El Seoud, O.A., Petri, D.F.S. 2007, *Langmuir*, 23, 12167.
19. Segura, R.L., Palomo, J.M., Mateo, C., Cortes, A., Terreni, M., Fernandez-Lafuente, R., Guisan, J.M. 2004, *Biotechnol. Progr.*, 20, 825.
20. Santos, J.C., Nunes, G.F.M., Moreira, A.B.R., Perez, V.H., de Castro, H.F. 2007, *Chem. Eng. Technol.*, 30, 1255.
21. Bornscheuer, U.T., Bessler, C., Srinivas, R., Krishna, S.H. 2002, *Trends Biotechnol.*, 20, 433.
22. Palomo, J.M., Munoz, G., Fernandez-Lorente, G. Mateo, C., Fernandez-Lafuente, R., Guisan, J.M. 2002, *J. Mol. Catal. B: Enzym.*, 19-20, 279.
23. Bastida, A., Sabuquillo, P., Armisen, P., Fernandez-Lafuente, R., Huguet, J., Guisan, J.M. 1997, *Biotechnol. Bioeng.*, 58, 486-493.
24. de Lathouder, K.M., Flo, T.M., Kapteijn, E., Moulijn, J.A. 2005, *Catal. Today*, 105, 443.
25. de Lathouder, K.M., Bakker, J., Kreutzer, M.T., Kapteijn, F., Moulijn, J.A., Wallin, S.A. 2004, *Chem. Eng. Sci.*, 59, 5027.
26. Ivanov, A.E., Schneider, M.P. 1997, *J. Mol. Catal. B: Enzym.*, 3, 303.
27. Balcao, V.M., Mateo, C., Fernandez-Lafuente, R., Malcata, F.X., Guisan, J.M. 2001, *Enzyme Microb. Technol.*, 28, 696.
28. Warmuth, W., Wenzig, E., Mersmann, A. 1995, *Bioprocess. Eng.*, 12, 87.
29. Miletić, N., Vuković, Z., Nastasović, A., Loos, K. 2009, *in press, J. Mol. Catal. B: Enzym.*
30. Dyal, A., Loos, K., Noto, M., Chang, S.W., Spagnoli, C., Shafi, K., Ulman, A., Cowman, M., Gross, R.A. 2003, *J. Am. Chem. Soc.*, 125, 1684.
31. Charusheela, A. Arvind, L. 2002, *Enzyme Microb. Technol.*, 30, 19.
32. Bertucci, C., Petri, A., Felix, G., Perini, B., Salvadori, P. 1999, *Tetrahedron: Asymmetry*, 10, 4455-4462.
33. Bhushan, I., Parshad, R., Qazi, G.N., Ingavle, G., Jamalpure, T.M., Rajan, C.R., Ponrathnam, S. Gupta, V.K. 2007, *J. Bioact. Compat. Polym.*, 22, 174.

34. Fernandez-Lorente, G., Terreni, M., Mateo, C., Bastida, A., Fernandez-Lafuente, R., Dalmases, P., Huguet, J., Guisan, J.M. 2001, *Enzyme Microb. Technol.*, 28, 389.
35. Martinek, K., Klibanov, A.M., Goldmacher, V.S., Tchernysheva, A.V., Mozhaev, V.V., Berezin, I.V., Glotov, B.O. 1977, *Biochim. Biophys. Acta*, 485, 13.
36. Reetz, M.T., Zonta, A., Simpelkamp, J. 1996, *Biotechnol. Bioeng.*, 49, 527.
37. Reetz, M.T. 1997, *Adv. Mater.*, 9, 943.
38. Reetz, M.T., Tielmann, P., Wiesenhofer, W., Konen, W., Zonta, A. 2003, *Adv. Synth. Catal.*, 345, 717.
39. Vidinha, P., Augusto, V., Almeida, M., Fonseca, I., Fidalgo, A., Ilharco, L., Cabral, J.M.S., Barreiros, S. 2006, *J. Biotechnol.*, 121, 23.
40. Kuncova, G., Szilva, J., Hetflejs, J., Sabata, S. 2003, *J. Sol-Gel Sci. Technol.*, 26, 1183.
41. Knezevic, Z., G. Kukic, M. Vukovic, B. Bugarski, B. Obradovic, 2004, *Process Biochem.*, 39, 1377.
42. Lalonde, J.J., Govardhan, C., Khalaf, N., Martinez, A.G., Visuri, K., Margolin, A.L. 1995, *J. Am. Chem. Soc.*, 117, 6845.
43. Cao, L.Q., van Rantwijk, F., Sheldon, R.A. 2000, *Org. Lett.*, 2, 1361.
44. Overbeeke, P.L.A., Govardhan, C., Khalaf, N., Jongejan, J.A., Heijnen, J.J. 2000, *J. Mol. Catal. B: Enzym.*, 10, 385.
45. Schoevaart, R., Wolbers, M.W., Golubovic, M., Ottens, M., Kieboom, A.P.G., van Rantwijk, F., van der Wielen, L.A.M., Sheldon, R.A. 2004 *Biotechnol. Bioeng.*, 87, 754.
46. Lopez-Serrano, P., Cao, L., van Rantwijk, F., Sheldon, R.A. 2002, *Biotechnol. Lett.*, 24, 1379.