Microbial lipases form versatile tools for biotechnology

Karl-Erich Jaeger and Manfred T. Reetz

Lipases are secreted into the culture medium by many bacteria and fungi. They catalyse not only the hydrolysis but also the synthesis of long-chain acylglycerols. Important uses in biotechnology include their addition to detergents, the manufacture of food ingredients, pitch control in the pulp and paper industry, and biocatalysis of stereoselective transformations. This makes them the most widely used class of enzymes in organic chemistry. Immobilization in hydrophobic sol–gel matrices and *in vitro* evolution are promising novel approaches to increasing the stability or enantioselectivity, respectively, of lipases.

Lipases (EC 3.1.1.3) catalyse both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids (Fig. 1). These reactions usually proceed with high regio- and/or enantioselectivity, making lipases an important group of biocatalysts in organic chemistry. The reasons for the enormous biotechnological potential of microbial lipases include the facts that they are (1) stable in organic solvents, (2) do not require cofactors, (3) possess a broad substrate specificity and (4) exhibit a high enantioselectivity. A number of lipases have been produced commercially, with the majority of them originating from fungi and bacteria. A recent publication of commercially available triacylglycerol lipases listed enzymes from 34 different sources, including 18 from fungi and 7 from bacteria[1]; Table 1 lists those microbial lipases that appear to be the most widely used in biotechnology. It should be noted that considerable confusion exists concerning the origin of particular lipases, resulting from changes in the systematic names of fungal and bacterial strains producing these lipases: *Candida rugosa* was formerly called *Candida cylindracea*; *Thermomyces lanuginosus* was *Humicola lanuginosa*; and *Pseudomonas glumae* and *Pseudomonas cepacia* have been renamed as *Burkholderia glumae* and *Burkholderia cepacia*. Furthermore, the *B. glumae* lipase is identical to the one from *Chromobacterium viscosum*. The steadily growing interest in microbial lipases is reflected by an increasing number of excellent monographs and review articles covering the molecular biology, biochemical properties and, in particular, the biotechnological applications of these enzymes[2–11]. Rather than presenting a comprehensive overview, this article aims to describe briefly the most important characteristics of lipases and highlighting a few important biotechnological applications.

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Three-dimensional structure of lipases and mechanism of lipolysis

The three-dimensional (3D) structures of the fungal lipase from *Rhizomucor miehei* and the human pancreatic lipase were determined in 1990[12,13]. Since then, eleven more lipase structures have been solved, which, with the exception of pancreatic lipase, are all of microbial origin[14]. These enzymes, which span a molecular weight range of 19 to 60 kDa, all exhibit a characteristic folding pattern known as the α/β-hydrolase fold[15]. The lipase core is composed of a central β sheet consisting of up to eight different β strands (β1–β8) connected by up to six α helices (A–F). The active site is formed by a catalytic triad consisting of the amino acids serine, aspartic (or glutamic) acid and histidine; the nucleophilic Ser residue is located at the C-terminal end of strand β5 in a highly conserved pentapeptide GXSXG, forming a characteristic β–turn–α motif named the 'nucleophilic elbow'. Substrate hydrolysis starts with a nucleophilic attack by the catalytic-site-Ser oxygen on the carbonyl carbon atom of the ester bond, leading to the formation of a tetrahedral intermediate stabilized by hydrogen bonding to nitrogen atoms of main-chain residues that belong to the so-called 'oxyanion hole'. An alcohol is liberated, leaving behind an acyl–lipase complex, which is finally hydrolysed with liberation of the fatty acid and regeneration of the enzyme.

Lipolytic reactions occur at the lipid–water interface, implying that the kinetics cannot be described by Michaelis–Menten equations, as these are valid only if the catalytic reaction takes place in one homogenous phase. Lipolytic substrates usually form an equilibrium between monomeric, micellar and emulsified states, resulting in the need for a suitable model system to study lipase kinetics. The monolayer technique[16] has been used extensively and, more recently, an oil-drop technology has been put forward in which lipase kinetics are monitored by automatic analysis of the profile of an oil drop hanging in water; the decrease in the interfacial tension between the oil and water caused by lipase hydrolysis is measured as a function of time[17]. The best-known phenomenon emerging from early

  0167 – 7799/98/$19.00. PII: S0167-7799(98)01195-0

kinetic studies of lipolytic reactions became known as 'interfacial activation', describing the fact that the activity of lipases is enhanced towards insoluble substrates that form an emulsion. Lipases, in contrast to esterases, were therefore defined as carboxylesterases acting on emulsified substrates.

The determination of their 3D structures seemed to provide an elegant explanation for interfacial activation: the active site of lipases was found to be covered by a surface loop, which was called the lid (or flap). Upon binding to the interface, this lid moves away, turning the 'closed' form of the enzyme into an 'open' form, with the active site now accessible to the solvent; at the same time, a large hydrophobic surface is exposed, which is thought to facilitate binding of the lipase to the interface[18]. More recently, it turned out that the presence of a lid-like structure is not necessarily correlated with interfacial activation[19]: lipases from *Pseudomonas aeruginosa* (Fig. 2), *B. glumae* and *Candida antarctica* B, and a coypu pancreatic lipase do not show interfacial activation but nevertheless have an amphiphilic lid covering their active sites[19]. This observation led the conclusion that the presence of a lid domain and interfacial activation are unsuitable criteria to classify an enzyme as a lipase. Therefore, the current definition is rather simple: a lipase is a carboxylesterase that catalyses the hydrolysis of long-chain acylglycerols[19].

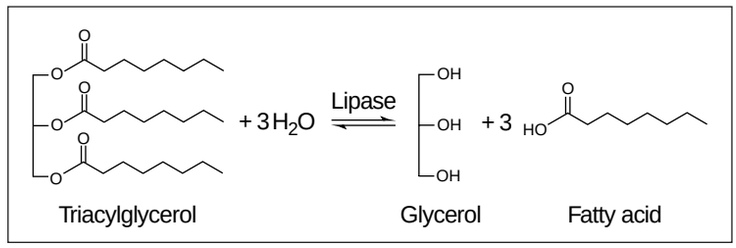


Figure 1

The catalytic action of lipases. A triglyceride can be hydrolysed to form glycerol and fatty acids, or the reverse (synthesis) reaction can combine glycerol and fatty acids to form the triglyceride.

Applications of lipases as hydrolases

Detergents

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. Enzyme sales in 1995 have been estimated to be US$30 million, with detergent enzymes making up 30%[6]. An estimated 1000 tons of lipases are added to the approximately 13 billion tons of detergents produced each year. In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase™, which originated from the fungus *T. lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced – Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes*, both produced by Genencor International.

The challenges that producers of detergent lipases need to meet include: (1) the high variation in the triglyceride content of fat stains, requiring lipases with low substrate specificity; (2) the relatively harsh washing conditions (pH 10–11 and 30–60°C), requiring stable enzymes; and (3) the effects of chemical denaturation and/or proteolytic degradation caused by detergent additives such as the surfactant linear alkyl benzene sulfonate (LAS) and proteases. Solutions to these problems are being sought by a combination of continuous screening for improved lipases and attempts to enhance lipase properties on the basis of protein engineering[5].

Food ingredients

The position, chain length and degree of unsaturation greatly influence not only the physical properties but also the nutritional and sensory value of a given triglyceride. Cocoa butter contains palmitic and stearic acids and has a melting point of approximately 37°C, leading to its melting in the mouth, which results in a perceived cooling sensation. In 1976, Unilever filed a patent describing a mixed hydrolysis and synthesis reaction to produce a cocoa-butter substitute using an immobilized lipase[21]. This technology is now commercialized by Quest-Loders Croklaan, based on immobilized *R. miehei* lipase, which carries out a transesterification reaction replacing palmitic acid with

Table 1. Examples of commercially available microbial lipases[1,4,8]

Origin	Organism producing lipase	Biotechnological applications	Commercialized by
Fungal	*Candida rugosa*[a]	Organic synthesis	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme, Sigma
	Candida antarctica A/B	Organic synthesis	Boehringer Mannheim, Novo Nordisk
	Thermomyces lanuginosus[b]	Detergent additive	Novo Nordisk, Boehringer Mannheim
	Rhizomucor miehei	Food processing	Novo Nordisk, Biocatalysts, Amano
Bacterial	*Burkholderia cepacia*[c]	Organic synthesis	Amano, Fluka, Boehringer Mannheim
	Pseudomonas alcaligenes	Detergent additive	Genencor
	Pseudomonas mendocina	Detergent additive	Genencor
	Chromobacterium viscosum[d]	Organic synthesis	Asahi, Biocatalysts

[a]Organism was formerly named *Candida cylindracea*
[b]Organism was formerly named *Humicola lanuginosa*
[c]Organism was formerly named *Pseudomonas cepacia*
[d]The lipase from *C. viscosum* is identical to the lipase from *Burkholderia glumae*

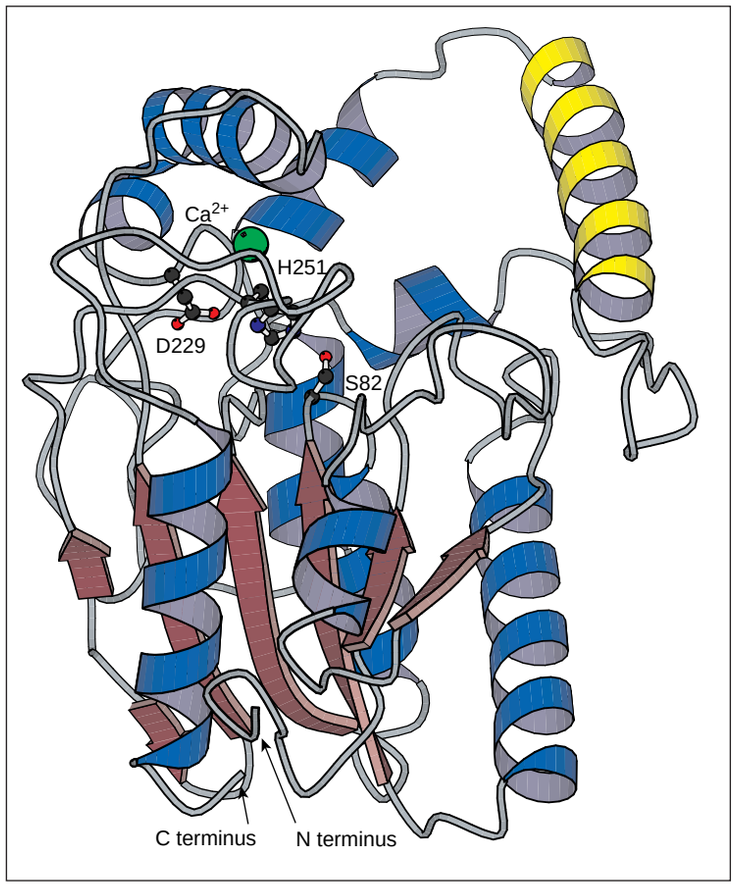


Figure 2

The structure of the lipase from *Pseudomonas aeruginosa* in a model built using the X-ray coordinates determined for *Burkholderia cepacia* lipase[20]. β strands are represented as arrows (dark red) and α helices as coils (blue or yellow); the yellow helix could form a 'lid' over the active site. The active-site residues Ser82, Asp229 and His251 are labelled, and the potential position of a Ca^{2+} ion is indicated by a green ball.

stearic acid to give the desired stearic–oleic–stearic triglyceride.

Polyunsaturated fatty acids (PUFAs; see Glossary) play an increasingly important role as biomedical and so-called 'nutraceutical' agents[22]. Many of them belong to the essential fatty acids, the uptake of which is required for membrane-lipid and prostaglandin synthesis, a fact that is often ignored when recommending a fat-free diet. Microbial lipases are used to enrich PUFAs from animal and plant lipids, such as menhaden, tuna or borage oil. Free PUFAs and their mono- and diglycerides are subsequently used to produce a variety of pharmaceuticals including anticholesterolemics, anti-inflammatories and thrombolytics[23].

Pulp and paper industry

Another application field of increasing importance is the use of lipases in removing the pitch from pulp produced in the paper industry. 'Pitch' is a term used to describe collectively the hydrophobic components of wood, namely triglycerides and waxes, which cause severe problems in pulp and paper manufacture[24]. Nippon Paper Industries in Japan developed a pitch-control method that uses a fungal lipase from *C. rugosa* to hydrolyse up to 90% of the triglycerides.

Other applications

A large number of additional hydrolytic applications have been described for microbial lipases, including flavour development for dairy products (cheese, butter, margarine, alcoholic beverages, milk chocolate and sweets), achieved by selective hydrolysis of fat triglycerides to release free fatty acids; these can act as either flavours or flavour precursors. In the future, the treatment of waste by lipases looks to become very important; this includes the breakdown of fat solids, the prevention or cleaning of fat films and the cleaning of fat-containing waste effluents.

Applications of lipases as synthetases

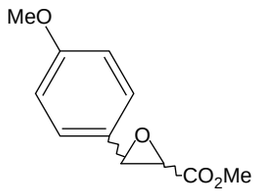
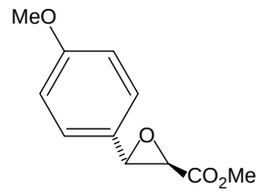
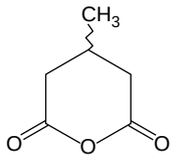
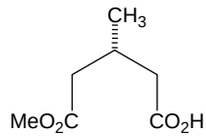
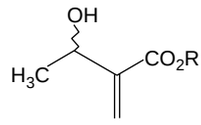
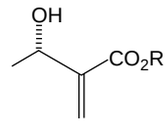
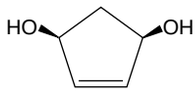
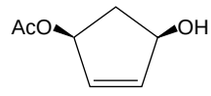
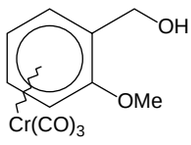
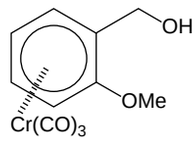
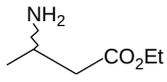
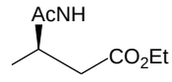
Lipases have been employed by organic chemists for a long time to catalyse a wide variety of chemo-, regio- and stereoselective transformations[2–4,8,25–27]. The majority of the lipases used as catalysts in organic chemistry are of microbial origin. One of the most exciting aspects of this fast-growing field is the possibility of enantioselective lipase catalysis on an industrial scale.

There are two basic types of enantioselective organic transformations amenable to lipase catalysis: (1) the reaction of prochiral substrates[2–4,25–27]; and (2) kinetic resolution of racemates[2–4,25–28]. Traditionally, prochiral

Glossary

Chiral	Geometrical attribute of a compound, describing a molecule that cannot be superimposed on its mirror image.
Enantiomer	One of a pair of molecules that are mirror images of each other and not superimposable.
Enantiomeric excess (ee)	The percentage excess of one **enantiomer** over the other in a mixture of two enantiomers, calculated as ee = |%*R* − %*S*|.
Enantioselective reaction	A chemical reaction or synthesis that produces the two **enantiomers** of a **chiral** product in unequal amounts.
Kinetic resolution	Partial or complete resolution of a **racemate** by virtue of unequal reaction rates of the **enantiomers** with a **chiral** agent, such as a biocatalyst.
***R* (*rectus*), *S* (*sinister*)**	Stereochemical descriptors describing the configuration of a given **chiral** molecule, following the rules developed by Cahn, Ingold and Prelog (the CIP convention).
Racemate	An equimolar mixture of two **enantiomeric** species.

Table 2. Examples of lipase-catalysed enantioselective reactions

Substrate	Source	Product	Yield	%ee	Ref.
	*Serratia marcescens*		45%	>98%	29
	*Pseudomonas* sp.		92%	87%	30
	*Pseudomonas* sp.		41%	>95%	31
	*Candida antarctica*		48%	>99%	32
	*Pseudomonas* sp.		47%	97%	33
	*Candida antarctica*		38%	95%	34

The first example shows a key transformation used in the synthesis of the calcium antagonist Diltiazem®; all others represent model reactions.
In the case of enantioselective reactions involving prochiral subtrates, the theoretical maximum yield is 100%, whereas, in the case of chiral resolution of a racemate, it is 50%.

or chiral alcohols and carboxylic-acid esters served as the two main classes of substrates but, over the years, the range of compounds has expanded rapidly to include diols, α- and β-hydroxy acids, cyanohydrins, chlorohydrins, diesters, lactones, amines, diamines, amino-alcohols, and α- and β-amino-acid derivatives[2–4,25–28] (Table 2). It is thus no exaggeration to note that the most important classes of functionalized organic compounds can, in principle, be prepared enantioselectively by lipase catalysis. Typical catalysts include lipases from the bacteria *P. aeruginosa*, *Pseudomonas fluorescens* and other *Pseudomonas* species, *B. cepacia*, *C. viscosum*, *Bacillus subtilis*, *Achromobacter* sp., *Alcaligenes* sp., and *Serratia marcescens*, as well as from fungi such as *C. antarctica* B and *C. rugosa*.

However, despite the large number of publications in this area, the number of industrial enantioselective processes based on lipase catalysis is limited[6,35,36]. Successful examples include the synthesis of chiral amines, catalysed by the lipase from *Burkholderia plantarii*[37,38], and the *Serratia marcescens* lipase-based production of (2*R*,3*S*)-3-(4-methoxyphenyl)methyl glycidate, which is used in the synthesis of the calcium antagonist Diltiazem™ (Ref. 29). In general, however, problems may arise from: (1) insufficient enantioselectivity; (2) limited enzyme activity; (3) difficulties in recycling

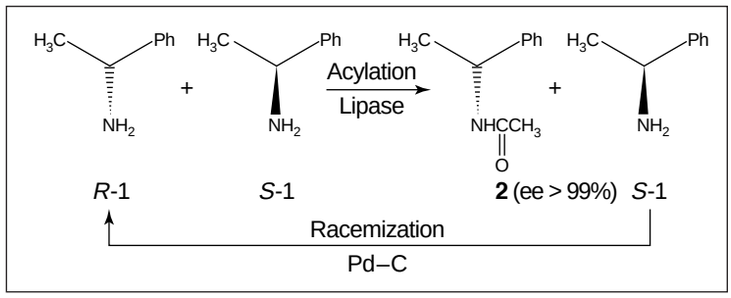


Figure 3

Dynamic kinetic resolution of racemic phenylethyl amine based on enantioselective lipase-catalysed acylation and racemization by palladium on charcoal (Pd–C).

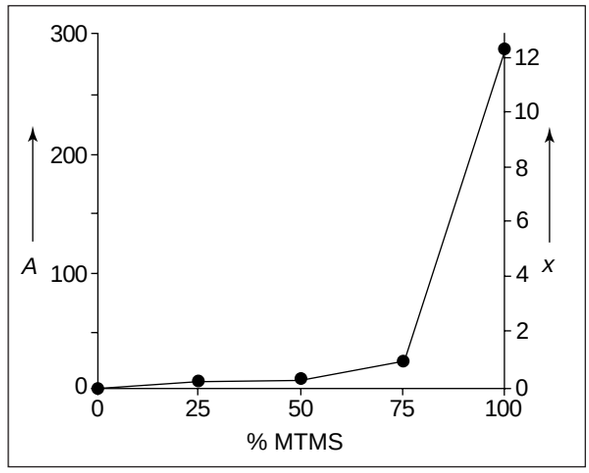


Figure 4

Dependence of the activity, *A* [μmol h^{-1} (mg lipase)$^{-1}$], and relative activity (relative to the use of nonimmobilized lipase powder), *x* of immobilized *Burkholderia cepacia* lipase on the composition of gels derived from tetramethoxysilane and methyltrimethoxysilane (MTMS).

the lipase; and (4) inherent practical limitations of the kinetic resolution arising from the fact that 50% conversion is the maximum possible. Nevertheless, solutions to these problems are emerging, which means that industrial applications are more likely in the near future. In addition to the optimization of reaction conditions such as the choice of solvent, temperature, acylating agent etc., the most important current developments include: (1) *in vitro* evolution as a method to increase enantioselectivity; (2) novel immobilization techniques, resulting in the enhancement of enzyme activity and stability in organic solvents[2–4,24–26,39]; (3) recycling techniques enabling multiple reuse of lipases[39]; and (4) methods for dynamic kinetic resolution in which the complete conversion of a racemate into a single enantiopure product is possible[27,40].

Lipase-catalysed dynamic resolution generally requires a second catalyst to induce the racemization of the enantiomer that is not accepted by the enzyme[27,40]. Most often, transition-metal catalysts are used, which must be compatible with the lipase. An example is the production of the *N*-acylated amine (Fig. 3) as the sole nitrogen-containing product of the reaction of the racemate using the lipase from *C. antarctica* and palladium on charcoal as the two catalysts[41].

Immobilization of lipases

As noted above, the industrial use of lipases as enantioselective catalysts in organic chemistry depends on their efficient immobilization. As studies in these areas are ongoing, it is currently difficult to assess the relative merits of the different approaches[2–4,24–27,39]. Unfortunately, in many studies concerning lipase immobilization, the scope and limitations of the stability and recyclability of the enzyme are not clearly defined.

A promising approach to enzyme entrapment makes use of inorganic matrices such as silica gel[42]. The so-called sol–gel process, initiated by the hydrolysis of $Si(OR)_4$, may be performed in the presence of the enzyme. Hydrolysis and condensation of the silicon precursor in the presence of an acid or base catalyst triggers crosslinking, with the formation of amorphous SiO_2, a porous inorganic matrix that grows around the enzyme in a three-dimensional manner. In the entrapped form, such enzymes as glucose oxidase, phosphatase, trypsin, aspartase, carbonic anhydrase, chinitase and monoamine oxidase have been shown to display activities of 30–100% relative to those of the natural, non-entrapped state[42]. Unfortunately, all attempts to extend this technology to lipases have been disappointing[43]. In a test reaction involving the esterification of lauryl acid with *n*-octanol, relative enzyme activities of less than 5% were generally observed[43].

As lipases are active at the lipid–water interface[18], we speculated that SiO_2 might not be the ideal matrix, and that silica gels modified by alkyl groups might offer a hydrophobic microenvironment that could interact with the lipase, perhaps triggering a phenomenon similar to classical interfacial activation[44]. Thus, monomers of the type $RSi(OCH_3)_3$ or disilanes such as $HO[(CH_3)_2SiO]_nSi(CH_3)_2OH$ were tested in the sol–gel process[39,43,44]; it has been known for a long time that the hydrolysis of such monomers induces a sol–gel process without the cleavage of the C–Si bonds.

In initial experiments, the lipase from *B. cepacia* was immobilized by the sol–gel process using $CH_3Si(OCH_3)_3$ (MTMS) and varying amounts of $Si(OCH_3)_4$ (TMOS)[43]. The gels obtained by this procedure displayed surprisingly high catalytic activities in the model reaction, depending upon the relative amounts of MTMS and TMOS; the relative catalytic activity reaches 1300% for a pure MTMS gel, which means that this material is 13 times more active than a conventional fine suspension (Fig. 4).

Consequently, other commercially available silane precursors, $R–Si(OCH_3)_3$, were also investigated. It was discovered that the nature of the alkyl group at silicon plays an important role, the relative lipase activity in the reactions increasing significantly as the nature of the alkyl group varies: $CH_3 < C_2H_5 < C_3H_7 < C_4H_9 < C_8H_{17}$. This correlates with the increase in hydrophobicity of these materials. Other lipases entrapped successfully using this method include those from *C. antarctica*, *Aspergillus niger*, *H. lanuginosa* and *Rhizopus arrhizus*. There is no general protocol regarding the optimal silane precursor or mixture of the precursors[39,44]; the entrapment of each lipase must be optimized individually. In most cases, the addition of stabilizing additives such as polyethylene glycol or polyvinyl alcohol is necessary for optimum enzyme performance. Relative enzyme activities of

1500–2000% are common, but values of up to 8800% (a factor of 88) have been observed. Many of the lipase-containing gels show specific activities of 200–300 μM h^{-1} (mg protein) (Refs 39,44). This compares well with other immobilized forms of lipases[2–4,24–27,39]. One of the prime virtues of sol–gel encapsulation is the ease of preparation compared with other new immobilization methods such as cross-linked enzyme crystals or lipid-coated enzymes.

The sol–gel entrapment of lipases has also been found to increase their chemical and thermal stability[39,44]. Immobilized lipase from *B. cepacia* in MTMS or MTMS–PDMS gels were used repeatedly (more than 30 times) in batch-esterification reactions of lauric acid with 1-octanol in isooctane, and the loss in long-term activity was only 10–15%. The first generation of these heterogeneous biocatalysts has recently been commercialized (Fluka Chemie, Switzerland). In some cases, the entrapped lipases show improved enantioselectivity in stereoselective reactions[39]. Further developments include fixation of the lipase-containing gels on glass beads for use as mechanically stable catalysts in fluid-bed reactors[39] and the simultaneous entrapment of lipases and magnetite to form magnetically separable heterogeneous biocatalysts[45].

Future technologies with lipases

Enzyme crystals

In addition to sol–gel encapsulation of lipases[39,43–45], several other novel immobilization methods have recently been reported. One interesting new technology uses lipases in the form of cross-linked enzyme crystals (CLEC). Lipases from *C. rugosa* and *B. cepacia* are crystallized to give microcrystals approximately 50–100 μm in length, which are subsequently cross-linked by the addition of glutaraldehyde and dried, preferably in the presence of a detergent[46]. These crystals have been used for the chiral resolution of commercially important organic compounds by ester hydrolysis giving high enantiomeric excesses (ee): *S*-ibuprofen (95% ee), *S*-naproxen (97% ee) and (−)-menthol (95% ee)[47]. In addition, CLECs can catalyse esterification and transesterification reactions in low-water organic solvents to achieve chiral resolution of racemic alcohols and acids with ee values in the range 95–99%. CLECs produced from *P. cepacia* and *C. rugosa* lipases were tested for their potential to catalyse the chiral resolution of various acids, alcohols and amines by acylation in organic solvent. They proved to be more active than the crude enzyme powders by a factor of between 10 and 90, determined by measuring the reaction rates in μmol min^{-1} mg (based on the weight of lipase protein)[48].

Lipid-coated enzymes

Lipase obtained from *Pseudomonas fragi* has been coated with a lipid monolayer at a ratio of about 150 lipid molecules per enzyme molecule. This lipase catalysed the esterification of racemic (*R*,*S*)-1-phenylethanol and lauric acid in isooctane with high enantioselectivity and conversion rate[49]. Lipid coating seems to render at least some lipases homogeneously soluble and stable in organic solvents (benzene, ethyl acetate, isooctane, isopropyl ether, dimethyl sulfoxide and ethanol). The enzymatic activity of *P. fragi* lipase coated with different glycolipids, zwitterionic, anionic and cationic lipids was measured as the initial rate of ester synthesis. Lipases coated with didodecyl *N*-D-glucono-L-glutamate and didocyl-*N*-D-glucono-D-glutamate showed the highest enantioselectivity at enzymatic activities of 50 and 58 μM sec^{-1} (mg protein) towards the preferred (*R*)-enantiomer of 1-phenylethanol[50].

In vitro *evolution of enantioselective lipases*

The enantioselective synthesis of chiral compounds is of rapidly increasing importance to the chemical industry: the total sales of therapeutics in 1995 has been estimated to be US$150 billion, US$60 billion of which resulted from chiral compounds. Chiral drugs with a current sales volume exceeding US$1 billion include amoxicillin (an antibiotic), captopril (an angiotensin-converting-enzyme inhibitor) and erythropoietin (the haematopoietic growth factor)[51]. Often, just one of the two enantiomers of a given pharmaceutical or agrochemical compound exerts the desired effect, forcing the US Food and Drug Administration (FDA) to require the evaluation of both chiral forms of all potential new drugs. So far, chiral compounds have been produced by chemical kinetic resolution using asymmetric catalysts or by biocatalysts identified through the screening of a large number of (often commercially available) enzymes or organisms. X-ray crystal structures of lipases have been used successfully to predict enantiopreference and to engineer novel catalytic properties[52]. Transaminases with increased enantioselectivities towards a β-tetralone have been identified upon screening of a mutant library[53] and, in other cases, efficient chemo- or biocatalysts have been identified[35,36] (see above), but a large number of synthetic

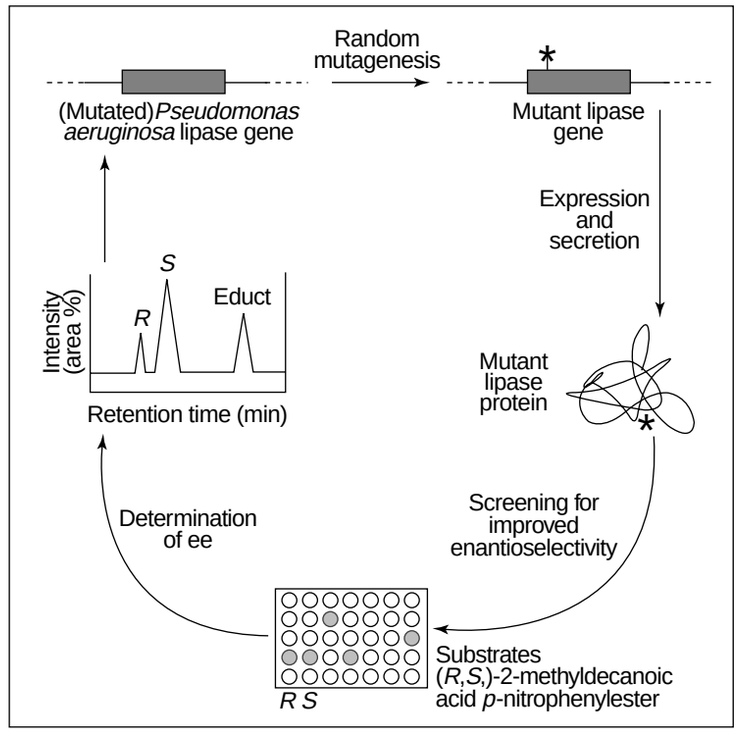


Figure 5

Strategy to create an enantioselective lipase by directed evolution. Intensity (area %) refers to the amount of *R*- and *S*-enantiomer as measured by chiral chromatography.

problems remained unsolved. Nevertheless, a solution has emerged that may well constitute a new direction in asymmetric catalysis: the creation of an enantioselective lipase by *in vitro* evolution[54]. This technique has been used previously to develop enzymes with improved thermostability, activity in organic solvents or substrate specificity[55]. The experimental protocol that has been developed[54] was as follows (Fig. 5):

- a lipase gene was chosen from *P. aeruginosa* because of previous experience with the overexpression and biotechnological applications of the enzyme[56–58];
- the gene was cloned into an overexpression plasmid and random mutations were introduced by error-prone PCR at a frequency corresponding to an average of one amino acid exchange per lipase molecule;
- mutant genes were amplified in *Escherichia coli* and the proteins expressed and secreted into the culture supernatant by *P. aeruginosa*;
- microtitre plates were loaded with samples of supernatant and samples of enantiomerically pure (*R*) or (*S*) 2-methyldecanoic acid *p*-nitrophenylester chosen as the substrate;
- the hydrolysis of each *R*–*S* pair of substrates was monitored spectrophotometrically at 410 nm;
- lipases with increased selectivity towards the (*S*)-enantiomer were further examined by gas chromatographic analysis on chirally modified capillary columns.

Only four generations of lipase mutants were needed to increase the enantioselectivity from 2% ee for the wild-type enzyme to 81% ee (Fig. 6).

DNA sequencing allowed the structural identification of the mutants, making further improvements in enantioselectivity possible (87% ee) by applying saturation mutagenesis at the identified positions. These results demonstrated that it is possible, in principle, to create enantioselective lipases (and other biocatalysts) by *in vitro* evolution for effective chiral resolution of a given substrate. Generalization to include the enantioselective synthesis of chiral compounds from prochiral substrates is likely to be successful, its success depending on: (1) the identification of genes encoding a biocatalyst suitable to catalyse a desired reaction; (2) the establishment of a functional microbial expression and secretion system; (3) the design of an efficient screening system for each substrate produced.

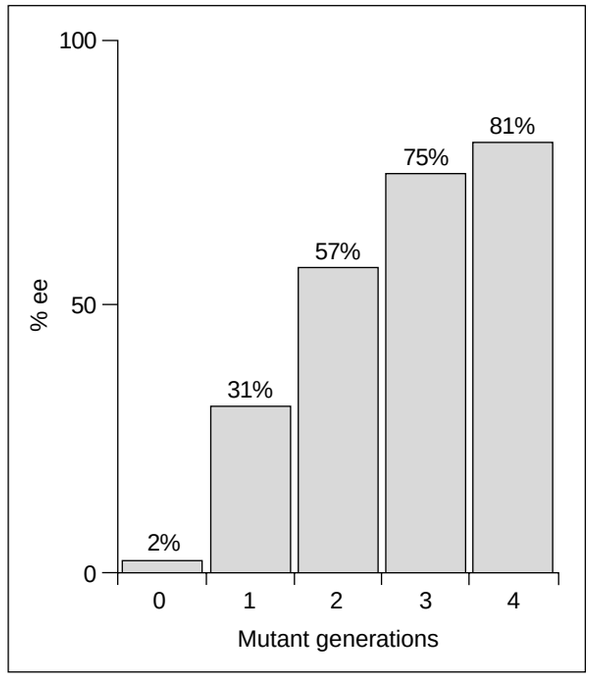


Figure 6

Sequential increase of enantioselectivity (as % ee) of *Pseudomonas aeruginosa* lipase as a result of mutagenesis experiments.

Conclusions

The biotechnological potential of microbial lipases is steadily increasing. New primary and 3D structures become available, and novel molecular-biological techniques provide the tools to tailor lipases for a variety of different applications. However, there are still only a few lipases used at an industrial scale in organic synthesis, presumably because of difficulties in establishing cost-effective scaling-up and downstream-processing protocols. In the near future, an important application area will be the increasing use of lipases as biocatalysts for the preparation of chiral compounds in enantiomerically pure form. The creation of enantioselective lipases by *in vitro* evolution will undoubtedly result in enzymes with novel and biotechnologically usable properties that may also allow applications at an industrial scale. It will be exciting to witness the future developments in basic research as well as biotechnological applications of these versatile microbial enzymes.

Acknowledgments

The authors would like to thank D. Lang (University of Gröningen, The Netherlands) for preparing Fig. 1. Work on bacterial lipases in the lab of K-E. Jaeger was supported by the EU in the framework of the BRIDGE (contract no. BIOT-CT 91-0272) and BIOTECHNOLOGY (contract no. BIOIV-CT 96-0119) programmes. Work in the lab of M. T. Reetz on immobilization and *in vitro* evolution was supported by the Max Planck Society.

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