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Microbial carboxyl esterases: classification, properties and application in biocatalysis

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Abstract

Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds and are widely distributed in animals, plants and microorganisms. Beside lipases, a considerable number of microbial carboxyl esterases have also been discovered and overexpressed. This review summarizes their properties and classification. Special emphasis is given on their application in organic synthesis for the resolution of racemates and prostereogenic compounds. In addition, recent results for altering their properties by directed evolution are presented.

Keywords: Carboxyl esterase; Microbial; Biocatalysis; Organic synthesis; Directed evolution

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1. Introduction

Esterases (EC 3.1.1.x) represent a diverse group of hydrolases catalyzing the cleavage and formation of ester bonds. They are widely distributed in animals, plants and microorganisms. Many of them show a wide substrate tolerance which led to the assumption that they have evolved to enable access to carbon sources or to be involved in catabolic pathways. Moreover, esterases also show high regio- and stereospecificity, which makes them attractive biocatalysts for the production of optically pure compounds in fine-chemicals synthesis [1–6]. The interest in these enzymes also resides in the fact that they do not require cofactors, are usually rather stable and are even active in organic solvents.

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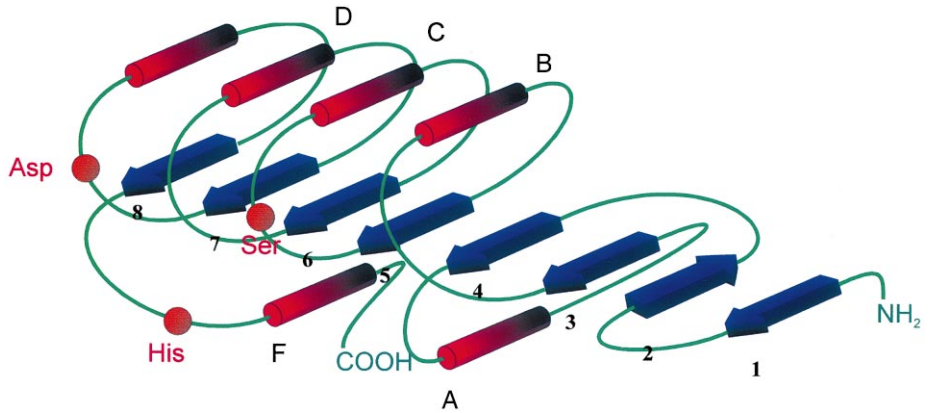

Fig. 1. Schematic presentation of the α/β-hydrolase fold. β-Sheets (1–8) are shown as blue arrows, α-helices (A–F) as red columns. The relative positions of the amino acids of the catalytic triad are indicated as orange circles.

Two major classes of hydrolases are of utmost importance: lipases (EC 3.1.1.1, triacylglycerol hydrolases) and 'true' esterases (EC 3.1.1.3, carboxyl ester hydrolases). The three-dimensional (3D) structures of both enzymes show the characteristic α/β-hydrolase fold [7] – a definite order of α-helices and β-sheets – which is also found in haloperoxidases and epoxide hydrolases (Fig. 1). The catalytic triad is composed of Ser-Asp-His (Glu instead of Asp for some lipases) and usually also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine. More recently, esterases have been identified containing a Gly-x-x-Leu motif [8] as well as enzymes showing high homology to class C β-lactamases [9]. The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases and is composed of four steps: First, the substrate is bound to the active serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl–enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol or ester in (*trans*-)esterification) forms again a tetrahedral intermediate, which after resolution yields the product (an acid or an ester) and free enzyme [1].

Lipases can be distinguished from esterases by the phenomenon of interfacial activation, which was only observed for lipases. Whereas esterases obey classical Michaelis–Menten kinetics, lipases need a minimum substrate concentration before high activity is observed. Structure elucidation revealed that this interfacial activation is due to a hydrophobic domain (*lid*) covering the active site of lipases – only in the presence of a minimum substrate concentration, i.e. a triglyceride phase or a hydrophobic organic solvent, the *lid* moves apart, making the active site accessible. Furthermore, lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze 'simple' esters (e.g. ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C_6 (Table 1). Both enzymes have been shown to be stable and active in organic solvents, but this feature is more pronounced with lipases. In a recent publication [10], a comparison of amino acid sequences and 3D-structures of lipases and esterases suggested that they can be distinguished by a pH-dependent electrostatic 'signature'; the active site of lipases displays a negative potential in the pH-range associated with their maximum activity (typically at pH 8), whereas esterases show a similar pattern, but at pH values around 6, which correlates with their usually lower pH-activity optimum [10].

Screening of esterases is usually performed either by employing chromophoric substances (e.g. *p*-nitrophenyl esters) or by using tributyrin-supplemented agar plates. Organisms producing active esterases are then identified by halo formation on plates containing dispersed water-insoluble substrates such as triglycerides. Hydrolysis of these dispersed lipid droplets leads to a clearing zone around the colonies. Then, lipases can be distinguished from carboxyl esterases by their substrate spectra, using *p*-nitrophenyl palmitate (cleaved by lipases) vs. *p*-nitrophenyl butyrate (cleaved by esterases and sometimes also by lipases).

In contrast to this traditional approach, modern molecular biology techniques allow for DNA library expression

Table 1
Differences between lipases and carboxyl esterases

Property	Lipase	Esterase
Preferred substrates	Triglycerides (long-chain), secondary alcohols	Simple esters, triglycerides (short-chain)
Interfacial activation/lid	Yes	No
Substrate hydrophobicity	High	High to low
Enantioselectivity	Usually high	High to low to zero
Solvent stability	High	High to low

screening, which also enables access to enzymes of 'non-culturable' organisms. By this strategy, for instance, the company Diversa (San Diego, CA, USA) identified 120 unique esterases/lipases from only 16% of the total DNA obtained from an alkaline soil sample [11]. Moreover, many of these enzymes are highly thermophilic, show broad substrate specificity and they belong to 21 protein families.

2. Classification

Classification of enzymes can be done either based on their substrate specificity or by sequence alignments. The former requires that all enzymes which need to be compared have been assayed with the same or at least related substrates, preferentially under similar reaction conditions. Although this would allow the (bio-)chemist to directly identify a suitable enzyme for a given synthetic problem, this is not a common strategy. Due to the increasing availability of sequence information in public databases, the comparison of amino acid sequences can provide a clearer picture about the evolutionary relationship between enzymes of different origin. On the other hand, it often turns out that high sequence homology cannot be related to the enzymes properties (substrate specificity, stereoselectivity, pH and temperature optima, solvent stability), and in some cases completely different types of reactions are catalyzed. For instance, a bromoperoxidase from *Streptomyces aureofaciens* shares ~55% sequence identity to an esterase from *Pseudomonas fluorescens* [12,13] but they share little substrate specificity.

Until a few years ago, it was believed that for all lipases and carboxyl esterases only the consensus sequence motif Gly-x-Ser-x-Gly (where x represents an arbitrary amino acid residue) occurs around the active site serine. Indeed, most lipases and esterases contain this motif (Table 2). More recently, a thorough comparison of 53 amino acid sequences of lipases and esterases revealed that other motifs also exist [14]. These have been discussed in detail by Arpigny and Jaeger [14] and only the most important findings will be reviewed here. For instance, some lipases and an esterase from *Streptomyces scabies* contain a GDSL (Gly-Asp-Ser-Leu) consensus sequence. Moreover, structure elucidation of this esterase revealed that it contains a catalytic Ser-His dyad instead of the common Ser-Asp-His triad [8]. The acidic side chain, which usually stabilizes the positive charge of the active site histidine residue, is replaced by the backbone carbonyl of Trp_{315} located three positions upstream of the His itself. The enzyme also has an α/β-tertiary fold, which differs substantially from the α/β-hydrolase fold. Other esterases in the GDSL group includes those from *Pseudomonas aeruginosa* (accession code: AF005091), *Salmonella typhimurium* (AF047014) and *Photorhabdus luminescens* (X66379), the first two being outer-membrane-bound esterases. Other enzymes show high homology to the mammalian hormone-sensitive lipase family. Here, conserved sequence blocks were found, which initially have been related to activity at low temperature. However, it was found that esterases from psychrophilic (e.g. *Moraxella* sp., X53869; *Psychrobacter immobilis*, X67712) as well as mesophilic (*Escherichia coli*, AE000153) and thermophilic (*Archeoglobus fulgidus*, AAE000985) origins belong to this family.

Members of family V, such as esterases from *Sulfolobus acidocaldarius* (AF071233) and *Acetobacter pasteurianus* (AB013096), share significant homology to non-lipolytic enzymes, e.g. epoxide hydrolase, dehalogenase and haloperoxidase. Rather small (23–26 kDa) enzymes are found in family VI, which includes an esterase from *P. fluorescens*, of which the structure is known [15]. The esterase is active as a dimer, has a typical Ser-Asp-His catalytic triad and hydrolyzes small substrates, but not long-chain triglycerides. Interestingly, ~40% homology to eukaryotic lysophospholipases is found for members of this family.

In contrast, esterases from family VII are rather large (~55 kDa) and share significant homology to eukaryotic acetylcholine esterases and intestine or liver carboxyl esterases (e.g. pig liver esterase). A *p*-nitrobenzyl esterase from *Bacillus subtilis* [16,17] and an esterase from *Arthrobacter oxydans* (Q01470) active against phenylcarbamate herbicides [18] belong to this group. In the last family, VIII, high homology to class C β-lactamases is observed. These enzymes contain a Gly-x-Ser-x-Gly motif and a Ser-x-x-Lys motif, but it has recently been demonstrated by site-directed mutagenesis studies of an esterase (EstB) from *Burkholderia gladioli* that the Gly-x-Ser-x-Gly motif does not play a significant role in enzyme function [9]. The most prominent member is an esterase from *Arthrobacter globiformis* (AAA99492) [19], which stereoselectively forms an important precursor of pyrethrin insecticides (see Section 3.1).

More information about the biochemical properties and preferred substrates of selected carboxyl esterases is summarized in Table 2.

3. Application in biocatalysis

3.1. Synthesis of optically pure compounds

Although a considerable number of microbial carboxyl esterases is known which have been overexpressed in suitable hosts (Table 2), only a few of them have been used for the synthesis of optically pure compounds. The major reasons for this are their limited commercial availability and their frequently observed moderate enantioselectivity. Several esterases have been available in recent years from various suppliers (e.g. Fluka, Amano, Jülich Fine Chemicals, Diversa, Roche Diagnostics, Thermogen).

Probably the best studied enzyme is the so-called carboxyl esterase NP (NP from naproxen, a non-steroidal

Table 2
Comparison of various (recombinant) microbial carboxyl esterases

Origin[a]	Biochemical properties	Specific substrates[b]	Remarks	Reference
B. gladioli ATCC10248 (EstB)	392 aa, 42 kDa	pNP-esters, triglycerides (up to C_6), deacylates, cephalosporins	S-x-x-K motif, β-lactamase-like	[9]
B. gladioli ATCC10248 (EstC)	298 aa, 32 kDa	pNP-esters (up to C_5), not triglycerides	G-x-S-x-G motif, homology to plant hydroxynitrile lyases	[65]
P. fluorescens DSM50106	36 kDa, $T_{opt.}$ 43°C	Lactones, ethyl caprylate, moderate enantioselectivity	G-x-S-x-G motif, homology to a haloperoxidase	[37]
P. fluorescens SIKW1	27 kDa, homodimer	pNP-esters, high enantioselectivity for α-phenyl ethanol, moderate *E* values for other alcohols and carboxylic acids	Low haloperoxidase activity, altered substrate specificity and improved enantioselectivity by directed evolution	[12,49,50,53,60,61,66]
Pseudomonas putida MR2068	29 kDa, homodimer, $T_{opt.}$ 70°C	Alkyl-dicarboxylic acid methylesters, high stereoselectivity ($E > 100$)	–	[58,67]
Bacillus acidocaldarius	34 kDa, $T_{opt.}$ 70°C	pNP-esters (best: hexanoate), moderate stereoselectivity (best: $E \sim 18$)	Homology to hormone-sensitive lipase	[68]
B. subtilis NRRL B8079	489 aa, 54 kDa, $T_{opt.}$ 52°C (66.5°C for best mutant)	*p*-nitrobenzyl ester of Loracarbef	Evolved by directed evolution for increased stability in DMF and thermostability	[16,17,38,55]
Bacillus stearothermophilus	–	pNP-esters, moderate enantioselectivity	Thermostable mutants	[69,70]
B. subtilis (ThaiI-8) [c]	32 kDa, $T_{opt.}$ 35–55°C	High enantioselectivity towards 2-arylpropionic acids	Structure known, more stable mutants by SDM[b]	[20]
Thermoanaerobacterium sp. JW/SL YS485	320 aa, 36 kDa	Xylose tetra acetate, cephalosporin C, MU-Ace[b]	G-x-S-x-G motif	[35]
Acinetobacter sp. ADP1	37 kDa	pNP-esters (best: hexanoate), benzyl esters	G-x-S-x-G motif, involved in catechol branch of β-ketoadipate pathway	[36]
Clostridium thermocellum	31 kDa	Feruloyl esters	Esterase activity within cellulosome	[33]
Pyrococcus furiosus DSM3638	$T_{opt.}$ 100°C, $t_{1/2}$ 50 min at 126°C	MU-Ace[b]	–	[71]
Lactococcus lactis[e]	258 aa, 30 kDa	pNP-esters (best: hexanoate), tributyrin, C_6-phospholipids	G-x-S-x-G motif, function unclear	[72]
Rhodococcus ruber DSM 43338[d]	Two esterases with opposite enantiopreference	Linalool-acetate ($E > 100$)	Two esterases with opposite enantiopreference	[59]
Rhodococcus sp. H1	34 kDa, tetramer	Heroin	G-x-S-x-G motif, conserved His_{86}	[73]
Rhodococcus sp. MB1	574 aa, 65 kDa, monomer	Cocaine	G-x-S-Y-x-G motif, homology to X-prolyl-dipeptidyl aminopeptidases	[74]
Streptomyces chrysomallus	42 kDa	pNP-esters (best: butyrate)	G-x-S-x-G motif, high homology to esterase from *A. globiformis*	[75]
Streptomyces diastatochromogenes	326 aa, 31 kDa	pNP-esters, moderate enantioselectivity	–	[76,77]
Orpinomyces sp. PC-2	313 aa, 35 kDa, $T_{opt.}$ 30°C	Xylose tetra acetate	High homology to other acetyl xylan esterases	[34]
Aspergillus awamori IFO4033[c]	275 aa, 31 kDa	Wheat bran, α-naphthol acetate	Homology to lipases from *Geotrichum candidum* and *Candida cylindracea*	[78]
Saccharomyces cerevisiae IFO2347	28 kDa, homodimer, $T_{opt.}$ 25°C	Isoamyl acetate, isobutyl acetate	–	[79]

[a]Overexpressed in *E. coli*, if not stated otherwise
[b]pNP, *p*-nitrophenyl; MU-Ace, 4-methylumbelliferyl-acetate; SDM, site-directed mutagenesis
[c]Non-recombinant purified enzymes
[d]Overexpressed in *L. lactis*
[e]Overexpressed in *B. subtilis*

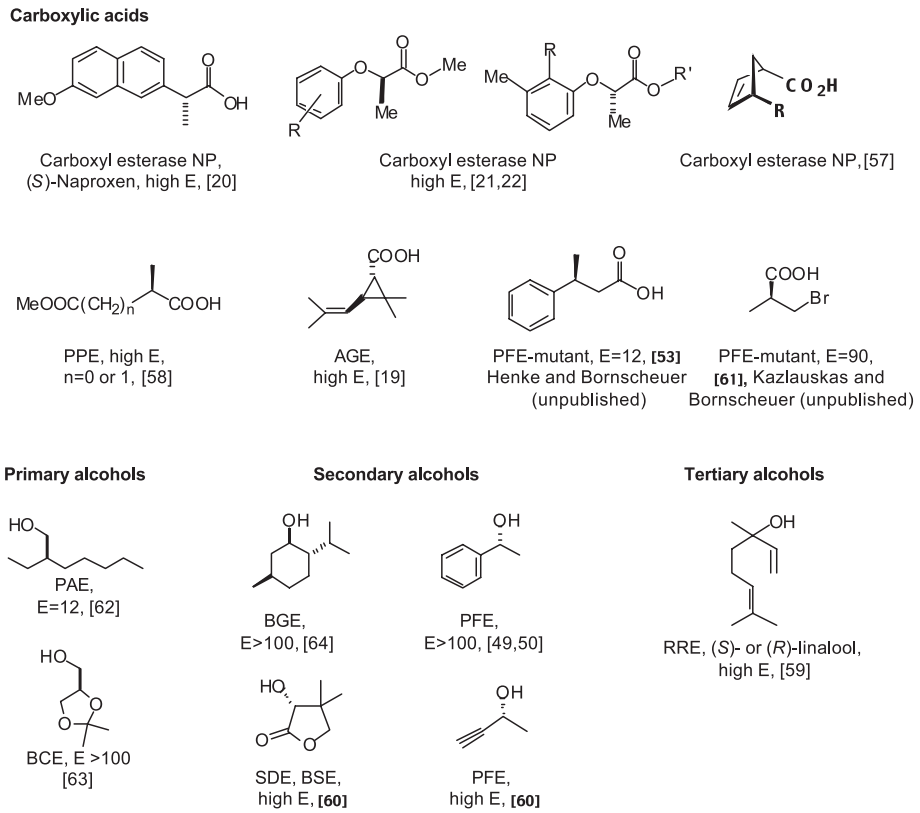

Fig. 2. Selected examples of chiral compounds obtained by carboxyl esterase-catalyzed kinetic resolutions. AGE, esterase from *A. globiformis*; BCE, esterase from *Bacillus coagulans*; BSE, esterase from *Bacillus stearothermophilus*; BGE, esterase from *B. gladioli*; PAE, esterase from *P. aeruginosa*; PME, esterase from *Pseudomonas marginata*; PPE, esterase from *Pseudomonas putida*; RRE, esterase from *Rhodococcus ruber*; SDE, esterase from *Streptomyces diastatochromogenes*. The *E* value is the enantioselectivity (often also named enantiomeric ratio), which reflects the ability of the enzyme to distinguish between the two enantiomers of a racemate and thus the rate with which each enantiomer is converted. *E* values above 100 allow the synthesis of optically pure product and substrate, *E* values >20 are sufficient to obtain the remaining substrate in high optical purity and acceptable yield. At $E=1$, racemic product is formed. *E* values can be calculated from the V_{max}/K_m values for each enantiomer, but are usually determined from % ee and conversion using the equations developed by Chen et al. [56]. A simple program to calculate the enantioselectivity is freely available at http://www.orgc.tu-graz.ac.at.

anti-inflammatory drug) originating from *B. subtilis* [20]. Besides naproxen, various other 2-arylpropionic acids are produced with high enantioselectivity (Fig. 2) [21,22]. Carboxyl esterase NP has a molecular mass of 32 kDa, a pH optimum between 8.5 and 10.5 and a temperature optimum between 35 and 55°C. Carboxyl esterase NP is produced as intracellular protein; its structure is unknown. In a pilot-scale process, (*R*,*S*)-naproxen methylester is hydrolyzed in the presence of Tween 80 to increase substrate solubility at pH 9.0. The (*S*)-acid is separated from the remaining (*R*)-methylester, and the latter is racemized using an organic base. This reaction yields (*S*)-naproxen with excellent optical purity (99% enantiomeric excess (ee)) at an overall yield of 95% [20]. Irreversible inactivation of carboxyl esterase NP was circumvented by site-directed mutagenesis replacing Lys_{34} with Glu, thus eliminating the positively charged target prone to the formation of a Schiff base. Carboxyl esterase NP was also used in the resolution of (*R*,*S*)-ibuprofen methylester and showed higher selectivity compared to lipase from *Candida rugosa* [23].

Another efficient kinetic resolution was achieved in the synthesis of (+)-*trans*-(1*R*,3*R*)-chrysanthemic acid, which is an important precursor of pyrethrin insecticides (Fig. 2). Here, an esterase from *A. globiformis* catalyzed the sole formation of the desired enantiomer (>99% ee, at 77% conversion). The enzyme was purified and the gene was cloned in *E. coli* [24]. In a 160-g scale process, hydrolysis was performed at pH 9.5 at 50°C. Acid produced was separated through a hollow-fiber membrane module and the esterase was very stable over four cycles of 48 h [19].

Further selected examples for the application of microbial carboxyl esterases in the synthesis of optically pure compounds are summarized in Fig. 2.

3.2. Other applications

Esterases can be also employed in reactions where chemo- or regioselectivity is of interest. The most prominent example is the use of carboxyl esterases in the release of ferulic acid from plant cell wall polysaccharides such as pectin or xylan. In xylans, ferulic acid is attached to ara-

binose residues, which are bound to the xylan backbone. In pectins, ferulic acid can be linked to galactose or arabinose in side chains. Ferulic acid thus obtained can be converted enzymatically into vanillin, a major flavor compound [25–27]. Feruloyl esterases have been isolated from a wide range of microorganisms [28–33]. Some of these esterases have been overexpressed in heterologous hosts and their properties are summarized in Table 2. In addition, the 2-*O* or 3-*O* positions of arabinose are often acetylated and the acetyl group can be also removed by the action of specific acetyl xylan esterases [34,35]. Properties of some of these recombinant enzymes are given in Table 2.

Microbial carboxyl esterases are also involved in the catabolism of aryl esters as shown for an enzyme found in *Acinetobacter* sp. [36], which releases an acid from benzyl esters. The resulting benzyl alcohols are then oxidized to benzoates by dehydrogenases and further metabolized in the β-ketoadipate pathway. For a lactone-specific esterase from *P. fluorescens*, it was proposed that the enzyme is involved in the hydrolysis of lactones formed by Baeyer–Villiger monooxygenases [37]. In the case of cyclohexanone, the resulting ω-hydroxy acids can then be further metabolized to adipate.

An application of considerable industrial interest is the mild removal of protecting groups as shown for a *p*-nitrobenzyl esterase from *B. subtilis*, which specifically removes this residue from the antibiotic Loracarbef [16]. As the enzyme is only weakly stable in dimethyl formamide, the esterase was improved by directed evolution (see Section 4.2).

4. Directed evolution of microbial esterases

4.1. Concept of directed evolution

Directed evolution (also called evolutive biotechnology or molecular evolution) is a technology developed in the early 1990s to generate desired enzyme variants or, more recently, to evolve metabolic pathways. Usually, random mutagenesis of the gene encoding the protein is performed by error-prone PCR, leading to huge libraries of mutants. Other methods are based on random recombination of DNA fragments such as DNA (or gene) shuffling, the Staggered Extension Process or random primer recombination. Libraries thus created are then assayed by high-throughput techniques to identify improved variants, as the huge number of mutants cannot be analyzed by common analytical tools such as gas chromatography and high-performance liquid chromatography. These are too time-consuming and expensive and therefore spectrophotometric methods are frequently used, especially photometric and fluorimetric assays performed in microtiter plate-based formats in combination with high-throughput robot assistance.

Prerequisites for directed evolution are the availability of the gene(s) encoding the enzyme(s) of interest, a suitable (usually microbial) expression system, an effective method to create mutant libraries which are then assayed by a suitable screening or selection system. In contrast to rational protein design, the knowledge of the 3D structure of the enzyme and its mechanism of catalysis are not necessary. It should also be emphasized, that a mutant library, once created, can be stored and subjected to new screening rounds if a new problem to be solved comes up.

Detailed overviews about methods for directed evolution as well as various examples for its successful application can be found in a considerable number of recent reviews [11,38–48].

4.2. Alteration of substrate specificity and enantioselectivity

Due to its very recent development, only a few examples for the directed evolution of esterases can be found. In my own group, we succeeded in altering the substrate specificity of an esterase from *P. fluorescens* (PFE) [49,50] by these methods. A sterically hindered 3-hydroxy ester not accepted as substrate by 20 wild-type hydrolases was stereoselectively hydrolyzed by a double-mutant of PFE generated using the mutator strain *Epicurian coli* XL1-Red [51,52]. The key to the identification of improved variants was an agar plate-assay system based on pH indicators, thus leading to a change in color upon hydrolysis of the ethylester. Parallel assay of replica-plated colonies on agar plates supplemented with the glycerol derivative of the 3-hydroxy ester was used to refine the identification, because only *E. coli* colonies producing active esterases had access to the carbon source glycerol, thus leading to enhanced growth and in turn larger colonies. Although this method was useful to turn an inactive wild-type enzyme into an active esterase, the best mutant showed only moderate enantioselectivity ($E \sim 5$).

Thus, in the next step, enantioselectivity of PFE was improved by directed evolution. Mutant libraries were created by error-prone PCR and by using the mutator strain. An extremely accurate determination of the enantioselectivity was achieved by using resorufin esters of (*R*)- or (*S*)-3-phenylbutyric acid, which allowed measurement of fluorescence in microtiter plates avoiding problems with interfering compounds present in the culture medium [53]. This led to the identification of variants, which exhibited enantioselectivities of $E = 5.2$–6.6 after a single round of mutation compared to an E of 3.5 for the wild-type enzyme. A further increase in enantioselectivity up to $E = 12$ was achieved by saturation mutagenesis at all three positions identified for the first-generation mutants (E. Henke and U.T. Bornscheuer, unpublished). However, combinations of the best variants by site-directed mutagenesis gave no further improvement (Figs. 2 and 3). It should be noted, that apparent E values (E_{app}) determined in microtiter plates were quite close to E_{true} values, which have been

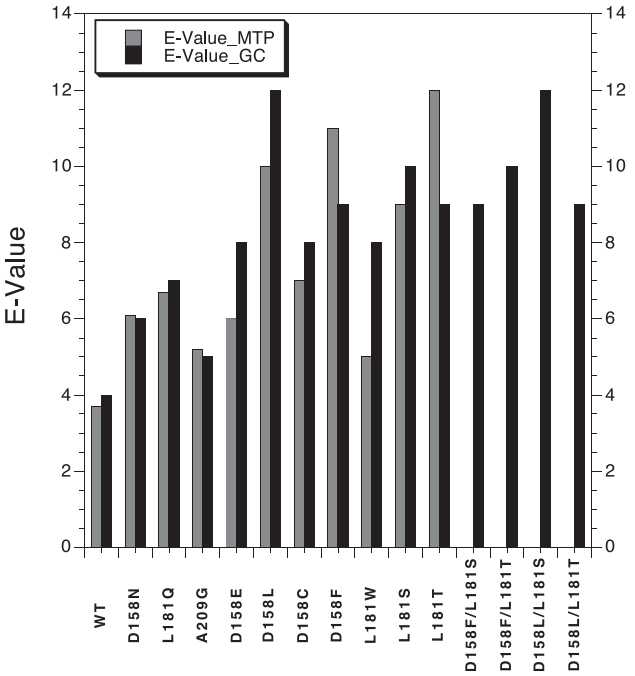

Fig. 3. Mutants obtained by directed evolution and saturation mutagenesis showing enhanced enantioselectivity in the resolution of 3-phenylbutyric acid derivatives. MTP: E values (E_{app}) determined in microtiter plates using the corresponding (R)- and (S)-resorufin ester [53]; GC: E values (E_{true}) calculated according to literature [56] from data determined by gas chromatographic analysis on a chiral column from samples obtained after esterase-catalyzed hydrolysis of (R,S)-3-phenyl butyric acid ethylester [50].

determined by gas chromatographic analysis of samples from resolutions of racemic mixtures of the corresponding ethylesters of 3-phenylbutyric acids. Here, competition between the two enantiomers is also included, whereas the microtiter plate-assay delivers only apparent E_{app} values.

Significantly higher increases in enantioselectivity were achieved for the resolution of methyl 3-bromo-2-methylpropionate (Fig. 2). Using a homology model of PFE, Trp_{29} and Phe_{199} were identified as positions for random mutagenesis. Libraries were then screened using Quick E [54], which is based on hydrolysis of optically pure (R)- or (S)-enantiomers in separate wells of microtiter plates in the presence of suitable pH indicators. The ratio of reaction rates for each enantiomer then yields E_{app}. Thus, mutant Trp29Leu was identified, which exhibited an $E = 90$ compared to $E = 12$ for wild-type PFE. This is, so far, the highest selectivity ever achieved by directed evolution techniques (U.T. Bornscheuer and R.J. Kazlauskas, unpublished), which now allows the synthesis of optically pure substrate and product of this compound.

4.3. Improvement of process stability and activity

Even if an enzyme with the desired substrate specificity and enantioselectivity is identified, its efficient application in an industrial process is often hampered by unsatisfactory process performance and stability. Directed evolution was successfully used to improve the stability and activity of an esterase from *B. subtilis*, which selectively cleaves the *p*-nitrobenzyl ester of Loracarbef, a cephalosporin antibiotic (see Section 3.2). Unfortunately, the wild-type enzyme was only weakly active in the presence of dimethylformamide (DMF), which must be added to dissolve the substrate. A combination of error-prone PCR and DNA-shuffling led to the generation of a variant with 150 times higher activity compared to the wild-type in 15% DMF [17,42]. Moreover, directed evolution also gave an increase in the thermostability of this esterase by ~14°C, thus making it more versatile in application [55].

5. Concluding remarks

It was shown in this review, that a significant number of microbial carboxyl esterases has been discovered so far and many of them have been overexpressed, enabling, in principle, production on a commercial scale. Moreover, our knowledge of their evolutionary relationships and the recent discovery of different consensus sequence motifs added considerably to our understanding of their functions. The large diversity of reactions and substrates handled by esterases in nature is still poorly explored. In part, this is caused by their limited commercial availability and the extensive use of many lipases in biocatalysis. These 'competing' existing enzymes often show higher enantioselectivity, stability in organic solvents and a broader substrate specificity than the esterases. However, modern methods of enzyme engineering – especially directed evolution – will certainly provide suitable carboxyl esterase variants with increased use in organic synthesis and other areas of application in the near future. The few examples of successfully evolved carboxyl esterases already show that this goal is indeed achievable.

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References

[1] Bornscheuer, U.T. and Kazlauskas, R.J. (1999) Hydrolases in organic synthesis – regio- and stereoselective biotransformations. Wiley-VCH, Weinheim.
[2] Phytian, S.J. (1998) Esterases. In: Biotechnology-Series, Vol. 8a (Rehm, H.J., Reed, G., Pühler, A., Stadler, P.J.W. and Kelly, D.R., Eds.), pp. 193–241. Wiley-VCH, Weinheim.
[3] Patel, R.N. (2000) Stereoselective Biocatalysis. Marcel Dekker, New York.
[4] Faber, K. (2000) Biotransformations in Organic Chemistry. Springer, Berlin.

[5] Drauz, K. and Waldmann, H. (1995) Enzyme Catalysis in Organic Synthesis, Vol. 1 and 2. Wiley-VCH, Weinheim.
[6] Wong, C.-H. and Whitesides, G.M. (1994) Enzymes in Synthetic Organic Chemistry. Pergamon Press, Oxford.
[7] Ollis, D.L. et al. (1992) The α/β hydrolase fold. Protein Eng. 5, 197–211.
[8] Wei, Y., Schottel, J.L., Derewenda, U., Swenson, L., Patkar, S. and Derewenda, Z.S. (1995) A novel variant of the catalytic triad in the *Streptomyces scabies* esterase. Nat. Struct. Biol. 2, 218–223.
[9] Petersen, E.I., Valinger, G., Sölkner, B., Stubenrauch, G. and Schwab, H. (2001) A novel esterase from *Burkholderia gladioli* shows high deacetylation activity on cephalosporins is related to β-lactamases and DD-peptidases. J. Biotechnol. 89, 11–25.
[10] Fojan, P., Jonson, P.H., Petersen, M.T.N. and Petersen, S.B. (2000) What distinguishes an esterase from a lipase: a novel structural approach. Biochimie 82, 1033–1041.
[11] Miller, C.A. (2000) Advances in enzyme discovery. Inform 11, 489–495.
[12] Pelletier, I. and Altenbuchner, J. (1995) A bacterial esterase is homologous with non-haem haloperoxidases and displays brominating activity. Microbiology 141, 459–468.
[13] Hecht, H.J., Sobek, H., Haag, T., Pfeifer, O. and van Pée, K.H. (1994) The metal-ion-free oxidoreductase from *Streptomyces aureofaciens* has an α/β hydrolase fold. Nat. Struct. Biol. 1, 532–537.
[14] Arpigny, J.L. and Jaeger, K.E. (1999) Bacterial lipolytic enzymes: classification and properties. Biochem. J. 343, 177–183.
[15] Kim, K.K., Song, H.K., Shin, D.H., Hwang, K.Y., Choe, S., Yoo, O.J. and Suh, S.W. (1997) Crystal structure of carboxylesterase from *Pseudomonas fluorescens*, an α/β-hydrolase with broad substrate specificity. Structure 5, 1571–1584.
[16] Zock, J. et al. (1994) The *Bacillus subtilis pnb*A gene encoding *p*-nitrobenzyl esterase: cloning, sequence and high-level expression in *Escherichia coli*. Gene 151, 37–43.
[17] Moore, J.C. and Arnold, F.H. (1996) Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents. Nat. Biotechnol. 14, 458–467.
[18] Pohlenz, H.D., Boidol, W., Schuttke, I. and Streber, W.R. (1992) Purification and properties of an *Arthrobacter oxydans* P52 carbamate hydrolase specific for the herbicide phenmedipham and nucleotide sequence of the corresponding gene. J. Bacteriol. 174, 6600–6607.
[19] Nishizawa, M., Shimizu, M., Ohkawa, H. and Kanaoka, M. (1995) Stereoselective production of (+)-*trans*-chrysanthemic acid by a microbial esterase: cloning, nucleotide sequence, and overexpression of the esterase gene of *Arthrobacter globiformis* in *Escherichia coli*. Appl. Environ. Microbiol. 61, 3208–3215.
[20] Quax, W.J. and Broekhuizen, C.P. (1994) Development of a new *Bacillus* carboxyl esterase for use in the resolution of chiral drugs. Appl. Microbiol. Biotechnol. 41, 425–431.
[21] Azzolina, O., Vercesi, D., Collina, S. and Ghislandi, V. (1995) Chiral resolution of methyl 2-aryloxypropionates by biocatalytic stereospecific hydrolysis. Il Farmaco 50, 221–226.
[22] Azzolina, O., Collina, S. and Vercesi, D. (1995) Stereoselective hydrolysis by esterase: a strategy for resolving 2-(*R*,*R*′-phenoxy)propionyl ester racemates. Il Farmaco 50, 725–733.
[23] Mustranta, A. (1992) Use of lipases in the resolution of racemic ibuprofen. Appl. Microbiol. Biotechnol. 38, 61–66.
[24] Nishizawa, M., Gomi, H. and Kishimoto, F. (1993) Purification and some properties of carboxylesterase from *Arthobacter globiformis*; stereoselective hydrolysis of ethyl chrysanthemate. Biosci. Biotech. Biochem. 57, 594–598.
[25] Falconnier, B., Lapierre, C., Lesage-Meessen, G., Yonnet, G., Brunerie, P., Colonna-Ceccaldi, B., Corrieu, G. and Asther, M. (1994) Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: identification of metabolic pathways. J. Biotechnol. 37, 123–132.
[26] Gasson, M.J. et al. (1998) Metabolism of ferulic acid to vanillin. J. Biol. Chem. 273, 4163–4170.
[27] Lesage-Meessen, G., Delattre, M., Haon, M., Thibault, J.-F., Colonna-Ceccaldi, B., Brunerie, P. and Asther, M. (1996) A two-step conversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. J. Biotechnol. 50, 107–113.
[28] Christov, L.P. and Prior, B.A. (1993) Esterases of xylan-degrading microorganisms: production, properties and significance. Enzyme Microb. Technol. 15, 460–475.
[29] Donaghy, J. and McKay, A.M. (1997) Purification and characterization of a feruloyl esterase from the fungus *Penicillium expansum*. J. Appl. Microbiol. 83, 718–726.
[30] Kroon, P.A., Williamson, G., Fish, N.M., Archer, D.B. and Belshaw, N.J. (2000) A modular esterase from *Penicillium funiculosum* which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module. Eur. J. Biochem. 267, 6740–6752.
[31] De Vries, R.P. et al. (1997) The faeA genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in degradation of complex cell wall polysaccharides. Appl. Environ. Microbiol. 63, 4638–4644.
[32] De Vries, R.P. and Visser, J. (1999) Regulation of the feruloyl esterase (faeA) gene from *Aspergillus niger*. Appl. Environ. Microbiol. 65, 5500–5503.
[33] Blum, D.L., Kataeva, I.A., Li, X.-L. and Ljungdahl, L.G. (2000) Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. J. Bacteriol. 182, 1346–1351.
[34] Blum, D.L., Li, X.-L., Chen, H. and Ljungdahl, L.G. (1999) Characterization of an acetyl xylan esterase from the anaerobic fungus *Orpinomyces* sp. strain PC-2. Appl. Environ. Microbiol. 65, 3990–3995.
[35] Lorenz, W.W. and Wiegel, J. (1997) Isolation, analysis, and expression of two genes from *Thermoanaerobacterium* sp. strain JW/SL YS485: a β-xylosidase and a novel acetyl xylan esterase with cephalosporin C deacetylase activity. J. Bacteriol. 179, 5436–5441.
[36] Jones, R.M., Collier, L.S., Neidle, E.L. and Williams, P.A. (1999) *areABC* genes determine the catabolism of aryl esters in *Acinetobacter* sp. strain ADP1. J. Bacteriol. 181, 4568–4575.
[37] Khalameyzer, V., Fischer, I., Bornscheuer, U.T. and Altenbuchner, J. (1999) Screening, nucleotide sequence and biochemical characterization of an esterase from *Pseudomonas fluorescens* with high activity toward lactones. Appl. Environ. Microbiol. 65, 477–482.
[38] Arnold, F.H. (1998) Design by directed evolution. Acc. Chem. Res. 31, 125–131.
[39] Arnold, F.H. and Volkov, A.A. (1999) Directed evolution of biocatalysts. Curr. Opin. Chem. Biol. 3, 54–59.
[40] Arnold, F.H. (1999) Unnatural selection: molecular sex for fun and profit. Eng. Sci. 40–50.
[41] Bornscheuer, U.T. (1998) Directed evolution of enzymes. Angew. Chem. Int. Ed. Engl. 37, 3105–3108.
[42] Arnold, F.H. and Moore, J.C. (1997) Optimizing industrial enzymes by directed evolution. Adv. Biochem. Eng. Biotechnol. 58, 1–14.
[43] Schmidt-Dannert, C. and Arnold, F.H. (1999) Directed evolution of industrial enzymes. Trends Biotechnol. 17, 135–136.
[44] Reetz, M.T. and Jaeger, K.-E. (1999) Superior biocatalysts by directed evolution. Top. Curr. Chem. 200, 31–57.
[45] Petrounia, I.P. and Arnold, F.H. (2000) Designed evolution of enzymatic properties. Curr. Opin. Biotechnol. 11, 325–330.
[46] Tobin, M.B., Gustafsson, C. and Huisman, G.W. (2000) Directed evolution: the 'rational' basis for 'irrational' design. Curr. Opin. Struct. Biol. 10, 421–427.
[47] Sutherland, J.D. (2000) Evolutionary optimisation of enzymes. Curr. Opin. Chem. Biol. 4, 263–269.
[48] Jaeger, K.E. and Reetz, M.T. (2000) Directed evolution of enantio-

selective enzymes for organic chemistry. Curr. Opin. Chem. Biol. 4, 68–73.

[49] Krebsfänger, N., Zocher, F., Altenbuchner, J. and Bornscheuer, U.T. (1998) Characterization and enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens*. Enzyme Microb. Technol. 22, 641–646.

[50] Krebsfänger, N., Schierholz, K. and Bornscheuer, U.T. (1998) Enantioselectivity of a recombinant esterase from *Pseudomonas fluorescens* towards alcohols and carboxylic acids. J. Biotechnol. 60, 105–111.

[51] Bornscheuer, U.T., Altenbuchner, J. and Meyer, H.H. (1999) Directed evolution of an esterase: screening of enzyme libraries based on pH-indicators and a growth assay. Bioorg. Med. Chem. 7, 2169–2173.

[52] Bornscheuer, U.T., Altenbuchner, J. and Meyer, H.H. (1998) Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of Epothilones. Biotechnol. Bioeng. 58, 554–559.

[53] Henke, E. and Bornscheuer, U.T. (1999) Directed evolution of an esterase from *Pseudomonas fluorescens*. Random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay. Biol. Chem. 380, 1029–1033.

[54] Janes, L.E. and Kazlauskas, R.J. (1997) Quick E. A fast spectrophotometric method to measure the enantioselectivity of hydrolases. J. Org. Chem. 62, 4560–4561.

[55] Giver, L., Gershenson, A., Freskgard, P.-O. and Arnold, F.H. (1998) Directed evolution of a thermostable esterase. Proc. Natl. Acad. Sci. USA 95, 12809–12813.

[56] Chen, C.S., Fujimoto, Y., Girdaukas, G. and Sih, C.J. (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. J. Am. Chem. Soc. 104, 7294–7299.

[57] Handa, S., Earlam, G.J., Geary, P.J., Hawes, J.E., Phillips, G.T., Pryce, R.J., Ryback, G. and Shears, J.H. (1994) The enantioselective synthesis of an important intermediate to the antiviral (-)-Carbovir. J. Chem. Soc. Perkin Trans. 1, 1885–1886.

[58] Ozaki, E. and Sakimae, A. (1997) Purification and characterization of recombinant esterase from *Pseudomonas putida* MR-2068 and its application to the optical resolution of dimethyl methylsuccinate. J. Ferment. Bioeng. 83, 535–539.

[59] Pogorevc, M., Strauss, U.T., Hayn, M. and Faber, K. (2000) Novel carboxyl esterase preparations for the resolution of linalyl acetate. Chem. Mon. 131, 639–644.

[60] Baumann, M., Hauer, B. and Bornscheuer, U.T. (2000) Rapid screening of hydrolases for the enantioselective conversion of 'difficult-to-resolve substrates'. Tetrahedron Asymmetry 11, 4781–4790.

[61] Liu, A.M.F. et al. (2001) Mapping the substrate selectivity of new hydrolases using colorimetric screening: lipases from *Bacillus thermocatenulatus* and *Ophiostoma piliferum*, esterases from *Pseudomonas fluorescens* and *Streptomyces diastatochromogenes*. Tetrahedron Asymmetry 12, 545–556.

[62] Hou, C.T. (1993) Screening of microbial esterases for asymmetric hydrolysis of 2-ethylhexyl butyrate. J. Ind. Microbiol. 11, 73–81.

[63] Molinari, F., Brenna, O., Valenti, M. and Aragozzini, F. (1996) Isolation of a novel carboxylesterase from *Bacillus coagulans* with high enantioselectivity toward racemic esters of 1,2-*O*-isopropylideneglycerol. Enzyme Microb. Technol. 19, 551–556.

[64] Marr, R., Gamse, T., Schilling, T., Klingsbichel, E., Schwab, H. and Michor, H. (1996) Enzymatic catalysis in supercritical carbon dioxide: comparison of different lipases and a novel esterase. Biotechnol. Lett. 18, 79–84.

[65] Reiter, B., Glieder, A., Talker, D. and Schwab, H. (2000) Cloning and characterization of EstC from *Burkholderia gladioli*. Appl. Microbiol. Biotechnol. 54, 778–785.

[66] Choi, K.D., Jeohn, G.H., Rhee, J.S. and Yoo, O.J. (1990) Cloning and nucleotide sequence of an esterase gene from *Pseudomonas fluorescens* and expression of the gene in *Escherichia coli*. Agric. Biol. Chem. 54, 2039–2045.

[67] Ozaki, E., Sakimae, A. and Numazawa, R. (1995) Nucleotide sequence of the gene for a thermostable esterase from *Pseudomonas putida* MR-2068. Biosci. Biotech. Biochem. 59, 1204–1207.

[68] Manco, G., Adinolfi, E., Pisani, F.M., Ottolina, G., Carrea, G. and Rossi, M. (1998) Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily. Biochem. J. 332, 203–212.

[69] Amaki, Y., Tulin, E.E., Ueda, S., Ohmiya, K. and Yamane, T. (1992) Purification and properties of a thermostabile esterase of *Bacillus stearophilus* produced by recombinant *Bacillus brevis*. Biosci. Biotech. Biochem. 56, 238–241.

[70] Amaki, Y., Nakano, H. and Yamane, T. (1993) Role of cysteine residues in esterase from *Bacillus stearothermophilus* and increasing its thermostability by the replacement of cysteines. Appl. Microbiol. Biotechnol. 40, 664–668.

[71] Ikeda, M. and Clark, D.S. (1998) Molecular cloning of extremely thermostable esterase gene from hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli*. Biotechnol. Bioeng. 57, 624–629.

[72] Fernandez, L., Beerthuyzen, M.M., Brown, J., Siezen, R.J., Coolbear, T., Holland, R. and Kuipers, O.P. (2000) Cloning, characterization, controlled overexpression, and inactivation of the major tributyrin esterase gene of *Lactococcus lactis*. Appl. Environ. Microbiol. 66, 1360–1368.

[73] Rathbone, D.A., Holt, P.-J., Lowe, C.R. and Bruce, N.C. (1997) Molecular analysis of the *Rhodococcus* sp. strain H1 her gene and characterization of its product, a heroin esterase, expressed in *Escherichia coli*. Appl. Environ. Microbiol. 63, 2062–2066.

[74] Bresler, M.M., Rosser, S.J., Basran, A. and Bruce, N.C. (2000) Gene cloning and nucleotide sequencing and properties of a cocaine esterase from *Rhodococcus* sp. strain MB1. Appl. Environ. Microbiol. 66, 904–908.

[75] Berger, R., Hoffmann, M. and Keller, U. (1998) Molecular analysis of a gene encoding a cell-bound esterase from *Streptomyces chrysomallus*. J. Bacteriol. 180, 6396–6399.

[76] Khalameyzer, V. and Bornscheuer, U.T. (1999) Overexpression and characterization of an esterase from *Streptomyces diastatochromogenes*. Biotechnol. Lett. 21, 101–104.

[77] Tesch, C., Nikoleit, K., Gnau, V., Götz, F. and Bormann, C. (1996) Biochemical and molecular characterization of the extracellular esterase from *Streptomyces diastatochromogenes*. J. Bacteriol. 178, 1858–1865.

[78] Koseki, T., Furuse, S., Iwano, K., Sakai, H. and Matsuzawa, H. (1997) An *Aspergillus awamori* acetylesterase: purification of the enzyme, and cloning and sequencing of the gene. Biochem. J. 326, 485–490.

[79] Fukuda, K., Kiyokawa, Y., Yanagiuchi, T., Wakai, Y., Kitamoto, K., Inoue, Y. and Kimura, A. (2000) Purification and characterization of isoamyl acetate-hydrolyzing esterase encoded by the IAH1 gene of *Saccharomyces cerevisiae* from a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 53, 596–600.