Chapter 12
Lipolytic Extremozymes from Psychro- and (Hyper-)Thermophilic Prokaryotes and Their Potential for Industrial Applications

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1 Introduction

Water insoluble lipids and fats compose a major part of earth's natural biomass. Lipolytic enzymes (lipases and esterases) are autocatalytic triacylglycerol acyl hydrolases catalyzing the release of free fatty acids and glycerol, monoacylglycerol and diacylglycerol from triglycerides (Fig. 12.1a). Lipases (EC 3.1.1.3) prefer water-insoluble long-chain acyl esters (>10 carbon atoms), while esterases (EC 3.1.1.1) catalyze the hydrolysis and synthesis of water-soluble short-chain fatty acids (<10 carbon atoms) (Fig. 12.2) (Bornscheuer 2002). In literature enzymes are mostly named "esterase" or "lipase" due to their preferred substrate. However, authors may refer to enzymes with preference for middle-chains length (C_{10}) as esterases or lipases, and there is no current agreement as to what chain length substrate preference delimits classification as a lipase versus esterase (Fuciños et al. 2011, 2014; Chow et al. 2012). Hence, regarding the differentiation based on substrate chain length, lipases and esterases can even be structurally engineered and transformed into one another.

Lipases are hydrophobic in nature and exhibit large hydrophobic surfaces at the active site region. Another feature of lipases is their interfacial activation, which is responsible for higher activity towards substrates at a water-micelle interface compared to substrate compounds dissolved in liquid solutions. Moreover, they do not only hydrolyze but also modify ester bonds including reverse esterification, and interesterification as well as aminolysis, alcoholysis (transesterification) and acidolysis (Wicka et al. 2013). Lipolytic enzymes usually exhibit enantio-, chemo- and regioselectivity making them superior over organic chemistry methods and valuable

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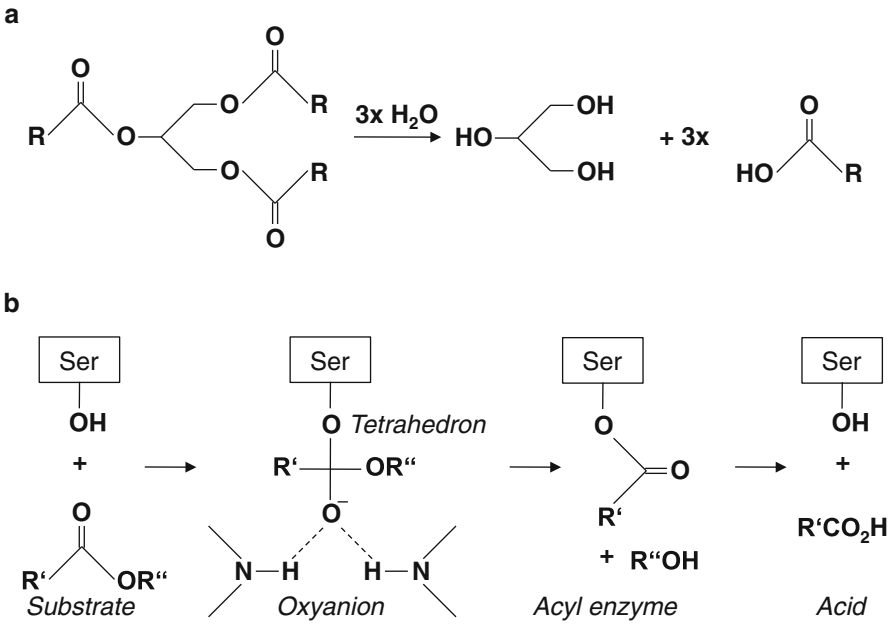

Fig. 12.1 Reaction scheme depicting lipolytic enzyme catalyzed hydrolysis of ester bonds to release three fatty acid residues and a glycerol (**a**) and illustration of reaction steps (**b**)

candidates for versatile applications. The physiological role of lipases in prokaryotes and eukaryotes is mainly the mobilization of lipids. These enzymes exhibit broad substrate specificity and solvent tolerance (organic solvents, ionic liquids, non-conventional solvents such as two-phase aqueous organic systems or microemulsion-based organogels) making them highly relevant for industrial applications including detergent, food and chemical industry as well as pharmaceutical, agrochemical and flavour sciences. Lipases also play a dominant role in the production of biofuels from lipids (Joseph et al. 2008; Hwang et al. 2014). Nowadays, lipolytic enzymes are considered to be one of the most important biocatalysts for biotechnological applications (Fig. 12.3) (Wicka et al. 2013). In particular, enzymes from extremophiles offer versatile ranges of applications including organic synthesis of chiral compounds, additives in food industries and in the biomedicine.

2 General Characteristics and Enzymatic Properties of Lipolytic Enzymes

Lipolytic enzymes belong to a large family of phylogenetically related biocatalysts. These enzymes have been identified in pro- and eukaryotic microorganisms, plants and animals. Most lipases and esterases consist of a compact and minimal domain

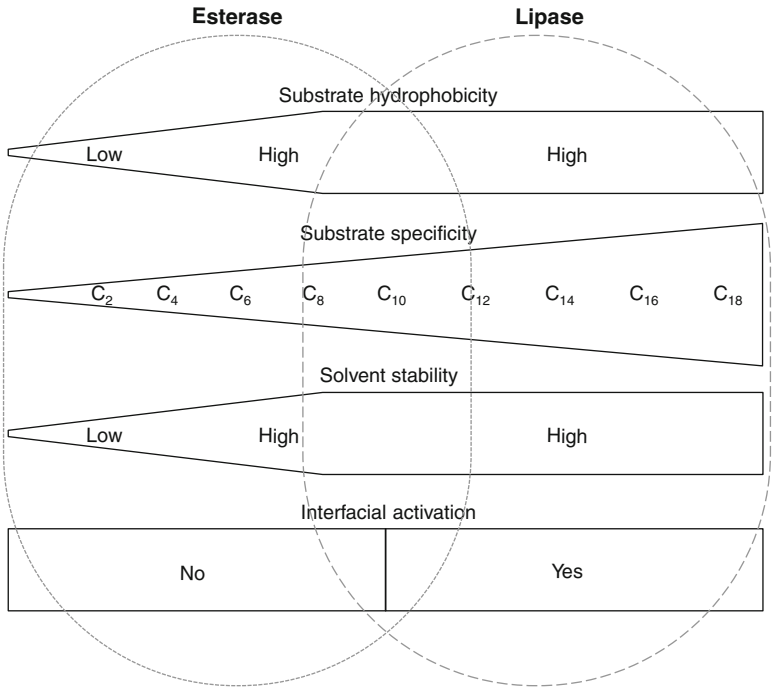

Fig. 12.2 Schematic representation of esterase and lipase properties with an overlap where features cannot be clearly assigned (Modified from Bornscheuer 2002)

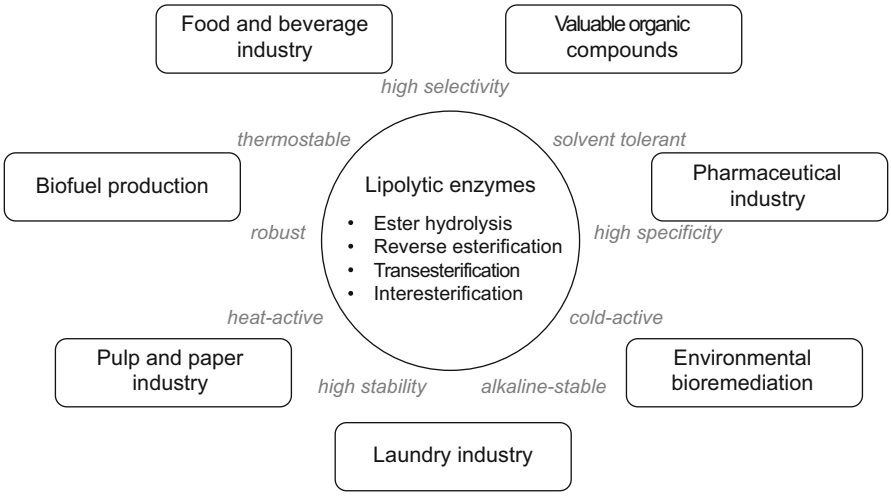

Fig. 12.3 Lipolytic enzyme catalyzed reactions, features and several applications

that can be clearly assigned to the α/β-hydrolase fold proteins often lacking additional structural domains. This typical fold consists of two layers of amphiphilic α-helices that enwrap a central hydrophobic core composed of eight β-sheets. However, there are some recent descriptions of variations including insertions of additional b-sheets in the conserved structural element (Arpigny and Jaeger 1999; Shaw et al. 2002; Siew et al. 2005). Their serine-protease like catalytic domain consists of a Ser-Asp-His triad, which is widespread in all serine hydrolases and is usually either solvent-exposed or closed by a flexible lid structure. The lid is composed of one or two α-helices that flips open in an activated conformation thereby guaranteeing access of a substrate to the active enzyme site. The lid was described as exclusive characteristic of lipases due to the movement of this domain in the presence of water-lipid interface (interfacial activation) (Bornscheuer 2002). The serine in the catalytic region acts as the nucleophile, histidine as the base and aspartate or glutamate as the acidic residue (Bornscheuer 2002). Moreover, this serine is embedded in a highly conserved pentapeptide Gly-X-Ser-X-Gly that is typical for the α/β-hydrolase superfamily (Bornscheuer 2002).

A classification for lipolytic enzymes based on amino acid sequence comparisons and some biological properties was proposed in 1999 (Arpigny and Jaeger 1999). Eight families (I–VIII) were specified with family I containing true lipases with the catalytic serine being embedded in the highly conserved motif Gly-His-Ser-X-Gly. Further six subgroups (I.1–I.6) were determined. Subfamily I.3 comprises enzymes from *Pseudomonas* sp. and others, while lipolytic enzymes from *Geobacillus* sp. belong to subfamily I.5 (Fig. 12.4). In family II (GDSL-family)

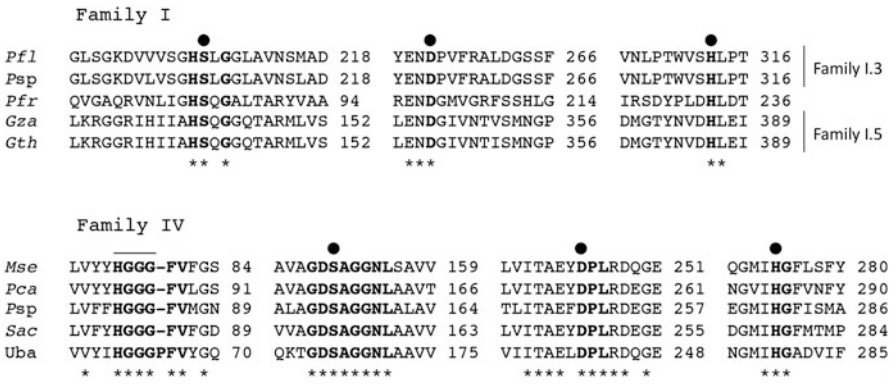

Fig. 12.4 Partial amino acid sequence alignments of conserved motifs of lipolytic enzymes assigned to family I, with two sequences grouped into subfamily I.3 and I.5 respectively, and family IV. Family I enzymes are depicted from *Pseudomonas fluorescens* (*Pfl,* AY694785), *Pseudomonas* sp. YY31 (*P*sp, AB642679), *Pseudomonas fragi* (*Pfr*, AJ250176), *Geobacillus zalihae* T1 (*Gza*, AY260764) and *Geobacillus thermoleovorans* YN (*Gth*, DQ298518). Family IV enzymes are depicted from *Metallosphaera sedula* DSM5348 (*Mse*, YP001191160), *Pyrobaculum calidifontis* (*Pca*, AB078331), *Pseudomonas* sp. B11-1 (*P*sp, AF034088), *Sulfolobus acidophilus* DSM10332 (*Sac*, AEW03609) and an uncultured bacterium (Uba, EF563989). Residues participating in the catalytic triad (•) and in oxyanion hole formation are highlighted (–)

Gly-Asp-Ser-(Leu) replaces the pentapeptide, while family III contains extracellular lipases from species of the genera *Streptomyces* and *Moraxella*. Family IV comprises the typical serine encompassing motif Gly-Asp-Ser-Ala-Gly-(Gly). This family is also described as the HSL (hormone-sensitive lipase) family due to high similarities to mammalian HSL. Lipolytic enzymes from psychrophiles (growth at 0–30 °C), mesophiles (30–45 °C) and thermophiles (50–80 °C) as well as from hyperthermophilic archaea (80–110 °C) could be assigned to HSL (Fig. 12.4). The pentapeptide is modified in family V to give Gly-X-Ser-(Met)-Gly-Gly, in family VI to Gly-Phe-Ser-Gln-Gly, in family VII to Gly-Phe-Ser-Gln-Gly-Gly and in family VIII to Ser-X-X-Lys (Arpigny and Jaeger 1999). In addition, other conserved amino acid arrangements were described for some of the different families. Since 1999 this classification has been extended by extensive elucidation of metagenomic sequences coding for novel lipolytic hydrolases showing low similarities to known enzymes (Kim et al. 2008; Nacke et al. 2011). Newly proposed families were rarely specific with regard to motifs or family names. Thereupon a continuation of the established family classification was proposed with family IX (PhaZ7), X (EstD), XI (LipG), XII (LipEH166), XIII (Est30) and XIV (EstA3). The conserved pentapeptides of these families were reported to be composed of Ala-His-Ser-Met-Gly, Gly-His-Ser-Leu-Gly, Gly-His-Ser-Leu-Gly-Gly, Gly-His-Ser-Leu-Gly, Gly-Leu-Ser-Leu-Gly-Gly and Cys-His-Ser-Met-Gly, respectively (Rao et al. 2011). Simultaneously, family X was identified as the LipR-cluster and consists of enzymes with the pentapeptide Gly-Tyr-Ser-Gly-Gly (Bassegoda et al. 2012b). Another new family (XV) was proposed for lipolytic enzymes exhibiting the serine-embedding motif Ser-His-Ser-Gln-Gly (Bayer et al. 2010). Subsequently, family XVI (Bacterial_Est97) further expanded the classification (Lenfant et al. 2013). Moreover, an alignment of proteins from different *Thermus* species (growth at 50–70 °C) resulted in the discovery of the putative pentapeptide Gly-Cys-Ser-Ala-Gly probably representing yet another family (Fuciños et al. 2011). These publications reflect the large number and the diversity of lipolytic enzymes from mesophiles and extremophiles.

The catalytic mechanism of α/β-hydrolases is composed of five reaction steps, beginning with the binding of an ester substrate and formation of a tetrahedral intermediate formed by catalytic serine mediated nucleophilic attack. An oxyanion hole is stabilized by two to three hydrogen bonds before the ester bond is cleaved. Afterwards, the alcohol moiety is released from the enzyme and finally the acyl enzyme is hydrolyzed (Joseph et al. 2008) (Fig. 12.1b). Among the most important properties of lipolytic enzymes for industrial applications are their thermostability and temperature range for optimal activity. Moreover, different lipolytic enzymes exhibit versatile properties with regard to their pH optima, tolerance towards detergents and metal ions, kinetics, fatty acid specificity and positional specificity. Structural modifications that are relevant for enzyme flexibility at certain conditions were investigated in cold- and heat-active lipolytic enzymes (extremozymes). In this context, it was hypothesized that lysine residues are replaced by arginine in enzymes from psychrophiles. Moreover, the number of prolines, loop structures, disulphide and salt bridges, aromatic-aromatic and hydrophobic interactions is reduced to adapt for cold environments and thermolability. In addition, an increased

number of glycine residues was speculated to be important for local mobility of peptide regions (Joseph et al. 2008). It is also well accepted that the flexible catalytic site of cold-active enzymes is more susceptible to heat-inactivation compared to the complete enzyme structure, while biocatalysts from thermo- and most mesophiles usually display highest activity near the protein denaturation temperature (Feller and Gerday 2003; Wi et al. 2014).

3 Lipolytic Enzymes from Psychrophiles

Psychrophiles are microorganisms that populate cold environments such as sea ice, deep sea, and snowfields. Their enzymes function more effectively at cold temperatures compared to their counterparts from meso- and thermophilic microorganisms (Elleuche et al. 2014). The most studied lipase from a psychrophilic microorganism is the highly enantioselective enzyme CalB, which has been isolated from the hemiascomycetous yeast *Candida antarctica*. CalB is only mentioned for completeness due to its eukaryotic origin and because of its importance as a model lipase (Joseph et al. 2008).

3.1 Diversity of Cold-Active Esterases and Lipases

Although most of earth's biosphere is cold and inhabited by psychrophilic microorganisms, only a few cold-active esterases and lipases have been isolated from bacterial strains thriving in permanently cold sea ice, soil or glaciers, in the deep sea or mountain regions (Al Khudary et al. 2010; Wu et al. 2013; Wi et al. 2014; Parra et al. 2015; Tchigvintsev et al. 2015). Other cold-active bacterial lipolytic enzymes were isolated from food samples e.g. *Serratia marcescens* lipase (Abdou 2003).

Most of the lipolytic enzymes that are considered to be "cold-active", display optimal activity around 30 °C and are rapidly inactivated at temperatures above 45 °C, but there are some exceptions, e.g. a lipase from the Alaskan psychrothrophic bacterium *Pseudomonas* sp. B11-1 that is optimally active at 45 °C (Choo et al. 1998). Moreover, such biocatalysts often retain activity around the freezing point of water. A cold-active lipase from a metagenomic library, derived from oil contaminated soil, retained activity even at −5 °C (Elend et al. 2007). While most cold-active lipolytic enzymes were purified and characterized from their natural hosts, there are sparse descriptions of heterologously expressed genes encoding lipolytic enzymes (Table 12.1). To provide cold-active lipolytic enzymes in quantitative amounts, recombinant enzymes have been produced in *E. coli* and other mesophilic heterologous hosts (Feller et al. 1996; Trincone 2011). Modern recombinant DNA-technology provides a tool to increase enzyme production of isozymes that can only be produced and purified in low yield. Cloning of lipase-encoding genes from psychrotrophic microorganisms to be expressed in *E. coli* had already been reported at the end of the 1980s and beginning of 1990s, when lipases from *P. fragi* and *Moraxella* TA144 were cloned and sequenced (Aoyama et al. 1988; Feller et al. 1991). Comparison

Table 12.1 Recombinant lipolytic enzymes from psychrophilic bacteria

Sources	T_{opt} (°C)	pH_{opt}	MW (kDa)	Preferred substrate	References
Esterases[a]					
Uncultured bacterium (Lipo1)	10	7.5	35.6	*p*NP-butyrate (C_4)	(Roh and Villatte 2008)
Bacillus pumilus ArcL5 (BpL5)	20	9.0	19.2	*p*NP-caprylate (C_8)	(Wi et al. 2014)
Uncultured bacterium (CHA2)	20	11	34.7	*p*NP-propionate (C_3)	(Hu et al. 2012)
Pseudoalteromonas sp. 643A (EstA)	20	7.5	23.0	*p*NP-butyrate (C_4)	(Dlugolecka et al. 2009)
Pseudoalteromonas arctica (EstO)	25	7.5	44.1	*p*NP-butyrate (C_4)	(Al Khudary et al. 2010)
Psychrobacter pacificensis (Est10)	25	7.5	24.6	*p*NP-butyrate (C_4)	(Wu et al. 2013)
Pseudomonas fragi (rPFL)	29	8.0	32.0	Tributyrin (C_4)	(Alquati et al. 2002)
Pseudomonas fluorescens (LipA)	30	9.5	42.0	(S)-ketoprofen ethyl ester (C_3)	(Choi et al. 2003)
Rhodococcus sp. (RhLip)	30	11.0	38.0	*p*NP-butyrate (C_4)	(De Santi et al. 2014)
Uncultured bacterium (LipA)	35	8.0	32.2	*p*NP-butyrate (C_4)	(Couto et al. 2010)
Pseudomonas sp. B11-1 (LipP)	45	8.0	33.7	*p*NP-butyrate (C_4)	(Choo et al. 1998)
Lipases[a]					
Pseudomonas fluorescens (LipB68)	20	8.0	50.2	*p*NP-caprate (C_{10})	(Luo et al. 2006)
Photobacterium lipolyticum M37 (M37 lipase)	25	8.0	38.0	*p*NP-caprate (C_{10})	(Ryu et al. 2006)
Uncultured bacterium (rEML1)	25	8.0	33.6	Trilaurin (C_{12})	(Jeon et al. 2009)
Colwellia psychrerythraea 34H (CpsLip)	25	7.0	35.0	*p*NP-laurate (C_{12})	(Do et al. 2013)
Pseudomonas sp. YY31 (LipYY31)	25–30	8.0	49.5	*p*NP-caprate (C_{10})	(Yamashiro et al. 2013)
Pseudomonas sp. TK-3 (LipTK-3)	25–30	8.0	50.1	*p*NP-caprate (C_{10})	(Tanaka et al. 2012)
Psychrobacter sp. 7195 (LipA1)	30	9.0	35.2	*p*NP-myristate (C_{14})	(Zhang et al. 2007)
Pseudomonas sp. 7323 (LipA)	30	9.0	64.4	*p*NP-caprate (C_{10})	(Zhang and Zeng 2008)
Uncultured bacterium (LipCE)	30	7.0	53.2	*p*NP-caprate (C_{10})	(Elend et al. 2007)
Pseudomonas sp. KB700A (KB-Lip)	35	8.0–8.5	49.9	*p*NP-caprate (C_{10})	(Rashid et al. 2001)

[a]Enzymes were assigned to the groups of esterases or lipases based on their substrate preferences

with wildtype enzymes revealed that such cold-active lipases conserved their main properties as recombinant proteins (Feller et al. 1991). Nowadays, cold environments have been shown to be a rich source of diverse cold-adapted enzymes. To identify novel lipolytic enzymes from cold environments, activity-based screenings were established starting with gene expression induced by a lipid-containing feedstock such as olive or soybean oil, milk, triolein, tributyrin or tricaprylin (Henne et al. 2000; Joseph et al. 2008; Wi et al. 2014). In a recent study, 23 unique active clones were isolated by screening 274,000 clones from gene libraries that were prepared from different seawater samples collected in the Barents Sea (Russia) and the Mediterranean Sea (Italy) with temperatures between 3 °C and 15 °C at the sampling sites. The catalytic properties of five highly active carboxylesterases were examined, displaying broad biochemical versatility including temperature optima at 15 °C or 30 °C and substrate preferences for *para*-nitrophenol- (*p*NP-) valerate (C_5), *p*NP-propionate (C_3) or α-naphthyl-propionate (C_3), and *p*NP-octanoate (C_8), respectively (Tchigvintsev et al. 2015). A different strategy was recently applied to identify lipase-encoding genes from Antarctic seawater. Consensus-degenerate hybrid oligonucleotide primers were designed to amplify novel genes from isolated genomic DNA, allowing the identification of a typical cold-active lipase that prefers *p*NP-caproate (C_{10}) at temperatures between 15 °C and 25 °C (Parra et al. 2015).

Among the members with the lowest temperature optimum is an esterase (Lipo1) from an uncultivated microorganism that is optimally active at 10 °C (Roh and Villatte 2008). Moreover, Lipo1 displayed a higher stability at 10 °C compared to 40 °C, making it a potential candidate for applications in organic chemistry. Screening of a genomic library of the deep sea psychrotrophic bacterium *Psychrobacter paci*fi*censis*, which was isolated from sediments in the Gulf of Mexico, revealed the existence of a cold-adapted and salt-tolerant esterase. The enzyme Est10 was optimally active at 25 °C and retained 55 % of its activity at 0 °C. Moreover, the catalytic activity and stability was even increased in the presence of high salt concentrations (between 2 and 5 M NaCl). As a typical esterase, this enzyme preferred *p*NP-butyrate (C_4) as substrate (Wu et al. 2013). Besides lipolytic enzymes from "true" psychrophilic bacteria, there are a number of candidates from mesophiles that exhibit cold-active enzymes including several lipases from different species of the genus *Bacillus* e.g. *Bacillus* sp. HH-01 (Kamijo et al. 2011). A further example is the cold-active esterase EstC from *Streptomyces coelicolor* A3(2). Genome mining revealed that this bacterium has at least 50 genes coding for putative lipolytic enzymes. EstC preferred *p*NP-valerate (C_5) as substrate and was optimally active at 35 °C. Moreover, this hydrolase retained 25 % of its activity when incubated at 10 °C (Brault et al. 2012).

3.2 *Improving Enzymatic Properties of Psychrophiles by Genetic Engineering*

Cold-active enzymes represent a promising target to increase thermal robustness or efficiency by molecular biology techniques including directed evolution and rational protein design (Bassegoda et al. 2012a; Joshi and Satyanarayana 2015). The

thermolabile nature of cold-active enzymes is highly amenable to sequence and structure modifications. Site-directed mutagenesis resulting in a single amino acid mutation (T103G) increased CalB's stability at high temperatures, but went along with 50 % reduced activity at the same time (Patkar et al. 1998). The lipolytic enzyme BpL5 from the arctic psychrophile *Bacillus pumilus* ArcL5 was optimally active at 20 °C and retained around 85 % of its activity at 5 °C. Site-directed mutagenesis of a serine based on structural model predictions significantly improved the catalytic activity for *p*NP-caprylate and tricaprylin (C_8), without affecting pH and temperature optima (Wi et al. 2014). In another approach, directed evolution was applied to enhance the organic solvent-stability of a cold-active lipase, Lip9, from *Pseudomonas aeruginosa* LST-03. This microorganism has tolerance of organic solvents itself and secretes a highly stable enzyme that has been produced in recombinant form in *E. coli*. In a first approach, Lip9 was purified from inclusion bodies and refolded by the assistance of a natural lipase-specific foldase from the same bacterium (Ogino et al. 2007). Enhancement of enzyme stability against organic solvents was evaluated by a developed screening assay using dimethyl sulfoxide (DMSO) in addition to the substrate tri-*n*-butyrin. Identified candidates were sequenced and tested with different organic solvents. Using this approach, the mutant enzyme LST-03-R65 was identified displaying a 9- to 11-fold increased stability in the presence of cyclohexane and *n*-decane. This enzyme accumulated amino acid mutations on the surface of the protein and it has been speculated that such amino acid substitutions might be beneficial to prevent organic solvents from penetrating the protein's interior (Kawata and Ogino 2009). Most efforts were undertaken to improve thermal stability of enzymes, because thermal denaturation is the major route for enzyme inactivation under industrial process conditions. In good agreement with mutations mediating stability towards organic solvents, amino acid residues on the protein surface were also shown to be relevant for thermal robustness. A lipase variant from *Bacillus subtilis* with nine amino acid substitutions displayed a 15 °C increased melting temperature in addition to a 20 °C shifted temperature optimum (Ahmad et al. 2008). The psychrophilic bacterium *Pseudomonas fragi* produced a cold-active lipase, PFL, with a temperature optimum at 29 °C (Alquati et al. 2002). PFL is even highly active and stable at 10 °C, but completely inactivated when incubated for short time periods at moderate temperatures, i.e. 19 min at 42 °C. Error-prone polymerase chain reaction led to the development of an enzyme variant with four amino acid substitutions that exhibited a fivefold increase in half-life at 42 °C and a 10 °C shift of the temperature optimum (Gatti-Lafranconi et al. 2008). Another strategy to predict stabilizing mutations would be to compare lipolytic enzymes from psychrophiles with their homologous meso- or thermophilic counterparts (Bassegoda et al. 2012a). In case of the multidomain carboxylesterase EstO from the arctic psychrophile *P. arctica*, it has been shown that the deletion of the non-catalytic OsmC domain resulted in an increased thermal stability of the functionally active α/β-hydrolase fold domain. Detailed investigation of the primary and secondary structure in combination with characterization experiments revealed that the OsmC domain is probably important for flexibility and adaptation to low temperatures (Al Khudary et al. 2010; Elleuche et al. 2011). Such genetically modified enzyme candidates are attractive for biotechnological applications in diverse industrial processes (Fig. 12.3).

3.3 Applications of Cold-Active Lipolytic Enzymes

Unusual specificities and high activity at low temperatures offer opportunities for cold-active lipolytic enzymes to be exploited and applied in versatile industrial fields. Lipases and esterases from psychrophilic microorganisms are mainly of interest for use in the organic synthesis of fragile chiral compounds, but they are also applied as additives in food industries (cheese manufacture, bakery) and as detergents (energy saving, cold washing) as well as in environmental biotransformations (bioremediations), biomedicine (pharmaceuticals) or molecular biology approaches (Joseph et al. 2008; Joshi and Satyanarayana 2015). Moreover, lipases were also shown to be of interest to prevent inclusion bodies formation in psychrophilic host organisms (Feller et al. 1996). Development of economically feasible industrial applications of cold-active lipolytic enzymes is currently hindered in some instances by their low specific activity, poor recyclability, and unavailability in purified form. However, there are several examples of cold-active lipolytic enzymes that are established in industrial processes, or that have been patented for diverse applications or that have been investigated with regard to their potential for industrial biotechnology. Such an application not only saves energy by using lower temperatures, but also allows for the reduction of the amount of chemicals and detergents that are harmful to the environment, i.e. enzymatic desizing of materials is environmentally favourable compared to the traditional utilization of acidic and oxidizing chemical compounds. Moreover, non-aqueous solvents offer opportunities for the biotechnological production of valuable fine chemicals catalyzed by lipolytic enzymes. This has been shown to be useful in the modification of oils and fats and for the production of sugar-based polymers or to produce surfactants, emulsifiers, structured lipids and wax esters or to synthesize fragrance compounds (Joseph et al. 2008). The cold-active prototype enzyme for industrial applications is CalB from the hemiascomycetous yeast *C. antarctica* that has been patented for applications in food, chemical and pharmaceutical applications. Interestingly, this enzyme is highly thermostable in non-aqueous solutions when immobilized on solid carriers (Koops et al. 1999).

In principle, lipases are most successful as ingredients in detergent solutions for laundry and in household dishwaters. Lipolytic enzymes applicable in detergents remain active and stable at alkaline conditions (pH 10–11) and at moderate temperatures (Wicka et al. 2013). Novozymes launched the first genetically engineered industrial enzyme – Lipolase® from the fungus *Thermomyces lanuginosa* produced in the filamentous ascomycete *Aspergillus oryzae* – in 1988. This enzyme is applicable in the laundry industry to remove clothing stains such as fats, butter, lipstick, sauces and salad oil. Furthermore, it has been engineered and Novozyme came up with second and third generation variants that have also found their way to the market in different detergent formulations. Another well-known example for a washing enzyme is Lipomax®, an engineered variant of *Pseudomonas alcaligenes* lipase that has been introduced by Gist-Brocades in 1995 (Joshi and Satyanarayana 2015). Moreover, lipolytic enzymes were used as additives in bleaching compositions, in contact lens cleaning or in liquid leather cleaning (Joseph et al. 2008).

It is also desirable in the food industry to carry out reactions at low temperatures. Thereby, contaminations and changes in the composition of ingredients and flavour can be avoided. Especially lipases from mould have been used to improve traditional chemical processes in food manufacture. The potential of lipolytic enzymes of prokaryotic origin has also been evaluated to improve the aroma or enhance the flavour of food, e.g. a lipase from psychrotrophic *Pseudomonas* strain P38 catalyzes ester synthesis of a flavouring compound with an organic solvent phase at low temperatures (Tan et al. 1996). Moreover, lipolytic enzymes are important to enrich manifold unsaturated fatty acids from animal and plant lipids or to change the position or replace the fatty acid attached to the glycerol backbone (Wicka et al. 2013).

Cold-active lipases have also great potential for the production of biodiesel via transesterification reactions. An enzyme from the psychrophilic bacterium *Pseudomonas fluorescens* B68, LipB68, was highly active at low temperatures and was capable of producing 92 % of biodiesel from soybean oil after 120 h of incubation at 20 °C. Running the process at lower temperatures allows substantial savings in energy consumption (Luo et al. 2006).

Lipolytic enzymes are also attractive for the pharmaceutical industry due to substrate promiscuity and the catalysis of regioselective reactions in various organic solvents. Compared to organic chemistry, enzyme catalyzed processes are environmentally favourable and offer a higher degree of selectivity in the production of optimally active chiral drugs (Fig. 12.5). Moreover, none-racemic chiral building block chemicals represent valuable compounds for the fine chemical industry. One of the most prevalent anti-inflammatory drugs is ketoprofen [(R,S)-2-(3-benzoylphenyl) propionic acid] whereof S-ketoprofen is pharmacologically active (de O. Carvalho et al. 2006). Further examples of non-steroidal anti-inflammatory drugs to combat human diseases that are processed by lipolytic enzymes are naproxen and ibuprofen (Hess et al. 2008). Moreover, a lipase from *S. marcescensis* is applied to produce the calcium channel blocking drug Diltiazem (Wicka et al. 2013). Although protein engineering of cold-active lipolytic enzymes has steadily improved enzyme properties, industrial applications have not been rapidly developed compared to heat-active enzymes from thermophiles (Joseph et al. 2008).

4 Lipolytic Enzymes from (Hyper-)Thermophiles

Thermophilic microorganisms grow at high temperatures between 50 °C and 80 °C and mainly include members of the *Bacteria* and *Archaea*, while only some eukaryotic moderate thermophiles (growth below 60 °C) have been identified. In addition, archaea dominate the group of hyperthermophiles that are even adapted to temperatures up to 110 °C. Typical environments that are inhabited by thermophilic microorganisms include hot springs, hydrothermal vents and volcanic islands. Although, thermostable enzymes (thermozymes) are found in mesophilic and thermophilic microorganisms, thermophiles produce enzymes that are comparatively robust and hence are preferably used for industrial applications (Hasan et al. 2005).

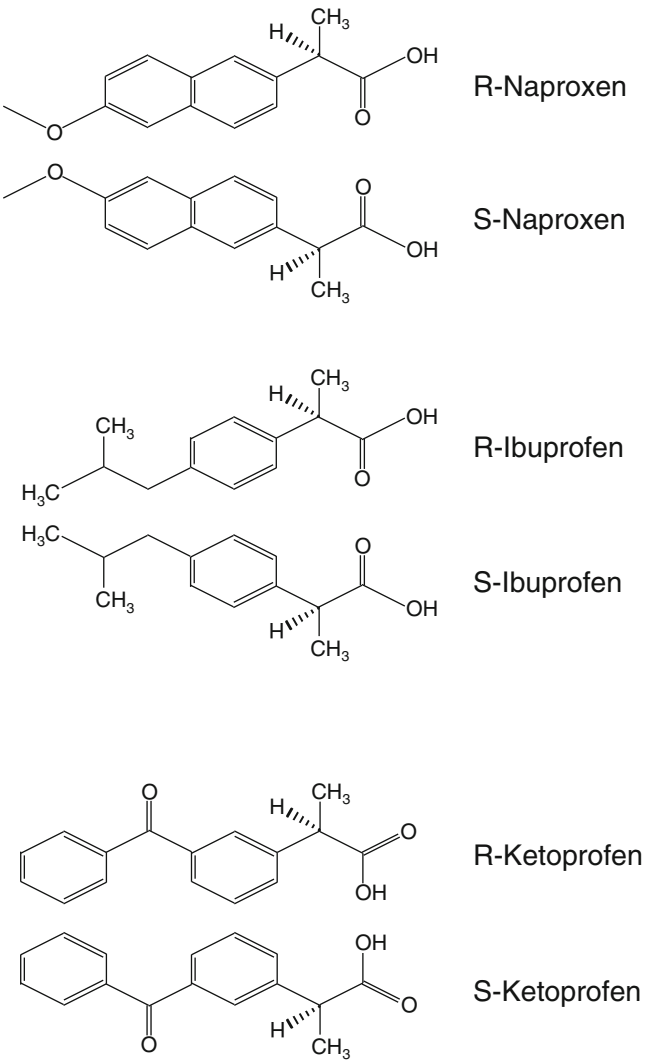

Fig. 12.5 Molecular structures of chiral drugs that can be produced by lipolytic enzyme catalyzed esterifications

4.1 Diversity of Heat-Active Lipases and Esterases

The recent growing demand and interest for enzymes from thermophilic microorganisms led to the identification and characterization of many heat-active lipases. Table 12.2 focuses on promising recombinant lipolytic enzymes from thermophilic bacteria. These enzymes showed the best catalytic performance between 55 °C and 80 °C and have molecular masses between 19 and 53 kDa. The highest temperature optimum of a characterized recombinant esterase EstTs1 from a thermophilic

Table 12.2 Recombinant lipolytic enzymes from thermophilic bacteria

Sources	T_{opt} (°C)	pH_{opt}	MW (kDa)	Preferred substrate	References
Esterases[a]					
Acidicaldus USBA-GBX-499	55	9.0	34	*p*NP-caproate (C_6)	(Lopez et al. 2014)
Thermus thermophiles HB27	58.2	6.3	37.7	*p*NP-caprate (C_{10})	(Fuciños et al. 2014)
Anoxybacillus sp. PDF1	60	8.0	26	*p*NP-butyrate (C_4)	(Ay et al. 2011)
Geobacillus thermoleovorans YN	60–65	9.5	29	*p*NP-acetate (C_2)	(Soliman et al. 2007)
Metagenome (activated sludge)	70	8.5	53	*p*NP-acetate (C_2)	(Shao et al. 2013)
Thermoanaerobacter tengcongensis MB4	70	9.5	50	*p*NP-caproate (C_6)	(Rao et al. 2011)
Thermosyntropha lipolytica	70	8.0	–	*p*NP-butyrate (C_4)	(Gumerov et al. 2012)
Thermotoga maritima	70	5.0–5.5	35.2	*p*NP-butyrate (C_4)	(Tao et al. 2013)
Thermus scotoductus SA-01	80	7.0	28.6	*p*NP-butyrate (C_4)	(du Plessis et al. 2010)
Lipases[a]					
Geobacillus sp. EPT9	55	8.5	44.8	*p*NP-palmitate (C_{16})	(Zhu et al. 2015)
Bacillus thermoleovorans ID-1	60	8.0–9.0	43	*p*NP-caprate (C_{10})	(Lee et al. 2001)
Bacillus thermoleovorans ID-1	60–65	9.0	19	*p*NP-caprylate (C_8)	(Lee et al. 2001)
Geobacillus sp. strain T1	70	9.0	43	Trilaurin (C_{12})	(Leow et al. 2007)
Geobacillus thermoleovorans YN	70	9.5	43	*p*NP-caprate (C_{10})	(Soliman et al. 2007)
Enrichment cultures (soil and water samples)	70	8.0	31.7	*p*NP-caprylate (C_8)	(Chow et al. 2012)
Enrichment cultures (soil and water samples)	75	8.0	36	*p*NP-caprate (C_{10})	(Chow et al. 2012)
Thermoanaerobacter thermohydrosulfuricus SOL1	75	8.0	34.2	*p*NP-caprate (C_{10})	(Royter et al. 2009)
Caldanaerobacter subterraneus subsp. *tengcongensis*	75	7.0	32.1	*p*NP-caprate (C_{10})	(Royter et al. 2009)
Fervidobacterium changbaicum	78	7.8	33	*p*NP-caprate (C_{10})	(Cai et al. 2011)

[a]Enzymes were assigned to the groups of esterases or lipases based on their substrate preferences

bacterium is 80 °C isolated from *Thermus scotoductus* (du Plessis et al. 2010). A recombinant lipase with a temperature optimum of 78 °C was derived from *Fervidobacterium changbaicum* (Cai et al. 2011). Two lipases purified directly from culture supernatant of *Thermosyntropha lipolytica* DSM1103 were described with highest activity at 96 °C (Salameh and Wiegel 2007). Within the scope of metagenomic approaches various novel lipolytic enzymes were discovered. Thermophilic microorganisms present in environmental samples can be enriched at elevated temperatures and used for metagenomic library construction. A metagenome can be isolated directly from suitable environments predominated by high temperatures to exploit the diversity and to gain access to non-cultivable organisms. Unique enzymes with low similarities to known proteins were found as shown for an esterase identified from a soil metagenome with 32–45 % identity to putative α/β-hydrolases (Choi et al. 2013). Another functional screening approach led to the identification of two genes encoding lipases that were optimally active at 70 °C and 75 °C (Chow et al. 2012).

The majority of characterized carboxylic ester hydrolases from thermophilic bacteria were described as esterases. Lipolytic enzymes are usually assigned to the class of esterases or lipases due to their substrate spectrum. Regarding the chain length of linked fatty acids within artificial substrates, some lipolytic enzymes display esterase and lipase activity (Fig. 12.2). TSLip1 from *Thermosynthropha lipolytica* exhibited the highest activity at 70 °C towards *p*NP-butyrate (C_4), minimal performance (<10 %) with the C_{10}-substrate, but activity increased again when *p*NP-palmitate (C_{16}) was used as substrate (Gumerov et al. 2012). Furthermore, various vegetable oils, such as soybean, olive, corn and sunflower oil were hydrolysed. A broad substrate spectrum was also reported at 70 °C for the recombinant lipase from *Geobacillus* sp. T1. Various natural oils and triacylglycerol substrates containing chain length between C_2 and C_{18} with an optimum towards C_{12} were hydrolyzed (Leow et al. 2007). Besides stability and maximal activity, substrate specificity could also be altered by single point mutations. A lipase mutant (N355K) exhibited highest activity toward *p*NP-palmitate (C_{16}) whereas the wildtype enzyme was most active toward *p*NP-laurate (C_{12}) with residual activity of approximately 15 % with palmitate (Sharma et al. 2014). A recombinant archaeal carboxylesterase displayed highest activity toward *p*NP-octanoate (C_8) with no residual activity towards *p*NP-butyrate (C_4). The C-terminal His-tagged protein showed similar results. When the gene was cloned with a N-terminal His-tag-encoding region, the enzyme exhibited a broader substrate spectrum with comparable activities towards *p*NP-butyrate (C_4) and *p*NP-octanoate (C_8). The substrate-binding pocket was located at the N-terminus, thus being somehow altered by the attached tag (Killens-Cade et al. 2014). Recombinant proteins may provide feature variations compared to the naturally occurring enzyme (Leow et al. 2007).

Generally, most bacterial lipolytic enzymes are optimally active at neutral or alkaline pH although they can act over a wide pH range (Gupta et al. 2004). Numerous thermostable lipolytic enzymes were reported to be active and stable exclusively at alkaline pH values. Esterases and lipases from metagenomes derived from not extreme habitats were often found to prefer alkaline conditions (Choi et al.

2013). Likewise, an esterase from the thermoacidophilic bacterium *Acidicaldus* sp. exhibited the highest activity at pH 9.0 and <20 % residual activity at pH 7.0. Only two heat-stable enzymes were described in the literature with slightly acidic pH optima including an esterase from *Thermus thermophilus* (pH optimum at 6.3) and an esterase from *Thermotoga maritima* with a pH optimum at 5.0–5.5 (Tao et al. 2013; Fuciños et al. 2014). The latter was described as first acid tolerant esterase from a thermophilic bacterium.

4.2 Hyperthermophilic Archaea as a Source for Lipolytic Enzymes

Thermophilic organisms grow at 50–80 °C, whereas hyperthermophiles grow between 80 °C and 113 °C. Archaea represent the group with the highest temperature optima (Egorova and Antranikian 2005). Accordingly, optimal temperature for heat-active biocatalysts from archaea often exceeds the one described for bacterial enzymes. Furthermore, they exhibit a high intrinsic thermal and chemical stability compared to bacterial proteins (Levisson et al. 2009). Some recombinantly produced and characterized archaeal lipolytic enzymes are shown in Table 12.3. Highest optimal temperature with 100 °C for activity was reported for the recombinant esterase from *Pyrococcus furiosus* with a half-life of 34 h at 100 °C (Ikeda and Clark 1998). The enzyme from *Sulfolobus solfataricus* P1 exhibited the highest activity at 80 °C and retained 41 % of its activity after 5 days of incubation at the same temperature (Park et al. 2006). The esterase from *Metallosphaera sedula* showed highest activity at 95 °C with C_8-substrates. This enzyme also showed a wide range for catalytic performance with residual activity even at 37 °C. Moreover, it exhibited more than 70 % residual activity after 6 h at 90 °C (Killens-Cade et al. 2014). *Sulfolobus acidophilum* esterase with an optimum at 70 °C appears comparatively low, but residual activity of >20 % was observed at 90 °C (Zhang et al. 2014). Enzymes from *Pyrobaculum* species and *Aeropyrum pernix* exhibited their highest activity at temperatures between 80 °C and 90 °C as well (Hotta et al. 2002; Gao et al. 2003; Shao et al. 2014). All esterases were active in a neutral or slightly alkaline pH range with one exception possessing activity at pH 10.0–11.0 (Table 12.3). This enzyme was active even at pH 12.0 being among the most alkaline pH range for hydrolases (Rusnak et al. 2005). No recombinant esterase from a hyperthermophilic organism was found with optimal activity below pH 7.0. An esterase from the extreme thermoacidophilic archaeon *Picrophilus torridus* (growth at 60 °C and pH 1.0–2.0) was reported to exhibit highest activity at pH 6.5 and 70 °C (Hess et al. 2008). An exceptional pH optimum of 2.0 at 50 °C with no residual activity at pH 5.0 was reported for an esterase from *Ferroplasma acidophilum* that grows at moderate temperatures and at pH 1.7 (Golyshina et al. 2006).

In the literature, no lipase with exclusive preference for long-chain fatty acid substrates (>C_8) was found to belong to the group of hyperthermophilic archaea.

Table 12.3 Recombinant lipolytic enzymes from hyperthermophilic archaea

Sources	T_{opt} (°C)	pH_{opt}	MW (kDa)	Preferred substrate	References
Archaeoglobus fulgidus DSM 4304	70	10.0–11.0	52.8	Methyl butyrate (C_4)/*p*NP-acetate (C_2)	(Rusnak et al. 2005)
Sulfolobus acidophilus DSM10332	70	8.0	34.1	*p*NP-butyrate (C_4)	(Zhang et al. 2014)
Pyrobaculum sp. 1860	80	9.0	23	*p*NP-acetate (C_2)	(Shao et al. 2014)
Pyrococcus furiosus	80	7.0	26.5	4-methylumbelliferyl heptanoate (C_7)	(Alqueres et al. 2011)
Archaeoglobus fulgidus DSM 4304	80	7.0–8.0	27.5	*p*NP-butyrate (C_4)	(Kim et al. 2008)
Thermococcus kodakarensis KOD1	85	8.0	29	*p*NP-butyrate (C_4)	(Cui et al. 2012)
Sulfolobus solfataricus P1	85	8.0	34	*p*NP-caprylate (C_8)	(Park et al. 2006)
Sulfolobus solfataricus	90	6.5–7.0	32	*p*NP-valerate (C_5)	(Morana et al. 2002)
Aeropyrum pernix K1	90	8.0	63	*p*NP-caprylate (C_8)	(Gao et al. 2003)
Pyrobaculum calidifontis VA1	90	7.0	34	*p*NP-caproate (C_6)	(Hotta et al. 2002)
Metallosphaera sedula DSM5348	95	7.0	33.2	*p*NP-caprylate (C_8)	(Killens-Cade et al. 2014)
Pyrococcus furiosus	100	7.6	–	4-methylumbelliferyl acetate (C_2)	(Ikeda and Clark 1998)

Nevertheless, some of the esterases shown in Table 12.3 were capable of hydrolyzing long-chain substrates to some extent. The enzyme from *Pyrococcus furiosus* exhibited 15 % residual activity toward *p*NP-palmitate (C_{16}) (Alqueres et al. 2011). The enzyme from *Aeropyrum pernix* hydrolyzed *p*NP-stearate (C_{18}) with 19 % activity compared to 100 % towards *p*NP-caprylate (C_8) (Gao et al. 2003). The lipolytic enzyme AFL from *Archaeglobus fulgidus* contained a small lid domain and an additional C-terminal lipid-binding domain (Chen et al. 2009). AFL was previously characterized and described as an esterase due to higher activity toward *p*NP-acetate (C_2) compared to the corresponding palmitate (Rusnak et al. 2005). Nevertheless, the extraordinary C-terminal domain of AFL was reported to be essential for binding long-chain substrates and thus, this enzyme was subsequently classified as true lipase due to structural findings (Chen et al. 2009). By unravelling structures of lipolytic enzymes, classification can be conducted more reliably than by investigation of artificial substrate preference. However, a lipase with preference for the long-chain substrate *p*NP-myristate (C_{14}) was recently identified from the mesophilic archaeon *Haloarcula* sp. G41 with optimal activity at 70 °C, pH 8.0 (Li and Yu 2014). This demonstrates the high potential of archaea as a source for novel lipases with unique features.

4.3 Enzyme Engineering

Thermal denaturation of proteins is reduced due to various features, such as higher rigidity or compact packing. However, higher rigidity is accompanied by less flexibility, which is thought to result in decreased activity. This assumption was recently disproved by identification of a lipase mutant with a more rigid active site than the wildtype but simultaneously enhanced activity and stability (Kamal et al. 2012). Different factors seem to contribute to the stability of thermoactive enzymes, such as higher amounts of disulphide bridges, hydrophobic interactions, hydrogen bonds or metal bindings (Vieille and Zeikus 2001). Exchange of a single amino acid (E315G) within a thermostable lipase showed that one residue close to the active site resulted in a decrease of thermal stability. In contrast to this substitution that enabled higher loop-flexibility, the N355K mutant enhanced the thermostability by formation of an additional hydrogen bond (Sharma et al. 2014). *Geobacillus* sp. EPT9 lipase contained a zinc-binding domain and higher percentages of proline and arginine compared to a lipase from a mesophilic *Bacillus* strain (Zhu et al. 2015). Proline was the most rigid amino acid and arginine was shown to participate in multiple non-covalent interactions. Disruption of the zinc-binding site of lipase L1 from *Geobacillus stearothermophilus* resulted in a shift of optimal temperature for enzyme activity from 60 °C to 45–50 °C with lower specific activity and in a decrease of thermal stability (Choi et al. 2005). Thus, the zinc-binding domain may participate in structural conformation of the active site. No ubiquitous generalized pattern for activity and stability at elevated temperatures can be determined. Nevertheless, it was shown that exchange of one residue might result in a small change of inner-protein interactions. This conformational deviation may lead to a significant decrease in stability and/or enzyme activity.

Several enzyme-engineering approaches were conducted to enhance thermal stability, resulting in mutants that exhibited maximal activity at higher temperatures. However, there are also some contrasting examples, e.g. one amino acid exchange resulted in the reduction of optimal temperature from 50 °C to 40 °C compared to a wildtype lipase, which was identified from a soil metagenome (Sharma et al. 2014). Hence, advantage can be taken of engineering robust enzymes from thermophiles or hyperthermophiles to create lipolytic enzymes suitable for processes that run at harsh conditions and moderate temperatures. For instance washing processes are favoured to function at lower temperatures nowadays to save energy. At the same time harsh conditions are provided under alkaline environment requiring stable fat cleaving enzymes (Jaeger and Reetz 1998). One of the latest achievements in developing new and improved lipolytic enzymes is the incorporation of non-canonical amino acids. Substitution of a high number of non-canonical amino acid residues in the heat-active lipase from *Thermoanaerobacter thermohydrosulfuricus* (TLL) resulted in a “cold-washing” enzyme that was activated without heat induction (Hoesl et al. 2011).

4.4 Applications of Heat-Stable Lipases and Esterases

Most industrial processes run at temperatures higher than 45 °C (Sharma et al. 2002). Higher operation temperature contributes to higher reaction rates of applied biocatalysts, increases solubility of substrates, and lowers viscosity and contamination risks. Furthermore, enzymes from thermophiles and hyperthermophiles are more stable under harsh denaturing conditions (Mozhaev 1993). High-level recombinant production of bacterial and archaeal enzymes has proven to yield sufficient amount of biocatalysts for industrial usage. Employing lipolytic enzymes for a conversion reaction implies milder conditions compared to treatment with chemicals, steam or pressure. The specificity of esterases and lipases contributes to reduction or elimination of unwanted side products and costs can be reduced due to unnecessary separation steps. It was shown that reaction temperature and incubation time were the major factors influencing the yield of produced menthyl butyrate by T1 lipase in a solvent-free system (Wahab et al. 2014). The high-yield synthesis was conducted with the recombinant enzyme without need of flammable or toxic solvents that have to be subsequently separated from the product. Nevertheless, when solvents are inevitable the lipolytic enzyme must show high tolerance. The lipolytic enzymes from *T. thermohydrosulfuricus* SOL1 and *Caldanaerobacter subterraneus* subsp. *tengcongensis* were resistant against several solvents or detergents up to a concentration of 99 % (Royter et al. 2009). Furthermore, the esterase from *T. thermohydrosulfuricus* SOL1 was reported to show high preference for esters of secondary alcohols and a high selectivity for R-enantiomers of pharmaceutically relevant substrates (Royter et al. 2009). This enzyme being active at high temperatures and resistant against organic solvents represents an attractive candidate for biotransformation in water-free media. The esterase Tm1160 from *Thermotoga maritima* showed high enantioselectivity hydrolyzing racemic ketoprofen ethyl ester (Tao et al. 2013). Likewise, the esterase from *Acidicaldus* sp. can be used to synthesize medically relevant S-enantiomers of naproxen and ibuprofen esters at harsh conditions (Fig. 12.5) (Lopez et al. 2014).

An example for industrial interesterification processes is the conversion of palm oil into cocoa butter fat substitute that exhibits a higher melting point and is therefore attractive as food, confection and cosmetic supplements. Cheap oils can be upgraded by enzymatic conversion into nutritionally important structured fats. High value products, such as human milk fat substitute or cocoa butter equivalents are commercially distributed (Hasan et al. 2005). Selective hydrolysis of fat triacylglycerides is also utilized for flavour development or flavour precursor formation (Jaeger and Reetz 1998). These compounds are difficult to isolate from parent sources and hence, industrial extraction procedures are often unprofitable. Terpene esters containing short-chain fatty acids, such as terpinyl esters, geraniol esters or citronellol esters, produced by lipolytic enzymes are applied in the beverage, food and pharmaceutical industry. Green synthetic routes for valuable compound production are favoured over chemical synthesis. Furthermore, new odour molecules are discovered by elucidating the wide range of action of lipolytic enzymes towards many substrates (Dhake et al. 2013).

Heat-stable lipases can also be applied within the scope of algae-based biofuel production. The amount of free fatty acids can be enhanced by cleavage of storage triacylglycerides. The use of thermostable lipolytic enzymes reduces time and energy consumption during the high temperature conversion of lipids to fuel (Killens-Cade et al. 2014). Furthermore, in the pulp and paper industry lipolytic enzymes are employed to remove hydrophobic components of wood, such as triacylglycerides and waxes, from the pulp at elevated temperatures (Jaeger and Reetz 1998).

5 Conclusion

Due to their versatile enzymatic properties, lipolytic extremozymes represent promising candidates to be used in several industries including organic synthesis of chiral compounds and pharmaceuticals, food and detergent industry and environmental bioremediations. A portfolio of cold- and heat-active esterases and lipases has been identified by modern molecular biology and microbiology approaches in extremophilic archaea and bacteria and are nowadays available for specific applications and engineering approaches. However, there are still a couple of barriers to overcome in the development of a tailor-made biocatalyst for certain industrial applications. Establishing novel and improved techniques to identify and engineer genes encoding biocatalysts and to produce proteins in recombinant and active form in high yield has made particular progress. Recent examples in the "omics" era are the development and allocation of appropriate expression hosts, directed evolution, gene shuffling, efficient cloning approaches, enzyme congeners containing non-canonical amino acids, fusion enzymes and site-directed mutagenesis (Sharma et al. 2002; Gatti-Lafranconi et al. 2008; Al Khudary et al. 2010; Merkel et al. 2010; Hoesl et al. 2011; Bassegoda et al. 2012a; Marquardt et al. 2014; Elleuche 2015; Joshi and Satyanarayana 2015).

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