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Review

Catalytic properties and potential applications of *Bacillus* lipases

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ABSTRACT

Up to date more than 70 lipases from the *Bacillus* and *Geobacillus* genera have been isolated, but for most of them only basic biochemical properties have been reported. In general, *Bacillus* lipases are easily produced and display high tolerance toward organic solvents, proving them useful in the synthesis of esters for food industry, cosmetics and biodiesel production. Many lipases preserve their activity at extreme temperatures and pH, and in the presence of surfactants, hydrogen peroxide, sodium hypochlorite, and therefore they can be applied in laundry formulations. *Bacillus* lipases display diverse selectivity to the chain length of the acid, and few enzymes show positional specificity. Several enzymes can be applied in the production of enantiopure compounds for the pharmaceutical industry due to their remarkable enantioselectivity. The immobilization experiments with *Bacillus* lipases, though a limited number, illustrate the vast possibilities for optimization of the properties of the biocatalysts for a particular application. The paper summarizes available experimental data on *Bacillus* and *Geobacillus* lipases and identifies areas for further research.

Contents

Abbreviations: CTAB, cetyltrimethylammonium bromide; DEPC, diethylpyrocarbonate; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTT, dithiothreitol; E600, diethyl *p*-nitrophenyl phosphate; EDTA, ethylenediamine-tetraacetic acid; IMAC, immobilized metal affinity chromatography; IPG, 1,2-*O*-isopropylidene-*sn*-glycerol; IPTG, isopropyl-β-D-1-thiogalactopyranoside; NAI, *N*-acetyl-imidazole; NBS, *N*-bromosuccinimide; Ni-NTA, Ni-nitrilotriacetic acid; PEG, poly(ethylene glycol); PHMB, *p*-hydroxymercury-benzoic acid; PMSF, phenylmethylsulfonyl fluoride; *p*NP, *p*-nitrophenyl; SDS, sodium dodecylsulfate; TAG, triacylglycerol.

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1. Introduction

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are enzymes, which under physiological conditions catalyze the hydrolysis of the ester bonds in the molecules of triglycerides liberating free fatty acids, diglycerides, monoglycerides and glycerol. Unlike true esterases (EC 3.1.1.1, carboxyl ester hydrolases), lipases are able to hydrolyze esters of long-chain fatty acids. The natural substrates of lipases are practically insoluble in water, so the reaction is catalyzed at the water–lipid interface and does not follow the classic Michaelis–Menten kinetics [1,2]. The catalytic mechanism of most lipases involves a step called "interfacial activation". In homogeneous aqueous medium, these enzymes are in closed, inactive conformation, when the active site cleft is isolated from the polar solvent by a lid formed by a helical segment of the polypeptide chain [3,4]. The inner side of this lid is built up of non-polar amino acid residues, matching the hydrophobic zone of the active site. However, in the presence of a second, lipidic phase, the lipase adsorbs at the interface. The lid undergoes conformational displacement, uncovering the inner hydrophobic part of the enzyme to the substrate, the non-polar solvent or a hydrophobic polymer carrier [5,6]. Lipases are monomeric proteins, sharing the α/β-fold, which consists of a central β-sheet surrounded by α-helices [7,8]. The catalytic centre of lipases is formed by the triad Ser-Asp (or Glu)-His, which is functionally analogous to the one in serine proteases, but differs in spatial organization. The catalytic serine residue belongs to a highly conservative pentapeptide. Another conservative structural element is the oxyanion hole built of backbone amide NH groups. In lipases undergoing interfacial activation, the oxyanion hole is formed upon transition from closed to open conformation.

Lipases are very attractive enzymes for biotechnology, because in low-water-content environment (organic solvent) they catalyze the reverse reaction of synthesis or transesterification, accepting a wide range of acyl donors and nucleophiles besides their natural substrates [9]. They also exhibit high tolerance to different factors of the environment (wide range of pH and temperature, organic solvents). This group has the potential to meet diverse requirements for a wide range of applications, because it comprises enzymes of broad or quite narrow selectivity toward the kind and the position of the fatty acids in the ester, as well as enantioselectivity [10]. Lipases are also very susceptible to immobilization unlike other enzymes, which is also beneficial for industry. Microbial lipases are the second largest group of industrial biocatalysts after bacterial amylolytic enzymes. Based on all qualities mentioned, lipases have been successfully applied as additives substituting digestive enzymes or in laundry formulations in combination with proteases; or as catalysts in food industry for modification of fats and oils (aiming at higher nutritional value, better texture/physical properties of the product), improving the flavor and texture of bread and cheese; or in the synthesis of flavor substitutes [11–13]. They are applied in paper pulp processing, in leather industry, for waste water treatment or in the field of fine organic synthesis for protection/deprotection of functional groups and in the resolution of racemic mixtures in the production of chiral reagents for the pharmaceutical industry [14,15]. Each particular application sets specific requirements to the biocatalyst to be used with respect to stability, selectivity, etc. Given all advantages of using enzymes as alternatives to chemical synthesis, this motivates the search and exploration of new biological sources, enzymes and new methods for fine rational modification of their properties for the industry.

Lipases are enzymes produced by bacteria, plants and animals. But microbial lipases are most suitable for industrial applications due to their low price, their simple production since they are mostly extracellular, susceptibility to expression in host microorganisms, diverse specificity, wide range of pH and thermal operational optima [15]. Bacteria are susceptible to genetic modifications (site-directed mutagenesis, directed evolution), which allow fine modification of the intrinsic properties of their lipases. The *Bacillus* genus represents a diverse group of microorganisms inhabiting some of the most extreme places on Earth – desert soil, polar ice, volcanic water, etc. [16–23]. As cells have adapted to survive in such extreme climatic conditions, their enzymes and lipases in particular have evolved and exhibit exceptional properties. A large number of *Bacillus* lipases have been isolated to date. In general, they have not been studied profoundly and in most cases only basic characteristics of the enzymes have been reported. The lipases of fewer than 10 *Bacillus* species have mainly been in the focus of intense research. They have been tested in diverse chemical reactions, their structures have been elucidated or theoretically modeled, and have been subjected to immobilization and genetic modification. The crystal structures of the lipases from *Geobacillus thermocatenulatus* (named BTL2) [24,25], *Bacillus subtilis* (lipA) [26–30], *Geobacillus zalihae* (named T1) [31]; two isoforms of the *Geobacillus stearothermophilus* lipase (L1 and P1) [7,8,23] and the monoacylglycerol lipase from *Bacillus* sp. H-257 [32] have been resolved so far. The aim of the present paper is to summarize all the available information on the studied *Bacillus* lipases, the optimal conditions for their production and catalytic reactions. This will provide a basis for identification of directions for further investigation and possible applications of these enzymes.

2. Microbiological classification

The genus *Bacillus* until recently represented a very large and heterogeneous group of bacteria [33,34]. It contained aerobic and facultatively anaerobic, rod-shaped, Gram-positive or Gram-variable endospore-forming bacteria. Over the years it has been progressively subdivided into novel genera: *Alicyclobacillus*, *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus*, *Virgibacillus*, *Salibacillus*, *Gracilibacillus*, *Ureibacillus* [33]. Most recently several existing thermophilic, aerobic, motile, rod-shaped, spore-forming *Bacillus* species, together with some novel at that time species, were reclassified to the new genus *Geobacillus*, such as *G. stearothermophilus*, *G. thermocatenulatus*, *Geobacillus thermoleovorans*, *Geobacillus kaustophilus*, *Geobacillus thermoglucosidasius*, *Geobacillus thermodenitrificans* and *Geobacillus* sp. TP10A.1 with *G. stearothermophilus* (formerly *Bacillus stearothermophilus*) as the type species. Subsequently, other species have been added to the genus [33,34].

Bacteria from the *Bacillus* and *Geobacillus* genera are the main sources of lipases within the *Bacillaceae* family. Having in mind the reorganization in the microbiological classification, the present paper will encompass *Bacillus* and *Geobacillus* lipases (i.e. lipases with *Bacillus* in their current name or basonym), reported in the literature. Despite the wide diversity in their properties, *Bacillus* lipases still constitute a group distinguishable from all other lipase groups. The individual enzymes will be referred to using their valid names and the differences between the subgroups will be pointed out.

3. Lipase classification and structural features

The catalytic serine residue of lipases belongs to a highly conservative pentapeptide. This sequence in combination with some basic physico-chemical properties of the enzymes was the basis for the classification of lipases into 8 families [1,35,36]. All *Bacillus* lipases have the conserved motif Ala-His-Ser-X-Gly (X is Met or Gln), containing the catalytic Ser residue. Ala replaces the Gly residue in the canonical pentapeptide of other microbial lipases [37]. The importance of this substitution was demonstrated by site-directed mutagenesis. The A76G variant of *B. subtilis* esterase lipB displayed marked temperature sensitivity with a half-life of 8 min at 45 °C, whereas the wild-type enzyme remained stable. It was shown that the side chain of the corresponding alanine (Ala-111) in *G. stearothermophilus* L1 lipase is pointing toward a hydrophobic core made of residues in the catalytic serine loop and contributes to the thermostability of the enzyme by tight packing [8]. *Bacillus* lipases are divided in two subfamilies in the current classification of lipases [1]. The enzymes show high sequence identity within the subfamily, but low sequence identity with the lipases from the other subfamily [1,7,35,38,39]. As a consequence, the two groups differ significantly in their biochemical properties.

3.1. I.4 subfamily

Lipolytic enzymes from *B. subtilis*, *Bacillus pumilus*, and *Bacillus licheniformis* were initially grouped in subfamily I.4 of true lipases [37]. The lipases of this group have the conservative peptide Ala-His-Ser-Met-Gly and comparatively low molecular weight (about 19–20 kDa). The X-ray structure of lipase A from *B. subtilis* showed that the overall conformation of the protein, the catalytic triad residues – Ser77, Asp133, and His156, and the residues forming the oxyanion hole (backbone amide groups of Ile12 and Met78) are in positions very similar to those of other lipases with known structure. However, no lid domain is present and the active site nucleophile Ser77 is solvent-exposed [26]. The oxyanion hole in *B. subtilis* lipase is preformed, unlike other lipases where the catalytic machinery is adjusted upon transition of the enzyme from closed to open conformation. In addition, it was found that *B. subtilis*, *B. pumilus* and *B. licheniformis* lipases lack stabilizing disulfide bridges. This suggests that these proteins have a flexible tertiary structure, which determines their biochemical properties [35].

The lipases from I.4 subfamily are not thermostable: their activities decrease significantly at temperatures above 45 °C. They exhibit extreme alkaline tolerance, with maximum lipase activities at pH values between 9.5 and 12.

3.2. I.5 subfamily

This family comprises mainly lipases from *Geobacillus* species, such as *G. thermocatenulatus*, *G. thermoleovorans*, *G. stearothermophilus* L1, *Geobacillus* sp. Tp10A.1. These are larger proteins with molecular weight of 40–45 kDa and higher. The lipase produced by *Bacillus* sp. GK8 has the highest reported molecular weight [40]. Their characteristic conserved pentapeptide is Ala-His-Ser-Gln-Gly. They have neutral or moderately alkaline pH optima (pH = 8.0–10.0) and very high temperature optima: they reach maximum activity at 60–75 °C [8]. These enzymes possess a hydrophobic lid and a Zn^{2+}-binding domain in addition to the calcium-binding site, which accounts for the higher molecular weight [24,41,42]. As evidenced by the crystal structure of *G. stearothermophilus* L1 lipase, the molecules of these enzymes feature tight side-chain packing and a more rigid structure, which most probably contribute to the unique activity and overall stability of the enzymes at high temperatures [8]. At low temperatures, the rigidity in the active site region limits enzyme activity. The temperature increase should provide the required flexibility for catalysis [8]. The lipases in I.5 subfamily possess cysteine residues, participating in the formation of disulfide bonds that may stabilize the protein against thermal inactivation [35].

4. Production of *Bacillus* lipases

4.1. Expression

Bacillus lipases have been mainly produced by submerged culture fermentation. Lipase secretion is associated with cell growth and maximum rate of lipase production is detected in the late exponential phase [43–48]. Commonly, a decrease in lipase activity is observed during the stationary phase, probably due to thermal instability of the enzyme and the presence of proteases in the culture medium [49,50]. *Bacillus* sp. strains DH4 and H1 were able to produce extracellular lipases in the stationary phase of fermentation [22,51]. Highest level of lipase production was detected in the stationary phase of growth of *G. thermoleovorans* CCR11 and of *B. licheniformis* in the presence of Tween 80 [35,50].

Bacillus cells are grown in a nutrient medium containing carbon, nitrogen and phosphorous sources and mineral salts, which are all specific to the organism [52]. Lipids like natural oils (olive, cottonseed, mustard, sesame, soybean, fish, rice bran, maize oil), triglycerides (triolein, tributyrin), or free fatty acids (oleic acid) are most often used as source of carbon alone or in combination with poly- and oligosaccharides (starch, maltose, mannose, glucose, xylose) or polyols (glycerol or mannitol). *Bacillus halodurans LBB2* cells were grown using methanol as a carbon source [53]. Yeast extract, peptone, casamino acid or tryptone are the commonly used organic nitrogen sources [20,21,39,40,44,48,54–65]. Peptone and tryptone are complex mixtures containing cofactors and amino acids that have been suggested to promote lipase secretion [43,66,67]. Tryptone supported good cell growth, but extracellular lipase production was low in *B.* sp. L2 cells, and casamino acid proved to be the best nitrogen source in this case [43]. Alanine, tryptophan and phenylalanine proved to be the best nitrogen sources for enzyme production in *G. stearothermophilus* AB-1 cells [68]. Often inorganic nitrogen salts are added, such as ammonium nitrate, sodium nitrate, potassium nitrate, ammonium sulfate, ammonium chloride, or ammonium phosphates, as well as urea [16,46,67,68]. However, addition of sodium nitrate and diammonium orthophosphate to the fermentation medium reduced lipase production in *B.* sp. strain 42 cells [66].

The production of lipases is mostly inducer dependent and in many cases oils act as good inducers [16,20,43,45,46,50,60,61,66,69–73]. Lipase activity was hardly detectable in the absence of 1% olive during cultivation of *Bacillus* sp. cells, although cell growth was similar [74]. However, addition of olive oil, Tween 20 and 80 had no effect on lipase production of *B. licheniformis* H1 [18]. *G. thermoleovorans* ID-1 produces two distinct thermostable lipases – BTID1-A (18 kDa) and B (43 kDa), but BTID1-A lipase could only be produced in the absence of a lipid substrate as an inducer [43]. Certain other compounds have been reported to stimulate the secretion of *Bacillus* lipases, such as bile salts [46], SDS, Tween 80 [35,51,55,59,62,71,75], Triton X-100. Tween 80 was reported to have no effect on the lipase production in *B. subtilis* and *Bacillus alcalophilus* B-M20 strains [18,76] and even an inhibitory effect on *B.* sp. strain L2 lipase production [43]. Glucose has a variable effect as well. It was reported to stimulate lipase production by *B. licheniformis* strains H1, *Bacillus* sp. GK 8 and SB-3 cells [18,40,69], *G. stearothermophilus* strains SB-1 and L1 [65], and others [58,64,77]. But it had an inhibitory effect on lipase production in *B. alcalophilus* B-M20 [76], *Bacillus megaterium* AKG-1 [46], *B.* sp. strains L2 and 42 [43,66], *B. subtilis* [60,61], and

G. thermoleovorans ID1 [49]. Glycerol enhanced lipase production of *B. subtilis* and *G. stearothermophilus* strain-5 cells [47,48,78], but in many other cases it had an insignificant or even a negative effect on lipase production, since it is the product of hydrolysis of triglycerides [18,68]. The lipase from *B.* sp. was poorly induced by oils, but its expression was stimulated by sugars and sugar alcohols, mainly galactose, lactose, glycerol, and mannitol [79].

The culture medium for *Bacillus* cell growth is usually neutral to slightly alkaline (pH 7.0–8.5). Cells of some *G. thermoleovorans* strains and *G. zalihae* [80] were grown optimally in slightly acidic environment (pH 6.0–6.5) [49,50,81,82]. Optimal pH for the lipase production in *B. alcalophilus* B-M20 was 10.6 and no cell growth was detected in neutral medium [76]. *B.* sp. RSJ-1 cells grew at pH 9.0 [59]. In a number of cases metal ions are also required for optimal lipase production. The nutrition medium for *Bacillus* sp. strain 42 cells contained a mixture of 0.01–0.04% magnesium, iron, and calcium ions. It was shown that the addition of iron ions was essential, but no individual ion or pair of ions could support that high level of lipase production [66]. In contrast, the expression of *Bacillus* sp. L2 lipase was completely inhibited by Mg^{2+} ions, while addition of Ca^{2+} and Fe^{3+} resulted in high lipase activity [43]. Sodium ions at 0.5% concentration stimulated lipase production in *Bacillus coagulans* [83]. Cells of *B.* sp. THL027 [77], *G.* sp. Tp10A.1 [84], *G. thermoleovorans* strains [81,82], *B. pumilus* B106 [71], and *B. licheniformis* MTCC-6824 [44] required higher concentration of NaCl in the culture medium. *B. alcalophilus* B-M20 tolerates up to 7.5% salinity, although high concentrations of NaCl and KCl inhibit lipase production [76].

The optimal growth temperature is a major property differentiating *Bacillus* from *Geobacillus* species. For example, *B. megaterium*, *B. subtilis* and *B. pumilus* cells grow optimally at moderate temperatures (20–40 °C), while *G. thermocatenulatus*, *G. thermoleovorans* and *G. stearothermophilus* strains, for example, grow and produce their lipases at 60–65 °C [49,81,82,85]. *B. alcalophilus* B-M20, *B.* sp. strains H1, J33, A30-1 and THL027 cells have similar temperature optima [21,51,76,77,86–88]. *B.* sp. strain L2 lipase was produced at 70 °C in neutral pH. Although at 75 °C cell growth was lower, higher lipase yield was obtained [43]. *Bacillus* cells grow in an aerobic environment and good aeration enhances lipase production, therefore shaking is an important factor as well [49,52,59,62,63,72]. Agitation rate was shown to influence cell growth and lipase expression yield of *B.* sp. strain L2 and *B. megaterium* AKG-1 [43,46]. 150 rpm was optimal to sustain cell growth and lipase production. No lipase production was detected under static conditions, whereas higher shaking rate decreased lipase activity by 50%, probably due to enzyme denaturation in an oxidative environment [43].

As a result of fermentation condition optimization, *G. stearothermophilus* strain-5 produced 176.6 U/ml extracellular lipase activity after 24 h; lipase activity determined using *p*NP palmitate as substrate [47]. The maximum lipase yield with *B. coagulans* cells was 0.2 U/ml (*p*NP palmitate) [83]. After 48 h of incubation in the presence of 1% sardine oil, *B. licheniformis* MTCC 6824 produced 13.6 U/ml lipase (*p*NP palmitate) [44]. *G. thermoleovorans* CCR11 produced 6000 U/ml lipase activity (*p*NP laurate) [50]. Lipase expression of *B. megaterium* AKG-1 was induced with 1% soybean oil and reached 1160 U/ml after 24 h (*p*NP laurate) [46]. For comparison, Alkan et al. reported rapid cell growth and very high levels of lipase production by solid state fermentation of *B. coagulans* cells on agricultural by-products [16]. This strategy proved to be economically very efficient. Lipase production was induced with 2% olive oil and starch and exceeded 140,000 U/g after 24-h cultivation at 37 °C and neutral pH [16]. The differences in lipase production yields can be attributed to bacterial strain properties, as well as culture medium composition. It should be noted, however, that measurement of lipolytic activity also strongly depends on the substrate and the experimental conditions of the test reaction, so comparison should be made with caution.

A large part of all *Bacillus* lipases described in the literature were produced by their host cells in very small amounts, which has lead to the development of a number of protocols for their expression in recombinant cells. There are two goals that need to be accomplished, namely high cell density and high-level gene expression, both strongly depending on the host strain and the expression vector [85]. The two major problems are proteolytic degradation by the host's own proteases and the accumulation of misfolded protein as inclusion bodies.

The fast growth rate and ease of cultivation make *Escherichia coli* very suitable for large-scale production of enzymes for industrial applications. Two *B. licheniformis* lipase genes were cloned into the pET20b(+) vector in-frame with a C-terminal region coding for 6 histidine residues and transferred into *E. coli* cells. Lipase expression, induced with IPTG, was increased by a factor of 36 compared with the native strain, and in addition the affinity tag facilitated enzyme purification [35,89]. The lipase from *G. stearothermophilus* P1 was expressed in *E. coli* cells upon induction with IPTG to a level that was 26,000-fold higher than those detected in cultures of either the wild-type strain or of the transformed *E. coli* M15[pREP4] in the absence of IPTG [90]. The lipase gene from *G. thermoleovorans* Toshki was cloned in the pET15b vector and expressed in *E. coli* under the control of the strong T7 promoter, leading to a 4.5-fold increase in lipase activity compared with the wild-type strain, or 350 U/ml (*p*NP palmitate) after 3 h of induction [85]. The lipase from *Bacillus sphaericus* 205y was expressed in *E. coli* cells under the control of the *lacZ* promoter, which resulted in 8-fold higher expression level, compared with the expression regulated by the native lipase promoter [91]. Lipase A from *G. thermoleovorans* YN was cloned and expressed in *E. coli* with a C-terminal 6xHis-tag under the control of the temperature-inducible λ promoter. Enzyme production was induced by heat shock and resulted in about 6000 U/ml lipase activity (against *p*NP palmitate) in the crude cell-free extract [92]. However, heterologous lipases are expressed intracellularly in *E. coli* cells, making enzyme purification complicated, thereby decreasing yields and increasing production cost. Moreover, this very often results in accumulation of the protein in an inactive form due to the limited space for protein storage [93]. So, the lipase from *Geobacillus* sp. T1 was expressed as a fusion protein with glutathione *S*-transferase. The expression level (11,041 U/l) was 279-fold higher as compared to the thermostable lipase from the wild-type host (150 U/l) [54,94]. The lipase T1 gene, lacking the signal peptide, was also co-expressed with the Bacteriocin Release Protein system in *E. coli*. The BRP-lipase was secreted to the culture medium, reaching 26.8 U/ml after 24 h of cultivation [95]. The fusion of a protein of interest to a large-affinity tag, such as maltose-binding protein, thioredoxin or glutathione *S*-transferase, can be advantageous in terms of increased expression, enhanced solubility, protection from proteolysis, improved folding and purification via affinity chromatography, but with a minimal effect on enzyme tertiary structure and biological activity [94]. The *G. thermocatenulatus* lipase BTL2 was weakly expressed under the control of its native promoter, so the gene of BTL2 was subcloned in the pCYT-EXP1 expression vector, which contains the temperature-inducible λP_L promoter [64,96]. Even higher expression level was achieved by fusing the BTL2 gene with the signal peptide of the OmpA protein, the major outer membrane protein of *E. coli*. Thus the enzyme was directed to the periplasm, thereby minimizing the risk of proteolysis and facilitating lipase purification, and enhancing expression yield – 660,000 U/g cells [97].

Saccharomyces cerevisiae and *B. subtilis* secrete recombinant lipases directly into the fermentation medium, thus requiring a more simplified purification procedure, compared with the intracellular production by *E. coli* [98]. They do not produce endotoxins

and pyrogens and are considered as safe organisms (GRAS) for the production of food and health-care products [65,99]. The wild-type *B. pumilus* B26 produces low levels of lipase activity – 0.5 U/ml of culture broth (olive oil as substrate). When the lipase gene was cloned into the pUC19 vector, the recombinant *E. coli* also showed very weak intracellular lipase activity. In *B. subtilis* extracellular lipase activity reached 8 U/ml after 20 h of cultivation (20-fold increase compared to the wild-type strain) [39]. The lipase from *B. subtilis* strain IFFI10210 was cloned and over-expressed in another *B. subtilis* strain with a final 100-fold higher yield than wild-type host [100]. The lipase from *G. stearothermophilus* L1 was expressed in *S. cerevisiae* using the signal sequence of α-amylase from *Aspergillus oryzae* and the galactose-inducible GAL10 promoter [65]. The enzyme was successfully secreted into the culture broth. In a batch reactor extra- and intracellular lipase activities were 24 and 13 U/ml, respectively. In a fed-batch culture at an early induction, extracellular lipase activity reached 820 U/ml (olive oil) [65]. Secretion of L1 lipase was further increased by fusing the mature lipase gene to the cellulose-binding domain (CBD) from *Trichoderma hazianum* endoglucanase II [98]. CBD was linked to the N-terminus of the lipase through a linker peptide from the endoglucanase. Also α-amylase signal peptide and the GAL10 promoter were used. Sevenfold increase in the amount of the secreted CBD-fusion protein (98 U/ml, olive oil) was observed in batch cultures, compared with L1 lipase in *S. cerevisiae* (14 U/ml). In high-cell density fed-batch cultures the activity of the fusion lipase reached 2200 U/ml. Therefore, CBD offers enhanced secretion of heterologous proteins in yeast and specific binding to a cheap cellulose matrix, thus facilitating enzyme purification and immobilization. The use of the flexible linker, separating the two functional domains, preserved specific activity of the fusion enzyme, as compared to the mature L1 lipase [98]. *B. subtilis* lipA (BSLA) was secreted efficiently from *S. cerevisiae*, when the BSLA gene without the signal sequence was fused to the PIR4 cell wall protein, yielding over 400 U/ml supernatant [60].

Pichia pastoris is another promising host for heterologous production of enzymes. It can grow to high cell density and secrete large amounts of protein. The gene of the lipase from *Bacillus* sp. L2 was cloned in-frame with α-factor secretion signal into the pPICZαA vector with a poly-histidine tag at the C-terminus for purification using affinity chromatography (IMAC). The lipase production in recombinant *P. pastoris* in shake flask culture was about 1400 times and 8 times higher than the wild-type bacteria and the recombinant *E. coli*, respectively [101]. The lipase from *G. thermocatenulatus* was also cloned into the pPICZαA vector under the control of the AOX1 promoter and transferred to *P. pastoris*. Expression was induced with methanol and reached 406 U/ml of lipase activity after 120 h of cultivation (*p*NP palmitate) [63].

4.2. Isolation and purification

The isolation and purification of *Bacillus* lipases follow the common steps. The cells or cell debris are removed by filtration or centrifugation. The culture broth is then concentrated by ultrafiltration, ammonium sulfate precipitation or extraction with organic solvents (ethanol, acetone, methanol). Often several chromatographic methods are applied as well (ion exchange, hydrophobic interaction, gel permeation chromatography) to yield the homogeneous lipase.

Due to the preference of lipases to hydrophobic surfaces, their molecules often aggregate or bind tightly to the column packing. This results in very low yields and an extremely complicated purification procedure. For example, the lipase from *B. subtilis* shows strong tendency to oligomerize due to the absence of the hydrophobic lid that covers the active site in other lipases. Surfactants like Triton X-100 or bile salts are usually added to the culture broth and the chromatographic buffers in order to prevent aggregation [96,102]. However, Kim et al. found that this surfactant can lead to inferior results. They yielded three times higher specific activity of *G. stearothermophilus* L1 lipase expressed in *E. coli*, when no Triton X-100 was added to the purification eluents. The use of Triton X-100 also slightly decreased the optimal temperature of the enzyme, probably due to its detrimental effect on enzyme structure or the physical state of the olive oil emulsion at high temperatures [103].

The capacity of lipases to engage in strong hydrophobic interactions was utilized in the purification of the *G. thermocatenulatus* lipase (BTL2) expressed in *E. coli* [104]. A lipase from *Pseudomonas fluorescens* (PFL), an enzyme with a strong tendency to dimerize, was immobilized via multipoint covalent attachment on glyoxyl-agarose in the presence of Triton X-100. Under these conditions the enzyme was fixed on the support with its active centre oriented toward the medium, allowing for specific adsorption of other lipase molecules. Only BTL2 out of a crude extract was selectively adsorbed on this glyoxyl-agarose-PFL and could be easily liberated using 0.5% Triton X-100 solution. This proved to be a very simple and cost-effective purification procedure with 95% activity yield in the adsorption step, 100% desorption of the target enzyme and multiple use of the immobilized preparation (the matrix was stable up to 4 months and could be used at least 10 times) [104].

In general, conventional purification protocols do not meet industrial requirements for large-scale downstream processing as they are time-consuming and not economical [105]. In this regard, Bradoo et al. reported a very effective three-step strategy for resolution and purification of two lipases from the crude extract of *G. stearothermophilus* SB-1, using aqueous PEG6000/phosphate two-phase system. First, total lipase activity completely partitioned to PEG top phase at pH 7.0. Acidic lipase was then extracted from this top phase into the bottom phase by addition of 25% phosphate at pH 4.0 and 5.27-fold purification was achieved. After that, the neutral lipase, remaining in the PEG phase, was eluted by the addition of 25% phosphate at pH 6.0 (15.25-fold purification). The two enzymes were recovered from the phosphate phase directly by immobilization on Accurel. The process was economical since recycling of both the salt and the PEG phase was achieved [105].

Several recombinant *Bacillus* lipases were purified in one step by immobilized metal ion affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) resin [35,96,106]. The recombinant 6×His-tagged *B. licheniformis* lipase was purified 80-fold with a yield of 72% [35]. The successful purification of the His-tagged lipase from *G. thermocatenulatus* BTL2 expressed in *E. coli* strongly depended on the pH of the buffers and the addition of surfactants (sodium cholate and dodecyl dimethylamine-*N*-oxide) to prevent BTL2 from aggregation. Thus, 98% lipase activity of the crude solution adsorbed on the Ni-NTA support and 60% were recovered (purification factor 6.7) [96]. In the case of *B. subtilis* lipases A and B, the C-terminal 6×His-fusions of both BSLA and BSLB exhibited insufficient binding affinity toward the Ni-NTA matrix and the purified samples were still contaminated [106]. In contrast, the 10×His-tag *N*-terminal fusion lipases tightly bound to the IMAC matrix and were purified to electrophoretic homogeneity in one step. The final yield was 95% for BSLA-10×His and 22% for BSLB-10×His. For comparison, the *B. subtilis* lipase was only partially purified by ultrafiltration and anion exchange chromatography with 0.63% activity recovery [61].

5. Biochemical properties of *Bacillus* lipases

5.1. pH of the reaction medium

The *Bacillus* genus is a source of a number of lipases active at particularly alkaline pH. While most *Bacillus* lipases are stable in

neutral to moderately alkaline media (pH = 7.5–9.0), a number of enzymes have a significantly higher pH optimum: *B. subtilis* FH5, *B. licheniformis* strains H1 and RSP-09, *B. alcalophilus* B-M20, *B. subtilis* lipA and lipB (Table 1) [18,37,76,89]. The recombinant *B. licheniformis* lipase has the highest pH optimum reported, exhibiting maximum activity at pH 10.0–11.5 [35]. This property, combined with stability in the presence of detergents, oxidizing agents, calcium ions, is advantageous for the use of these enzymes as additives in detergent formulations.

A number of *Bacillus* lipases have a pH optimum in the acidic pH range (pH < 6.0). The lipase produced by *B. megaterium* CCOC-P2637 is optimally active at pH = 6.0 and the one produced by *Bacillus* sp. shows maximal activity at pH = 5.5–7.2 [74]. The lipase from *G. stearothermophilus* SB-1 has a pH optimum in the extremely acidic range (pH = 3.0) [105]. Lipases with an acidic pH optimum are required in leather industry for removal of fats, which hinder the access of the chemicals used in leather processing to the skin. Lipases can replace expensive and toxic organic solvents and surfactants in this application.

Generally, *Bacillus* lipases are stable at pH = 7.0–9.0. A number of enzymes have a broad interval of pH stability, covering the acidic range. For example, *Bacillus* sp. lipase retains full activity after 24-h incubation at pH ranging from 5.0 to 11.0 [74]. The lipase from *B.* sp. A30-1 retains 90–95% of its activity after 15 h at pH = 5.0–10.5, similar to *G. stearothermophilus* L1 lipase [21,103,107]. *B. megaterium* 370 lipA is stable for 1 h at room temperature and pH 4.0–12.0 [38]. The lipase from *B. sphaericus* 205y keeps its activity in the range of pH 5.0–13.0 [108].

In contrast, *B. subtilis* 168 lipase is stable in extremely alkaline medium [48]. It keeps its initial activity after 24 h incubation at pH 12.0 and retains 65% activity at pH 13.0, which can be sufficient for an effective (industrial) biotransformation. The lipase from *B. alcalophilus* B-M20 loses only 20% of its activity after 1 h at 60 °C and pH 11.0 [76]. The lipase of *B. licheniformis* SB-3 retains 50% activity at pH 3.0 after 30 min at 50 °C [69].

5.2. Temperature

As mentioned above, the majority of *Bacillus* lipases, *Geobacillus* in particular, are thermostable and have their temperature optima around 45–50 °C (Table 1). Several enzymes demonstrate outstanding temperature activity and stability profiles. The lipase from *Bacillus* sp. H1 shows maximal activity at 70 °C and preserves nearly 80% of it after 20-h incubation at 60 °C (pH 7.0) [51]. The enzymes from *G. stearothermophilus* MC7 and *G. thermoleovorans* ID1 are optimally active at 75 °C and are half-inactivated after 30 min at 70 °C [49,109]. *B.* sp. A30-1 lipase preserves 50% of its initial activity after 8-h heating at 75 °C, even in the absence of substrate [21].

A hyperthermostable enzyme was reported – the lipase from *G. stearothermophilus* SB-1 has $\tau_{1/2}$ = 15 min at 100 °C (pH = 6.0) and $\tau_{1/2}$ = 25 min at 100 °C (pH = 3.0) [69]. Thermostable lipases with broad substrate selectivity are applied in the processing of paper and pulp, for removal of pitch – a hydrophobic component of wood, composed mainly of triglycerides and waxes.

5.2.1. Thermostability

Thermostability is a desired property for most industrially applied biocatalysts. Thermophilic lipases often show higher resistance to chemical denaturation, which makes them ideal tools in industrial and chemical processes where relatively high reaction temperatures and/or organic solvents are used [50]. The elevated reaction temperature provides higher conversion rate; minimal risk of microbial contamination; higher solubility of the substrates and lower viscosity of the reaction medium, favouring mass transfer. Thermostability is dependent on the structure of the enzyme, the environment (solvent), pH, the presence of additives (substrate, ions), and immobilization. Some comparative studies reported that several different structural features such as loop stability, compactness, secondary structure and core packing distinguish thermophilic proteins from mesophilic ones [110]. Stabilizing factors for proteins are: increase of the surface charge, presence of many proline residues, salt bridge network, fewer glycine and labile (Asn, Gln, Met, Cys) residues, more disulfide bridges and hydrogen bonds and stabilization by metals [59]. The typical mesophilic enzyme from *B. subtilis* (LipA) was subjected to site-directed mutagenesis. One combination of nine point mutations (all located on the protein surface and most of them in non-regular secondary-structure elements) resulted in a remarkable enhancement of 15° in the melting temperature and a 20-degree increase in the temperature optimum of activity of the lipase variant (Topt = 55 °C) compared to wild type (Topt = 35 °C). The wild-type enzyme was half-inactivated after 2.8 min at 55 °C, whereas the mutant had $\tau_{1/2}$ = 300 min at 66 °C [30]. In another study on *B. subtilis* lipA the less-packed residues with no water-contact were targetted aiming at increasing inner packing to design a thermostable lipase [111]. A set of mutants of Gly residues to Ala and Ala to Val were created and analyzed for improved thermostability. Mutants A38V, G80A, A146V and G172A were stable after 1-h incubation at 45 °C, conditions under which the wild-type lipase lost its activity drastically. It was observed, however, that the increase in thermal stability of the mutants was accompanied by a decrease in their catalytic activity, both attributed to protein rigidity [111].

A number of enzymes require the presence of metal ions, such as calcium ions, for the maintenance of their stable and active structures. These ions are bound to specific binding sites on the surface of the molecules and restrict local flexibility or unfolding [59,112]. Thermostability of most *Bacillus* lipases is enhanced in the presence of calcium ions. For example, the purified *Bacillus* sp. RSJ-1 lipase had a half life of 150 min at 60 °C. In the presence of 1 mM Ca^{2+} $\tau_{1/2}$ increased to 210 min [59]. The crystal structure of the lipase from *G. stearothermophilus* L1 (Topt = 68 °C) revealed the existence of a second, zinc-binding domain. The zinc-coordinated extra domain makes tight interactions with the loop extended from the C-terminus of the lid helix, providing evidence for understanding the temperature-mediated lid opening (activation of the enzyme) [8]. In contrast, the thermostability and catalytic activity profile of the lipase from *B. pumilus* B26 in the presence of calcium ions was identical with the profile in the absence of Ca^{2+} [39].

Thermostability of *B. subtilis* lipA strongly depends on the pH of the medium. At pH below 6 this lipase slowly denatures upon heating, but the activity and the native structure of the enzyme are completely recovered upon cooling. At high pH and temperature above 45 °C the protein rapidly and irreversibly aggregates and precipitates out of the solution [113]. The authors identified specific inter- and intra-molecular ionic interactions at pH below 6.0, that trap the molecule in a conformation that allows its complete refolding upon cooling.

Dramatic improvement of lipase behavior at extreme temperatures can be achieved through the wide variety of immobilization techniques. The practical examples with *Bacillus* lipases are discussed below.

5.3. Inhibitors

Most of the lipases from *Bacillus* genus are strongly inhibited by PMSF, which irreversibly binds to the catalytic Ser residue. It was observed that the activity of the thermostable lipase of *G. thermoleovorans* ID1 lipase B was not affected by PMSF [73]. EDTA does not have a significant effect on the activity of *Bacillus* lipases (Table 1). It even slightly enhanced the hydrolytic activity of the lipases from *B. licheniformis*, and *B.* sp. strains BP6 and H1 [35,51,114,115]. EDTA inactivated dramatically the lipases from *B.*

Table 1
Biochemical properties of *Bacillus* and *Geobacillus* lipases.

Lipase origin[a], Activity test	Molecular weight (kDa)	pH optimum and stability[b]	*T* optimum and stability[b]	Selectivity	Solvents[b]	Detergents, inhibitors[b]	Metal ions[b]	Reference
Bacillus alcalophilus BM-20 Hydrolysis of olive oil		10.0–11.0 1 h, pH 11 (80%)	60 °C 1 h, 75 °C, pH 10.5 (70%)				50 mM Ca^{2+} (150%)	[76]
Bacillus atrophaeus SB-2 Hydrolysis of olive oil		7.0 pH 3.0 (50%)	50 °C °C	TAGs: C18:0 > C14:0 > C4 > C12:0 > C10:0 > C18:1 > C8 Natural oils	50% (v/v) diethylether (120%); butanol (70%); *i*-propanol (70%); chloroform (60%); hexane (60%); methanol (40%); acetone (30%); glycerol (10%); ethanol (0%)			[69]
Bacillus cereus C7 Hydrolysis of olive oil		8.0 Stable at pH = 7.2–9.0	60 °C Stable 1 h, *T* = 4–60 °C			0.5% (w/v) sodium deoxycholate (123%); sodium taurocholate (120%); cholic acid (90%) 1% (w/v) sodium hypochlorite (100%); hydrogen peroxide (100%) 0.2% (w/v) SDS (106%); Tween 20 (95%); benzokonium chloride (95%); Tween 80 (94%); Span 40 (94%); PEG (86%); Brij 35 (84%); Triton X-100 (50%); CTAB (33%) Trypsin (100%) 0.2% (w/v) commercial detergents (50–80%); Ariel (108%)	1 mM Ca^{2+} (204.0%); Zn^{2+} (141.5%) 0.1 mM Co^{2+} (212.6%); Cu^{2+} (183.0%); Mg^{2+} (146.7%) 1–5 mM Mg^{2+} (100%) 0.1 mM Fe^{3+} (75.0%); Sn^{2+} (53.6%); Mn^{2+} (30.1%); 5 mM Mn^{2+} (0%); Fe^{3+} (0%); Sn^{2+} (0%)	[67]
Bacillus cereus C71 Hydrolysis of *p*NP butyrate	42	9.0 3 h, 33 °C, pH = 8.5–10.0 (>70%)	33 °C 3 h, pH 9.0, *T* = 20–35 °C (>90%)	*p*NP esters: C4 > C8 > C12 > C2 > C16 C18		0.5% (w/v) Triton X-100 (160%); Tween 40 (153%); Tween 80 (141%); Tween 20 (128%) 0.05% (w/v) SDS (86%)	10 mM Na^{+}; Ca^{2+} (98%); Mg^{2+} (88%); K^{+} (75%); Ni^{2+} (71%); Co^{2+} (65%); Mn^{2+} (61%) 1 mM Zn^{2+} (49%); Cu^{2+} (39%)	[55]
Bacillus coagulans Hydrolysis of *p*NP palmitate		7.0 1 h, pH = 5.0–8.0 (72%)	37 °C 1 h, *T* = 50 °C (70%)				0.1% (w/v) Ca^{2+} (105%); Mg^{2+} (76%); Cu^{2+} (71%); Zn^{2+} (71%); Fe^{3+} (56%); Mn^{2+} (32%); Ni^{2+} (26%)	[16]
Bacillus coagulans BTS-1 Hydrolysis of *p*NP palmitate		8.5	45 °C					[70]
Bacillus coagulans BTS-3 Hydrolysis of *p*NP palmitate	31	8.5 Stable at pH = 8.0–10.5	55 °C 20 min, *T* = 70 °C (75%) $\tau_{1/2}$ (55 °C) = 2 h $\tau_{1/2}$ (60 °C) = 30 min	*p*NP esters: C16 > C12 > C8 > C2, C1			1 mM Hg^{2+} (458%); K^{+} (386%); Mg^{2+} (357%); Fe^{3+} (251%); Na^{+} (100%); Al^{3+} (86%); Mn^{2+} (0%); Zn^{2+} (0%)	[57]
Bacillus coagulans MTCC-6375 Hydrolysis of *p*NP palmitate	103	8.5 180 min, pH 10.5 (116%)	45 °C 20 min, *T* = 55 °C (50%)	*p*NP esters: C8 > C16:0 > C1 > C2 > C12:0		150 mM EDTA (13%)	5 mM Hg^{2+} (117.3%); Al^{3+} (117.2%); Fe^{3+} (115.3%); Mg^{2+} (115.2%); Ca^{2+} (112.0%); K^{+} (106.4%); Co^{2+} (101.4%); $Na_2MoO_4{\cdot}H_2O$ (95.5%); Mn^{2+} (25.3%); Zn^{2+} (22.4%) 1 mM Cu^{2+} (104.4%); Na^{+} (109.2%); Mn^{2+} (101.4%); Co^{2+} (39.7%)	[56]

Table 1 (*Continued*)

Lipase origin[a], Activity test	Molecular weight (kDa)	pH optimum and stability[b]	*T* optimum and stability[b]	Selectivity	Solvents[b]	Detergents, inhibitors[b]	Metal ions[b]	Reference
Bacillus coagulans ZJU318	32	9.0 pH = 7.0–10.0	45 °C 1 h, *T* = 40 °C (93.8%)			No effect: EDTA; Triton X-100 Inhibit: SDS; Brij 30; Tween 80	Inhibit: Ag^{+}; Cu^{2+}	[160]
Bacillus licheniformis Hydrolysis of *p*NP palmitate	25	10.5 4 h, *T* = 30 °C, pH 7.0–12.0 (>80%)	55 °C 30 min, *T* = 45 °C (90%)	*p*NP esters: C6 > C8 > C16, C18 > C14		PMSF (40%); 2-mercaptoethanol (100%) 1 mM EDTA (125%); 0.5 mM DTT (142%)	1 mM Ca^{2+} (98%); Mg^{2+} (95%); Sn^{2+} (92%); Cu^{2+} (60%); Fe^{2+} (60%); Mn^{2+} (60%); Ba^{2+} (60%); Ni^{2+} (60%); Zn^{2+} (<30%); Co^{2+} (<30%); Hg^{2+} (0%)	[35]
Bacillus licheniformis H1 Hydrolysis of olive oil		10.0 30 min, 4 °C, pH 12.0 (65%)	55 °C 15 min, 70 °C (100%) $\tau_{1/2}$ (70 °C) = 30 min				10 mM Ca^{2+} (120%); Cu^{2+} (45%); Fe^{3+} (45%)	[18]
Bacillus licheniformis MTCC 6824 Hydrolysis of *p*NP palmitate	74.8	8.0	45 °C $\tau_{1/2}$ (45 °C) = 82 min $\tau_{1/2}$ (50 °C) = 75 min $\tau_{1/2}$ (55 °C) = 48 min	TAGs: C4 > C12:0 > C16:0 > C18:0 > C18:1	50% (v/v) methanol (72%); *i*-propanol (63%); ethanol (44%); acetone (31%); *n*-hexane (14%); ethyl acetate (9%); acetonitrile (6%); chloroform (5%)	sorbitol (100%) polypropylene glycol (67.8%); ethylene glycol (53.8%); glycerol (71.6%) 20 mM EDTA (67%) 10 mM urea (71%); 2-mercaptoethanol (69%); thiourea (57%); NBS (48%); PMSF (40%)	30 mM Ca^{2+} (118%); Mg^{2+} (112%) 20 mM K^{+} (108%) 10 mM Mn^{2+} (93%); Na^{+} (92%); Hg^{2+} (86%); Co^{2+} (81%); Zn^{2+} (74%); Cu^{2+} (69%); Fe^{2+} (66%)	[44]
Bacillus licheniformis RSP-09 Hydrolysis of *p*NP palmitate	24	10.0 1 h, *T* = 37 °C, pH = 6.0–11.0 (100%)	40 °C 1 h, *T* = 45 °C, pH 10.0 (95%) 1 h, 60 °C, pH 10.0 (7%)	*p*NP esters: C4 > C10 > C12:0 > C16 > C18:0	25% (v/v) DMSO (96%); *n*-hexane (65%); toluene (58%); DMF (57%); *i*-octane (54%); benzene (53%); acetonitrile (23%)	1 mM EDTA (100%); DTT (100%); PMSF (23%) 1% (w/v) Triton X-100 (90%); Tween 20 (67%); Tween 80 (64%); SDS (35%)		[89]
Bacillus licheniformis SB-3 Hydrolysis of olive oil		7.0 pH 3.0, 50 °C (50%)	50 °C	TAGs: C18:0 > C14:0 > C18:1 > C12:0 > C10:0 > C4 > C8 Natural oils	50% (v/v) diethylether (118%); butanol (70%); *i*-propanol (65%); chloroform (60%); hexane (50%); methanol (40%); acetone (30%); glycerol (30%); ethanol (0%)			[69]
Bacillus megaterium 370 lipA Hydrolysis of *p*NP laurate	19.5	7.0 1 h, r.t., pH = 4.0–12.0	45 °C 40 days, pH 7.0, *T* = 4–30 °C 1 h, 50 °C (9.1%)	*p*NP esters: C4 > C5 > C6 > 8 > C10 > C12:0 > C16:0 > C18:0		0.05% SDS (800%) 1 mM EDTA (100%); 0.4% SDS (100%) 2.5 mM capric acid (160%) 5 mM myristic acid (286%); lauric acid (232%) 25 mM capric acid (90%) Inhibit: NBS; urea; PMSF; phytic acid; >1% Triton X-100 20 mM EDTA (40%)	1 mM Ag^{+} (52%); Hg^{2+} (0%)	[38,114]
Bacillus megaterium AKG-1 Hydrolysis of *p*NP laurate	35	7.0	55 °C 1 h, 60 °C (>70%) $\tau_{1/2}$ (70 °C) = 30 min		10% (v/v) *i*-propanol (170%) 20% (v/v) acetone (142%); DMSO (132%); ethanol (80%); hexane (78%); chloroform (57%); methanol (42%); toluene (41%)	0.1% (w/v) deoxycholic acid (176%); sodium deoxycholate (131%); litocholic acid (124%); rhamnolipid (124%); Brij 52 (122%); cholic acid (113%); DTT (100%); PCMB (100%); Brij 30 (96%); Tween 80 (94%); Triton X-100 (91%); Brij 76 (82%); SDS (58%)	0.5 mM Li^{+}; Ca^{2+}; K^{+} (100%) 1.0 mM Ca^{2+} (91%); Li^{+} (80%); Pb^{2+} (79%); Ni^{2+} (71%); K^{+} (67%); Mg^{2+} (63%); Cu^{2+} (57%); Mn^{2+} (57%); Co^{2+} (56%); Zn^{2+} (17%); Hg^{2+} (17%)	[119]

Table 1 (*Continued*)

Bacillus megaterium CCOC-P2637 Hydrolysis of *p*NP palmitate	40	6.0 pH 3.0 (60%) pH 4 (73%) 1 h, T = 29 °C, pH = 5.0–8.0 (100%)	55 °C $\tau_{1/2}$ (50 °C) = 42 min $\tau_{1/2}$ (60 °C) = 23 min	*p*NP esters: C16 > C6 > C4 > C2 TAGs: C4 > C8 > C18:1	80% (v/v) ethanol (195%); acetone (127%) 50% (v/v) *i*-propanol (176%) 100% (v/v) *n*-heptane (121%); toluene (107%); butanol (102%); hexane (101%); *i*-propanol (97%); *i*-octane (67%); ethanol (44%); acetone (21%)			[45]
Bacillus pumilus B26 Hydrolysis of olive oil	19.2	8.5	35 °C	TAGs: C3 > C4 > C6 > C2 > C8 > C10 > C16, C14, C18		5 mM EDTA (100%)	10 mM Ca^{2+} (100%)	[39]
Bacillus pumilus B106 Hydrolysis of *p*NP acetate		8.0 1 h, pH = 7.0–9.0 (>65%)	50 °C 1 h, T = 30–50 °C (>55%)		10% (v/v) methanol (138%) 20% (v/v) methanol (127%) 10% (v/v) DMSO (91%); ethanol (88%); *i*-propanol (81%) 30% (v/v) methanol (63%)			[71]
Bacillus sp. Hydrolysis of olive oil	22	5.6–7.2	60 °C 30 min, T = 30–65 °C, pH 5.6 (90–100%)	*sn*-1,3 positional specificity Me esters: C18:1 > C18:2 > C18:3 > C12 > C4 > C2 TAGs: C8 > C10 > C4 > C18:1 > C12 > C14 > C16 > C6 > C18:0 > C2 > C3	20% (v/v) acetone (160%); *n*-hexane (60%) 10% (v/v) *n*-hexane (100%) >70% (v/v) acetone (0%)	1 mM PCMB; EDTA (100%)	1 mM Mg^{2+}; Ca^{2+}; Fe^{2+}; Sr^{2+}; Cd^{2+}; Sn^{2+}; Ba^{2+} (100%) 1 mM Cu^{2+}; Zn^{2+}; Hg^{2+} (30%)	[74]
Bacillus sp. Lip1 Hydrolysis of *p*NP laurate	60	8.5 pH = 7.0–9.0 (>80%)	65 °C $\tau_{1/2}$ (70 °C) = 85 min $\tau_{1/2}$ (80 °C) = 23 min	TAGs: C4 > C6 > C10 > C18:0 > C12 > C8 > C18:1 > C16		1 M urea (100%) 0.1 M PMSF (<60%) 5; 10 mM eserine (67%; 0%) Trypsin; chymotrypsin (100%); thermolysin (50%) Inhibit: EDTA; DEPC; β-mercaptoethanol; DTT	Activate: 10 mM Na^{+}; K^{+}; Mg^{2+} No effect: 10 mM Li^{+}; Ca^{2+} Inhibit: 10 mM Hg^{2+} (0%)	[19]
Bacillus sp. Lip2 Hydrolysis of *p*NP laurate	60	8.0 8.0–8.5	60 °C $\tau_{1/2}$ (70 °C) = 45 min $\tau_{1/2}$ (80 °C) = 17 min	TAGs: C16 > C4 > C18:0 > C8 > C18:1 > C12 > C6 > C10	30% (v/v) benzene (207%); hexane (201%); ethylene glycol (95.7%); methanol (60.3%); ethanol (57%)	100 mM DTT (109.9%); SDS (94.3%); DEPC (47.3%); EDTA (36.8%); PMSF (35.1%) 5 mM eserine (97.7%)	Activate: 10 mM Ca^{2+}; K^{+}; Li^{+}; Mg^{2+} inhibit: 10 mM Hg^{2+} (0%); Cu^{2+}; Fe^{2+}; Ba^{2+}; Co^{2+}; Cd^{2+}	[161]
Bacillus sp. strain 42 Hydrolysis of olive oil					25% (v/v) benzene (105%); *n*-hexane (104%); *n*-hexadecane (104%); tetradecane (94.5%); *i*-octane (93.4%); decanol (90%); toluene (84.7%); *p*-xylene (79.7%); dodecanol (71%); propyl acetate (63.6%); ethyl acetate (46.4%); 1-propanol (34.5%)			[66]
Bacillus sp. strain 398 Hydrolysis of olive oil	50	8.2 1 h, T = 37 °C, pH = 6.0–11.0 (>80%)	65 °C 30 min, pH 7.0, T = 30–60 °C (100%) $\tau_{1/2}$ (65 °C) = 30 min	TAGs: C8 highest *p*NP esters: C6 highest Preference for *sn*-1 and *sn*-3				[20]

Table 1 (*Continued*)

Lipase origin[a], Activity test	Molecular weight (kDa)	pH optimum and stability[b]	*T* optimum and stability[b]	Selectivity	Solvents[b]	Detergents, inhibitors[b]	Metal ions[b]	Reference
Bacillus sp. A30-1 Hydrolysis of corn oil	65	9.5 15 h, pH 5–10.5 (90–95%)	60 °C $\tau_{1/2}$ (75 °C) = 8 h	TAGs: C12:0 > C10:0 > C18:1 > C18:2 > C14:0, C8 > 16:0 > C18:0 > C20:0 > C22:0 Natural oils		5 U/ml subtilisin (60%) 1 h 0.5% (w/v) H_2O_2 (100%); 18 h (60%) 5 h 4% (w/v) H_2O_2 (75%)		[21]
Geobacillus sp. ARM Hydrolysis of olive oil								[52]
Bacillus sp. BP-6 LipA Hydrolysis of *p*NP laurate	19	MUF-butyrate 7.0 1 h, r.t., pH = 4.0–12.0	45 °C 40 days, pH 7.0, *T* = 4–30 °C	*p*NP esters: C4 > C5 > C6 > C8 > C10 > C12 > C16 > C18		5 mM myristic acid (292%); lauric acid (231%) 2.5 mM capric acid (156%) 1 mM EDTA (110.1%) 10 mM PHMB (120.5%); NAI (105.8%); NBS (63.7%); PMSF (10.2%) 0.4% SDS (115.6%) 1% Triton X-100 (89.2%) 10% (w/v) Triton X-100 (164%); Triton X-114 (149%); Tween 80 (100%); Tween 20 (99%); Tween 40 (90.5%)	1 mM Fe^{2+} (110%); Mn^{2+} (102.3%); Pb^{2+} (102.2%); Zn^{2+} (100.4%); Cu2+ (91.2%); Ag^{+} (45.9%); Hg^{2+} (26.3%) 10 mM NH_4Cl (119.5%); Ba^{2+} (108.3%); Mg^{2+} (103.6%); Ni^{2+} (99.5%); Co^{2+} (98.8%); Ca^{2+} (88.1%)	[114,115]
Bacillus sp. DH4 Hydrolysis of *p*NP laurate		9.0 6 h, 50 °C, pH 9.0 (>145%)	50 °C 1 h, *T* = 60 °C (95.8%) 1 h, *T* = 70 °C (90%)			0.2% SDS (2%); 1% (w/v) sodium cholate (92.25%) 1% (w/v) commercial detergents (65–85%) 1 h with 400 mg/l chlorine (80%)		[22]
Bacillus sp. GK 8 Hydrolysis of *p*NP laurate	108	8.0 1 h, pH = 6.0–10.0 (90%)	45–50 °C $\tau_{1/2}$ (60 °C) = 2 h $\tau_{1/2}$ (65 °C) = 40 min $\tau_{1/2}$ (70 °C) = 18 min	TAGs: C4 > C18:1 > C12 > C16 > C10 > C6 > C8	Activate: 30% (v/v) Glycerol; ethylene glycol 10% (v/v) DMSO No effect: 10% (v/v) isoamyl alcohol; ethanol Inhibit: 10% (v/v) dioxane; acetonitrile; acetone	0.1% (v/v) DTT (107.4%); Tween 80; Tween 60; sodium taurocholate (100%); eserine (95.8%); SDS (92.6%); EDTA (85.3%); PMSF (35.8%); DEPC (30.5%); Brij-35 (0%); cetrimide (0%); Triton X-100 (0%)	activate: 10 mM Mg^{2+}; Ba^{2+}; Na^{+} (NaCl; Na_2CO_3) No effect: 10 mM K^{+}; Li^{+}; Ca^{2+} Inhibit: 10 mM Hg^{2+} (80%); Co^{2+}; Zn^{2+}; Ni^{2+}	[40]
Bacillus sp. H1 Hydrolysis of β-naphtyl caprylate	19.4	7.2 pH 9.0 (74%)	70 °C 20 h, pH 7.0, *T* = 60 °C (77%) $\tau_{1/2}$ (70 °C) = 3 h	β-naphtyl esters: C4 > C8 > C10 > C12		1 mM EDTA (142%) 4.8–38.3 mM sodium taurocholate (100–104%)	1 mM Ba^{2+} (132%); Co^{2+} (107%); Ca^{2+} (107%); Na^{+} (107%); K^{+} (106%); $Ni(NO_3)_2$ (105%); Mg^{2+} (102%); $Al(NO_3)_3$ (29%); $FeSO_4$ (25%); Hg^{2+} (<1%)	[51]
Bacillus sp. H-257 Hydrolysis of monolauryl glycerol	25	6.0–8.0 10 min, pH 7.0–10.0, 70 °C	75 °C 10 min, *T* = 60°C (90%)	*p*NP esters: C4 > C8 > C2 > C12 > C16 1-Monoacylglycerols: C12 > C8 > C14 > C18:3 > C18:2 > C18:1 > C16:0 > C18:1 > C4 > C18:0 > C2 Monoacylglycerol lipase		0.05% (w/v) Triton X-100 (100%) 5 mM cholate (99.5%) 1% Triton X-100 (89.5%) 1 mM cholate (97.5%) 5 mM deoxycholate (83.1%)	No effect: 10 mM Na^{+}; K^{+}; Li^{+}; Mg^{2+}; Mn^{2+}; Ca^{2+}	[58]

Table 1 (*Continued*)

Bacillus sp. J33 Hydrolysis of *p*NP laurate	45	8.0–8.5	60 °C 30 min, T = 60 °C (100%) 30 min, T = 70 °C (50%) $\tau_{1/2}$ (37 °C) = 40 h $\tau_{1/2}$ (60 °C) = 5 h	TAGs: C12=C4 > C6 > C16 > C10 = C8 = C18:1 > C18:0	60% (v/v) benzene; hexane; DMSO (120–140%) 30% (v/v) ethylene glycol; glycerol; DMSO (100%) 30% (v/v) methanol; propanol; acetonitrile; dioxane (50%)	0.1% (v/v) Tween 20 (160%); deoxycholic acid (140%); Triton X-100 (140%); Tween 80 (120%) No effect: sodium taurocholate; cetrimide 10 mM EDTA (60%); PMSF (40%); SDS (0%)	Activate: Mg^{2+} (163%); Na^{+} (163%); Li^{+} (123%) No effect: Ca^{2+}; Fe^{2+}; K^{+} Inhibit: Hg^{2+}; Cd^{2+}	[87,116]
Bacillus sp. L2 Hydrolysis of olive oil	45	8.0	70 °C $\tau_{1/2}$ (60 °C) = 3.5 h $\tau_{1/2}$ (65 °C) = 106 min	TAGs: C16 > C12 > C14, C10 > C18:1 > C18:0 > C8 > C6 > C4 > C2 Natural oils		1 mM 2-mercaptoethanol (98.6%); DTT (77.5%); PMSF (8.7%); pepstatin A (3.2%); EDTA (0.4%)	10 mM Ca^{2+} (510%) 1 mM Ca^{2+} (395%); Na^{+} (301%); Cu^{2+} (222%); Mn^{2+} (209%); Fe^{3+} (114%); K^{+} (100%); Zn^{2+} (61%); Mg^{2+} (26%); K^{+} (53); Fe^{3+} (33%); Mn^{2+} (31%); Cu^{2+} (0%)	[101]
Geobacillus sp. Mk1 A.1 Hydrolysis of *p*NP esters		9.0	50 °C $\tau_{1/2}$ (51 °C) = 101 min $\tau_{1/2}$ (67 °C) = 50 min	pNP esters: C10				[127]
Geobacillus sp. OK4 A.2 Hydrolysis of *p*NP esters		9.0	50 °C $\tau_{1/2}$ (51 °C) = 26 min $\tau_{1/2}$ (67 °C) = 17 min	pNP esters: C10				[127]
Geobacillus sp. RD-2 Hydrolysis of *p*NP butyrate	43.0	7.5 Stable at pH = 6.0–11.0	55 °C $\tau_{1/2}$ (65 °C) = 8 h					
Bacillus sp. RS-12		8.0	50–55 °C $\tau_{1/2}$ (65 °C) = 60 min $\tau_{1/2}$ (70 °C) = 18 min $\tau_{1/2}$ (75 °C) = 15 min			Oxidizing; reducing; chelating agents Surfactants (80–100%)	Activate: Ca^{2+}; Na^{+}; Ba^{2+}	[162]
Bacillus sp. RSJ-1 Hydrolysis of *p*NP butyrate	37	8.0 2 h, pH = 8.0–9.0 (83%)	50 °C $\tau_{1/2}$ (60 °C) = 150 min $\tau_{1/2}$ (65 °C) = 90 min $\tau_{1/2}$ (70 °C) = 55 min $\tau_{1/2}$ (75 °C) = 45 min			1 mM EDTA (100%); Triton X-100 (100%) 1 mM sodium citrate (96%); Tween 80 (92%); Tween 20 (82%); ascorbic acid (74%); $(NH_4)_2S_2O_8$ (72%); 2-mercaptoethanol (64%); deoxycholate (54%) Commercial detergents (>55%) 0.1% H_2O_2 (93%); 0.2% H_2O_2 (74%)	1 mM Ca^{2+} (116%); NaCl (112%); Ba^{2+} (103%); Mg^{2+} (102%); KCl (100%); NaH_2PO_4 (91%); Ni^{2+} (88%); Mn^{2+} (63%); Cu^{2+} (56%); Cs^{2+} (49%); Co^{2+} (47%); KI (47%); Zn^{2+} (47%); Sn^{2+} (46%)	[59]
Geobacillus sp. T1 Hydrolysis of olive oil	43.0	9.0 30 min, pH 9.0–11.0, T = 50 °C (>60%)	70 °C $\tau_{1/2}$ (65 °C) = 5 h 15 min $\tau_{1/2}$ (70 °C) = 70 min	TAGs: C12 > C14 > C10 > C16 > C8 > C18:1 > C6 > C18 > C4 > C2 Natural oils		1 mM Tween 20 (122%); Tween 40 (101%); Tween 60 (126%); Tween 80 (188%); Triton X-100 (72%); SLS (48%); SDS (5%) 5 mM PMSF (11%) 1 mM pepstatin (14%)	1 mM Ca^{2+} (100%); Na^{+} (99%); Mn^{2+} (87%); K^{+} (81%); Mg^{2+} (81%); Cu^{2+} (49%); Fe^{3+} (39%); Zn^{2+} (22%)	[94,54]
Bacillus sp. TG43 Hydrolysis of *p*NP stearate	44.5	Native enzyme 9.0 30 min, T = 60 °C, pH 9.0 (80%)	60 °C	*p*NP esters: short-chain substrates				[163,164]

Table 1 (*Continued*)

Lipase origin[a], Activity test	Molecular weight (kDa)	pH optimum and stability[b]	*T* optimum and stability[b]	Selectivity	Solvents[b]	Detergents, inhibitors[b]	Metal ions[b]	Reference
Bacillus sp. THL027 Hydrolysis of olive oil	69	7.0	70 °C 1 h, *T* = 60–75 °C (>80%)	TAGs: C8 > C4, C6, C10, C12 > C14:0 > C16:0 > C18:0 Natural oils *sn*-1,3 positional specificity	50% (v/v) *i*-propanol (87%); methanol (87%); ethanol (76%); acetonitrile (76%); acetone (65%)	inhibit: EDTA; 1.0% SDS (90%) (2 h incubation)	1 mM Na^+ (100%); Cu^{2+}; Co^{2+} (>85%); K^+ (>75%); Fe^{3+} (54%); Ca^{2+} 1 mM Mg^{2+}; Mn^{2+}; Zn^{2+}; NH_4^{2+} (>65%)	[77]
Geobacillus sp. Tp10A.1 LipA Hydrolysis of *p*NP laurate	45	8.0	60 °C 80 °C (>50%)	*p*NP esters: C8, C10, C12 > C6, C14 > C18:0 > C16 > C4		0.1% (w/v) taurocholic acid (145%); Triton X-100 (128%); Tween 80 (0%) 1% (w/v) Brij 35 (20%); Tween 20 (0%)		[84]
Geobacillus sp. TW1 Fusion protein with glutathione *S*-transferase Hydrolysis of *p*NP butyrate		7.0–8.0 15 min pH = 6.0–9.0 (>80%)	40 °C 15 min *T* = 50 °C (80%)			1 mM EDTA (78%); β-mercaptoethanol (86%); SDS (72%); PMSF (98%); DTT (97%) 0.1% (w/v) Triton X-100 (93%); Chaps (95%); Tween 20 (86%)	1 mM Ca^{2+} (152%); Fe^{3+} (149%); Fe^{2+} (137%); Mn^{2+} (99%); Cu^{2+} (91%); Li^+ (88%) 5 mM Mg^{2+} (286%); Zn^{2+} (140%); Mn^{2+} (0%); Cu^{2+} (0%)	[88]
Bacillus sphaericus 205y Hydrolysis of olive oil	30	7.0–8.0 Stable *T* = 37 °C, pH = 5.0–13.0	55 °C 30 min, 60 °C (28%)	TAGs: C10 > C8 > C12 > C16 > C14, C6, C4, C18, C2 *sn*-1,3 positional specificity	100% (v/v) DMSO (176%); methanol (120%); *p*-xylene (120%); *n*-decane (110%); no solvent (100%); ethanol (96%); hexadecane (60%); 1-decanol (55%); *n*-dodecane (43%); toluene (40%); acetonitrile (26%); *n*-butanol (23%)	PMSF (33%); β-mercaptoethanol (47%); pepsin A (24%); EDTA (90%)	activate: 1 mM Na^+ (112%); Mg^{2+}; Ca^{2+}; Ba^{2+} No effect: 1 mM K^+ Inhibit: 1 mM Fe^{3+} (94%); Zn^{2+} (90%); Cu^{2+} (48%);	[108]
Geobacillus stearothermophilus strain-5 Hydrolysis of *p*NP palmitate		8.0 30 min pH = 6.0–9.0 (>96%)	60–65 °C 15 min *T* = 30–60 °C (>90%) 15 min *T* = 70 °C (85%)		50% (v/v) diethylether (92%); butanol (67%); hexane (63%); methanol (31%); *i*-propanol (25%); ethanol (23%); toluene (22%); propanol (15%); chloroform (15%); acetone (13%); glycerol (113%);			[78,47]
Geobacillus stearothermophilus AB-1 Hydrolysis of tributyrin		7.5 1 h, pH = 7.0–8.0 (>80%)	35 °C					[68]
Geobacillus stearothermophilus JC Hydrolysis of *p*NP palmitate	43.2	9.0 Stable 24 h pH = 5.0–11.0	55 °C 30 min *T* = 40–55 °C (>80%)	*p*NP esters: C2 < C4 < C6 < C8 < C10 > C12 > C16 > C14	ethanol (145%) *i*-propanol (100%) methanol (70%); DMSO (70%); acetonitrile (70%) acetone (<50%); butanone (<50%)	10 mM Triton X-100 (168.8%); Tween 80 (23.8%); SDS (13.3%)	1 mM Ca^{2+} (168.3%); Mn^{2+} (69.4%); Zn^{2+} (61.8%)	[165]
Geobacillus stearothermophilus L1 Hydrolysis of olive oil	43	9.0–10.0 Stable 24 h, pH = 5.0–11.0	60 °C 30 min, *T* = 30–60 °C (>80%)	*p*NP esters: C8 > C10 > C12 > C14 > C16 > C18 > C6 > C3 > C4, C2 TAGs: C3 > C4 > C12 > C14, C16 > C6, C10 > C8 > C18:1, C18:3 > C18:2 > C18:0 > C2		1 mM PMSF (90%); EDTA (89%); iodoacetamide (85%); E600 (71%) 1% (v/v) 2-mercaptoethanol (89%); sodium deoxycholate (83%); Triton X-100 (83%); SDS (2%)	1 mM Ca^{2+} (100%); Mg^{2+} (90%); Mn^{2+} (89%); Zn^{2+} (85%); Fe^{2+} (81%); Hg^{2+} (25%); Cu^{2+} (13%)	[65,106,107]

Table 1 (*Continued*)

Geobacillus stearothermophilus MC7 Hydrolysis of *p*NP palmitate, tributyrin	62.5	7.5–9.0 30 min, T = 60 °C, pH = 7.5–10.5 (>80%)	75–80 °C $\tau_{1/2}$ (70 °C) = 30 min	*p*NP esters: C16 > C2 TAGs: C4 > C10 > C18:1 > C18:0 > C12, C16 > C2	50% (v/v) solvent: methanol (80%); *i*-propanol (42%); ethanol (27%); acetone (42%); glycerol (42%); *n*-hexane (15%); butanol (0%); toluol (0%); chloroform (0%); diethylether (0%)	5 mM sorbitol (110%); iodacetamide (100%); DTT (75%); PCMB (30%); NBS (46%); PMSF (0%) 0.001 mM $PEG_{70.000}$ (230%) 0.1 mM PEG_{2000} (200%); PEG_{4000} (213%); PEG_{6000} (230%) 1 mM PEG_{400} (173%) 2.7 mM Span 20 (130%); Span 40 (120%); Span 80 (120%) 0.027 mM Span 60 (120%); Tween 60 (130%) Inhibit: Tween 20; Tween 40; Tween 80	5 mM Ca^{2+} (110%); Na^{+} (100%); K^{+} (95%); Li^{+} (83%); Mn^{2+} (67%); Mg^{2+} (67%); Hg^{2+} (46%); Co^{2+} (36%); Zn^{2+} (13%); Fe^{2+} (10%); Cu^{2+} (0%)	[109,123]
Geobacillus stearothermophilus P1 Hydrolysis of *p*NP caprate, olive oil	43.2	8.5	55 °C $\tau_{1/2}$ (55 °C) = 7.6 h	*p*NP esters: C10 > C8 > C12 > C14 > C16:0 > C18:0, C6 > C4 TAGs: C8 > C10 > C12 > C14 > C6 > C16:1 > C18:3, C16:0 > C18:1 > C18:2 > C18:0, C20:1 > C4, C10:0 > C22, C22:0		1 h incubation with 1% (w/v) CHAPS (58%); Triton X-100 (50%); sodium deoxycholate (42%); SDS (37%); Tween 20 (14%) 30 min incubation with 10 mM β-mercaptoethanol (80%); DTT (74%); EDTA (69%); PMSF (2%); 1-dodecanesulfonyl chloride (5%); 1-hexadecane-sulfonyl chloride (0%)	10 mM Ca^{2+} (92%); Na^{+} (90%); Mg^{2+} (90%); Cs^{+} (84%); K^{+} (72%); Li^{+} (71%); Cu^{2+} (63%); Mn^{2+} (41%); Zn^{2+} (1.6%); Fe^{2+} (0.76)	[23,90]
Geobacillus stearothermophilus SB-1 Hydrolysis of olive oil		6.3 pH 3.0, 50 °C (70%)	50 °C 30 min, 100 °C, pH 6.0 (40%) $\tau_{1/2}$ (100 °C) = 25 min (pH 3.0) $\tau_{1/2}$ (100 °C) = 15 min (pH 6.0)	TAGs: C18:0 > C14:0 > C12:0 > C10:0 > C8 > C18:1 > C4 Natural oils	50% (v/v) glycerol (130%); butanol (76%); diethylether (67%); hexane (67%); acetone (58%); *i*-propanol (55%); chloroform (34%); methanol (11%); ethanol (11%)			[69,166]
Bacillus subtilis LipA Hydrolysis of *p*NP laurate	19.3	10.0	35–40 °C	TAGs: C8:0 > C6 > C10 > C4 > C3 > C12 > C18:1 > C2 *p*NP esters: C14:0 > C8 > C12:0 > C16:0 > C6 > C4 > C18:0		2.5–5.0 mM capric; lauric; myristic acid (150%) Inhibit: 20–25 mM capric acid; lauric acid; myristic acid		[37,114]
Bacillus subtilis LipB	19.5	10.0	35–40 °C	TAGs: C8:0 > C6 > C4 > C10 > C3 > C2 > C12 *p*NP esters: C8 > C14:0 > C12:0 > C10, C6 > C18:0 > C4				[37]
Bacillus subtilis Hydrolysis of olive oil						1 h at 45 °C: Triton X-100 (75%); protease (75%); Tween 20 (50%); Tween 80 (50%); SDS (25%); EDTA (0%)		[61]

Table 1 (*Continued*)

Lipase origin[a], Activity test	Molecular weight (kDa)	pH optimum and stability[b]	*T* optimum and stability[b]	Selectivity	Solvents[b]	Detergents, inhibitors[b]	Metal ions[b]	Reference
Bacillus subtilis 168 LipA Hydrolysis of tributyrin	19	10 24 h, pH = 13 (65%)	35 °C 30 min, *T* = 55 °C (0%)	*p*NP esters: C8 > C12 > C4 > C16, C18 TAGs: C8 » C4 > C16 > C12 > C2 C18 (0%)	60% (v/v) DMSO (0%) 30% (v/v) DMSO (140%); *i*-propanol (0%); acetone (0%); ethanol; pyridine	0.1 mM PMSF (30%) 1 mM PMSF (0%)	10 mM Ca^{2+} (294%); Cu^{2+} (45%); Mn^{2+} (32%); Zn^{2+} (37%)	[48]
Bacillus subtilis EH 37 Hydrolysis of olive oil		8.0	60 °C 60 min, *T* = 60 °C (100%) 30 min, *T* = 70 °C (100%)		15 mM *i*-propanol (107%); acetone (102%); DMSO (100%); *n*-hexane (98%) 30 mM *n*-hexane (115.0%); acetone (99.8%); ethanol (80.0%); methanol (76.0%); DMF (76.0%)		1 mM Ca^{2+} (116%); Mg^{2+} (107%); Zn^{2+} (102%); Fe^{3+} (95.8%); Co^{2+} (95%) 10 mM Ca^{2+} (94.5%); Zn^{2+} (91.0%); Mg^{2+} (90.0%); Co^{2+} (86%); Fe^{3+} (85.0%)	[120]
Bacillus subtilis FH5 Hydrolysis of *p*NP laurate	62; 24	10.0	15–40 °C (100%) 60 °C (80%)		20% (v/v) ethanol (90%); acetone (87%) 30% (v/v) *i*-propanol (90%)	1 mM SDS (16.2%); EDTA (19.3%); PMSF (9.75%)	1 mM Mg^{2+} (141.0%); Ca^{2+} (136.7%); Fe^{2+} (134.5%); Mn^{2+} (134.5%); Ba^{2+} (107.3%); Hg^{2+} (105.0%); K^{+} (100%); Cu^{2+} (95.8%); Na^{+} (91.0%); Zn^{2+} (86.8%); Co^{2+} (84.2%)	[62]
Bacillus subtilis IFFI10210 Hydrolysis of *p*NP caprylate	24	8.5 20 h, *T* = 4 °C, pH = 6.5–10.0 (>90%)	43 °C 20 h, *T* = 20–40 °C (>90%) 20 h, *T* = 45 °C (75%)	*p*NP esters: C8 > C3 > C12 C16, C18 (0%)		7 mM sodium taurocholate (148%) Inhibit: >10 mM sodium taurocholate	10 mM Ca^{2+} (116%); K^{+} (112%); Mg^{2+} (110%); Co^{2+} (15%); Fe^{2+} (9%); Cu^{2+} (0%) 50 mM K^{+} (102%); Mg^{2+} (98%); Ca^{2+} (80%)	[100]
Geobacillus thermocatenulatus (DSM 370) Hydrolysis of olive oil	16	8.0	65–70 °C	Lower preference for *sn*-2 than for *sn*-1,3		0 min incubation: 0.1% (w/v) taurocholic acid (129%); CHAPS (123%); octylglucoside (113%) 90 min incubation: 0.1% (w/v) lubrol PX (75%); Brij 35 (71%); Triton X-100 (62%); Tween 80 (0%); Tween 20 (0%)		[72]
Geobacillus thermocatenulatus BTL2 recombinant, *E. coli* Hydrolysis of olive oil, *p*NP palmitate, tributyrin	43	8.0–9.0 12 h, *T* = 30 °C, pH = 9.0–11.0 (90–100%)	60–70 °C 30 min, pH 9.0, *T* = 30–50 °C (>80%)	pH 7.5 TAGs: C4 > C6 > C8 > C10 > C12 > C16, C18> C2 > C14 *p*NP esters: C10 > C12 > C14 > C16 > C8 > C6 > C2 > C4 pH 8.5 TAGs: C4 > C18 > C16 > C12 > C8	0 min incubation 30% (v/v) methanol (118%); ethanol (100%); acetone (100%); *i*-propanol (100%) 30 min incubation 30% (v/v) *i*-propanol (95%); ethanol (90%); acetone (82%); methanol (80%)	0 min incubation 0.1% lubrol PX (139%); Tween 20 (137%); Triton X-100 (119%) 1% (w/v) CHAPS (184%); octylglucoside (180%); Brij 35 (163%); Triton X-100 (0%); Tween 80 (0%) No effect: 10 mM EDTA; 1 mM NaN_3; PMSF	10 mM Ca^{2+}; Mg^{2+} (100%); Mn^{2+} (69%) 1 mM Ag^{+} (72%)	[117]
Geobacillus thermocatenulatus BTL2 recombinant, *Pichia pastoris* Hydrolysis of *p*NP palmitate	43	8.0–9.0	65–75 °C 30 min, pH 8.0, *T* = 50 °C (100%) 30 min, pH 8.0, *T* = 70 °C (18%)	pH 7.5 TAGs: C4 > C8 > C6, C10 > C16 > C12, C14 > C18 > C2	1.5 h incubation 30% (v/v) methanol (84%); acetone (84%); *i*-propanol (53%)	1.5 h incubation 1% (w/v) Triton X-100 (130%); Tween 80 (106%); Tween 20 (93%); cholate (80%); SDS (5%)		[63]

Table 1 (*Continued*)

Geobacillus thermoleovorans CCR11 Hydrolysis of *p*NP laurate	11	9.0–10.0 26 h, $T = 30\,^{\circ}C$, pH-5.0–11.0 (>80%)	60 °C 1 h, $T = 50$–$60\,^{\circ}C$ (75%) 1 h, $T = 70\,^{\circ}C$ (0%)	*p*NP esters: C10 > C6 > C12 > C16 > C18 > C3 > C4 > C2	0 h incubation 70% (v/v) methanol (121%); ethanol (102%); acetone (89.2%); *i*-propanol (89.2%); butanol (0%) 1 h incubation 70% (v/v) *i*-propanol (101%); ethanol (98.6%); acetone (97.7%); methanol (92.4%)	1 mM Triton X-100 (108.7%); 2-mercaptoethanol (95.3%); EDTA (61.7%); PMSF (17.3%); Tween 20 (0%); Tween 80 (0%); SDS (0%)	0 h incubation 1 mM Ca^{2+} (159.2%); K^{+} (100.4%); Na^{+} (98.3%); Ba^{2+} (94.9%); Li^{+} (94.0%); Mg^{2+} (93.6%); Hg^{2+} (24.9%)	[50]
Geobacillus thermoleovorans GE-7 Hydrolysis of olive oil, *p*NP palmitate								[81]
Geobacillus thermoleovorans ID-1 LipA Hydrolysis of tricaprylin	18	9.0	60–65 °C 30 min, $T = 60\,^{\circ}C$ (>75%)	TAGs: C8 > C10 > C14 > C12 > C16 > C18 C4, C6 (0%) *sn*-1,3 positional specificity	1% (v/v) ethanol (58%); *i*-propanol (47%); DMSO (46%)	1% (w/v) SDS (97%); 2-mercaptoethanol (74%); DTT (52%); EDTA (32%); PMSF (8%)	1 mM Ca^{2+} (87%); Na^{+} (80%); Mg^{2+} (75%); Co^{2+} (62%); K^{+} (62%); Zn^{2+} (55%); Mn^{2+} (55%); Hg^{2+} (54%); Fe^{2+} (35%); Ag^{+} (34%); Cu^{2+} (32%)	[73]
Geobacillus thermoelovorans ID-1 LipB Hydrolysis of tricaprylin	43	8.0–9.0	60 °C $\tau_{1/2}$ (70 °C) = 30 min	TAGs: C10, C8, C16 > C12 > C6 > C14 > C18 > C4	1% (v/v) *i*-propanol (130%); DMSO (126%); ethanol (124%)	1% (w/v) 2-mercaptoethanol (135%); DTT (129%); PMSF (124%); EDTA (55%)	1 mM Na^{+} (124%); Mn^{2+} (124%); K^{+} (120%); Ca^{2+} (121%); Co^{2+} (119%); Ag^{+} (109%); Hg^{2+} (87%); Fe^{2+} (64%); Zn^{2+} (63%); Cu^{2+} (56%)	[73]
Geobacillus thermoleovorans ID-1 Hydrolysis of *p*NP butyrate	34	7.5	75 °C 30 min $T = 60\,^{\circ}C$ (73%) 30 min $T = 70\,^{\circ}C$ (50%)	*p*NP esters: C6 > C4 > C10 > C14 > C12 > C16 > C8 > C18 TAGs: C8 > C10 > C12 > C14 > C16 > C6 > C18 > C4		1 mM EDTA (92%); β-mercaptoethanol (84%); DTT (88%); PMSF (64%); SDS (30%)	1 mM Zn^{2+} (126%); Ca^{2+} (118%); Mn^{2+} (102%); K^{+} (102%); 1 mM Li^{+} (95%); Na^{+} (95%); Mg^{2+} (95%)	[49]
Geobacillus thermoleovorans Toshki Hydrolysis of *p*NP palmitate	43.1	8.0	65 °C 1 h $T = 70\,^{\circ}C$ (80%) 1 h $T = 80\,^{\circ}C$ (60%)					[85]
Geobacillus thermoleovorans YN Hydrolysis of *p*NP palmitate	43	7.5–9.5	65 °C 30 min $T = 70\,^{\circ}C$ (100%)	*p*NP esters: C10 > C6 > C4 > C2 > C16 > C12 > C18 > C14 Me esters: C10 > C8 > C12 > C6 > C14 > C16, C18 > C3 > C4 TAGs: C8 > C16 > C14 > C18 > C4, C12 > C2	1% (v/v) ethanol (98.8%); DMSO (94.1%); *i*-propanol (93.7%)	1% (w/v) DTT (96.5%); SDS (99.5%); EDTA (98.1%)	1 mM Ca^{2+} (102.1%); Cs^{2+} (97.9%); Mg^{2+} (96.1%); Na^{+} (94.8%); Zn^{2+} (14.4%)	[92]
Geobacillus zalihae T1 Hydrolysis of olive oil			70 °C 30 min $T = 60\,^{\circ}C$ (>90%)					[80]

[a] Bacteria are listed in alphabetical order by the species and strain name.
[b] Values in brackets represent residual lipolytic activity.

coagulans MTCC-6375; *B. subtilis* strains FH5, J33, and L2; and *B. subtilis* [56,61,62,87,101,116]. This means that these enzymes require a metal ion to stabilize their active conformation. Reducing agents like DTT and β-mercaptoethanol affect mainly the activity of lipases belonging to I.5 subfamily, since disulfide bridges support the conformation of the active site and contribute for the thermostability of the enzymes.

5.4. Metal ions

Metal ions and salts are of special importance in thermostable enzymes. These ions are bound to specific binding sites on the surface of molecules and have a structural role [112]. By coordination, they keep together and fix the conformation of certain flexible segments of the polypeptide chain. In general, calcium ions activate *Bacillus* lipases by 10–50% or preserve the activity of the enzymes at high temperatures. The activity of *B. cereus* C7 lipase was doubled in the presence of 1 mM Ca^{2+} [67]. The lipase from *B.* sp. L2 retained 510% activity in 10 mM Ca^{2+} [101]. Apart from the structural role, the positive effect of Ca^{2+} is also attributed to formation of insoluble Ca-salts of the fatty acids released during hydrolysis, thereby suppressing product inhibition [48]. There are several lipases that are not affected by the presence of calcium ions [39,40,55,103,117–119]. The lipase from *B. subtilis* tolerates the presence of 50 mM Ca^{2+}. Some *Bacillus* lipases are slightly inhibited by calcium (see Table 1). The effect of potassium and magnesium ions is variable. In most cases enzymes preserve their initial activities or are weakly activated by 5–20% in the presence of up to 10 mM K^+. Potassium ions had a significant stimulating effect on the activity of *B. coagulans* BTS-3: 1 mM K^+ resulted in 386% lipase activity [57]. Zinc ions have inhibitory effect on most *Bacillus* lipases tested, as do in most cases Co^{2+}, Mn^{2+}, Fe^{2+} and Fe^{3+}, Cu^{2+}, Na^+. Zn^{2+} only activated *Bacillus* sp. EH37 [120], *G. thermoleovorans* ID1 [49], *B. cereus* C7 [67]. Mercury (II) ions are strong inhibitors for *Bacillus* lipases. They probably interact with the sulfur-containing groups of the protein, thus altering the conformation of the enzyme. Several lipases are comparatively resistant to Hg^{2+} ions: *B. licheniformis* MTCC-6824 [44], *B.* sp. GK8 [40], *B. subtilis* FH5 [62], and *G. thermoleovorans* ID1 lipB [73]. Interestingly, Hg^{2+} activated *B. coagulans* BTS-3 lipase nearly 4.5-fold [86] and *B. coagulans* MTCC-6375 [56]. It has been demonstrated that the effect of metal ions on enzyme activity depends on the their concentration and time of incubation [50,72,90,117].

5.5. Solvents

Lipase behavior in various solvents is related with their efficiency in both synthetic and hydrolytic reactions. Esterification and transesterification are carried out in low-water-content media using non-polar solvents, in order to suppress the reverse reaction of hydrolysis and the spontaneous non-enzymatic hydrolysis of products. *Bacillus* lipases are very stable in hydrophobic organic solvents. Their activity is even slightly increased in the presence of 10–50% (v/v) of diethyl ether, *n*-hexane, *n*-heptane, octane, cyclohexane, benzene and toluene. Non-polar solvents probably shift the equilibrium from closed to open conformation of the enzyme and also modify the solubility of the substrates and products in the reaction medium.

Polar water-miscible solvents are more destabilizing to proteins than water-immiscible solvents, as they remove the solvation water from the enzyme [50]. Good stability in polar solvents is also related with substrate inhibition in the synthesis of flavor esters and the production of biodiesel, where one of the reactants is usually methanol or ethanol. Most lipases from the *Bacillus* genus are inactivated by alcohols, although a few lipases retain full activity in polar solvents. For example, the lipases from *B. megaterium* AKG-1 and *B. coagulans* BTS-3 are stable in *i*-propanol [119;121]; those from *B. megaterium* CCOC P2637 [45] and *G. thermoleovorans* ID-1 [49] tolerate ethanol and *i*-propanol; and the lipases from *B. pumilus* B106 [71], *G. thermocatenulatus* [117] and *G. thermoleovorans* CCR11 [50] are stable in methanol. *B. sphaericus* lipase retains 176% activity in DMSO, a solvent which strongly destabilizes enzymes [122]. It has been suggested that polar solvents may prevent lipases from aggregating, resulting in increased reaction rate [45]. The effect of the solvent on enzyme activity strongly depends on the time of incubation [50,63,117]. Stability in various solvents is greatly improved by immobilization of enzymes.

5.6. Surfactants

Surfactants are applied in lipase-catalyzed reactions because they increase the water–lipid interfacial area (improving the stability of the emulsion); increase substrate solubility; stabilize the open conformation of the enzyme and prevent it from aggregation, all leading to greatly improved catalytic performance [118]. The effect of surfactants has been tested on very few of the isolated *Bacillus* lipases. It can be concluded that in general *Bacillus* lipases are very tolerant to the presence of detergents, as their activity is weakly affected (either positively or negatively) compared to other microbial lipases [118].

Generally, *Bacillus* lipases tolerate the presence of small amounts of Triton X-100 or are inactivated weakly. In low concentrations (below 1%, w/v) Triton X-100 increased the activity of a number of *Bacillus* lipases by 40–60% [55,63,84,87,116,117]. The lipase from *B.* sp. DH4 retained 164% activity in the presence of 10% (w/v) of this surfactant [22]. SDS is an inhibitor of *Bacillus* lipases with an exception of *B. megaterium* 370 lipase, which is activated by a factor of 8 in the presence of 0.05% of it [38]. *Bacillus* lipases were reported to be activated in the presence of free fatty acids, cholates, Tweens and spans, but again the increase is in the range of 10–60%. The activity of the lipase from *Geobacillus* sp. T1 was increased by 88% in the presence of 1 mM Tween 80 [94]. The lipases from *B. megaterium* and *B.* sp. BP6 were strongly activated (up to 290% residual activity) by capric, myristic and lauric acid in low concentrations (2.5–5 mM) unlike other bacterial lipases [114]. In high concentrations the effect of the surfactants on lipase activity is negative, probably because they block the access of the substrate to the active site. An important factor is again the time for incubation.

In some cases surfactants may improve the thermostability of enzymes without altering their activity. *G. stearothermophilus* MC7 lipase preserved 60% of its initial activity after 30 min incubation at 75 °C, whereas the half-life of the pure enzyme at 70 °C was 0.5 h [123]. The thermostability of *B. licheniformis* MTCC6824 was enhanced by the combination of sorbitol and Ca^{2+} ions [44]. The incubation of the free lipase from *B.* sp. J33 at 70 °C for 1 h decreased its activity to 33%. In the presence of glycerol and ethylene glycol practically no loss of activity was observed after 2 h at 70 °C. In the presence of sorbitol, the activity of the enzyme even increased during incubation [116]. Some of the detergents are lyoprotectant. For example, the lyophilized *G. stearothermophilus* MC7 lipase preserved 80% of its initial activity in the presence of polyethylene glycols of molecular weight from 400 to 70,000, whereas the native enzyme retained only 30% residual activity [123].

5.6.1. Laundry detergents

Necessary properties of the enzymes added in laundry formulations are optimal activity in alkaline medium and elevated temperatures and good stability in a complex mixture containing high concentrations of Ca^{2+} ions, surfactants, oxidizing agents (peroxide, perchlorates), and proteases. Several *Bacillus* lipases were tested and demonstrate exceptional stability and high potential for such application. The lipase from *Bacillus cereus* C7 retained

60% or higher activity in the presence of a number of commercial detergents. The lipase was highly stable in the presence of 1% (v/v) hydrogen peroxide and 1% (w/v) sodium hypochlorite after 1-h incubation. Bleach stability is an important property and bleach stable enzymes are not very common. The *B. cereus* lipase was also found to be stable in the presence of trypsin and could be used in combination with proteases in detergent formulations [67]. *B.* sp. DH4 lipase was found to be stable in both ionic and nonionic surfactants. The enzyme was inactivated by 20–40% in the presence of laundry detergents and retained almost 80% activity after being exposed to chlorine for 1 h [22]. The lipase from *Bacillus* sp. RSJ-1 retained more than 90% activity at 60 °C and pH 9.0. It was stable in the presence of some non-ionic surfactants (Triton X-100, Tween 80 and Tween 20) and retained 93% and 74% activity at 0.1 and 0.2% concentration of H_2O_2, respectively. The lipase was found to be highly stable (more than 90%) in the presence of Advanced Ariel Compact, Henko (Stain Champion), Gain Super Soaker, etc. and 1 mM $CaCl_2{\cdot}2H_2O$ [59]. The activity of *Bacillus* sp. L2 lipase was maximal at 70 °C and pH 8.0 and increased to 500% compared to the control in the presence of calcium ions, but it was completely inactivated by pepstatin A (3.2% residual activity) [101]. *B. coagulans* BTS-1 lipase was pretreated with trypsin and α-chymotrypsin at 37 °C for 20 min and its hydrolytic activity was increased by approximately 13–43% in comparison to the untreated bacterial lipase [124]. The lipase from *B. sphaericus* 205y was inactivated by proteolytic digestion by pepsin A (24% residual activity) [108]. The lipase from *B. subtilis* retained 75% of its activity after treatment with a protease [61].

5.7. Selectivity

Lipases are distinguished from esterases by their ability to hydrolyze long-chain fatty acid esters in addition to short-chain TAGs. *Bacillus* lipases are a versatile group with respect to selectivity (Table 1). The apparent acyl chain length selectivity varies significantly with the nature of the leaving group and may differ in the test of hydrolysis of TAGs and *p*NP esters. For example, *B. megaterium* CCOP 2637 lipase hydrolyzes preferably long-chain *p*NP esters and short-chain TAGs (tributyrin was best) [45]. The activity of *B.* sp. lipase was higher in the hydrolysis of long-chain methyl esters (C18:1) and medium-chain TAGs (C8, C10) [74]. The selectivity of BTL2 was shifted from medium-chain to long-chain TAGs by a change in the pH [117]. The preference for short chain esters can be exploited in the production of flavors for food and cosmetics or possibly for flavor developing in food products (cheese, bread, etc.) Broad selectivity toward the chain length of the fatty acid is an advantage for lipases in waste water treatment and in laundry formulations.

Several *Bacillus* lipases were tested in the hydrolysis of natural oils and show very good activity. This illustrates their broad selectivity, which makes them useful for modification of natural oils (improving the texture and nutritional value) and production of a wide range of saturated and (poly-)unsaturated fatty acids. The lipase of *B.* sp. A30-1 hydrolyzed with the highest rate corn oil, olive oil, cottonseed oil, coconut oil and soybean oil (102–110% relative to olive oil) and displayed significant activity toward beef tallow (75%) [21]. The lipases from *Geobacillus* sp. T1 and *B.* sp. L2. showed preference for corn, palm, soybean, and olive oil, which are all rich in unsaturated fatty acids [94,101]. The best substrate for lipase T1 was sunflower oil. Hydrolytic activity toward coconut oil was low, as it contains more than 80% of total saturated fatty acids. The lipases from *G. stearothermophilus* SB-1 and *B. atrophaeus* SB-2 displayed 4-fold and 2-fold higher activity, respectively, in the hydrolysis of neem oil as compared to olive oil [69]. They efficiently hydrolyzed jasmine and rose oils and could be applied in interesterification reactions in the manufacture of personal care products. *G. stearothermophilus* SB-1 is suitable for fat liquefaction as it hydrolyzes saturated long-chain fatty acids. Short chain fatty acid hydrolysis by *B. atrophaeus* SB-1 and *B. licheniformis* SB-3 lipases could be exploited in developing flavors for food preparations and cosmetic products [69].

Most of *Bacillus* lipases do not show specificity toward the position of the acyl chain in the triglyceride molecule. The lipases from *G. thermoleovorans* ID-1 (lipase A) [73], *B. subtilis* 168 [48], *G. thermocatenulatus* [90,125], *Bacillus* sp. THL027 [77] and *Bacillus* sp. [74] are *sn*-1,3-specific enzymes. Enzymes with positional specificity can be applied in the synthesis of structured triglycerides used in clinical nutrition.

Bacillus lipases hydrolyze/synthesize esters of a broad range of acids and nucleophiles. Their tolerance toward the structure of the substrate has been utilized mainly in the resolution of racemic mixtures of pharmacologically important substances.

5.7.1. Enantioselectivity

Enantiopure compounds for the pharmaceutical industry are currently obtained by chiral chemical synthesis using transition metal catalysts or by enzymatic resolution of racemic mixtures. Hydrolases are a very important group of enzymes applied for this purpose. Only few lipases produced by *Bacillus* cells were studied for enantioselectivity in very few model reactions (Fig. 1) and several enzymes demonstrated excellent enantioselectivity. The lipase from *B. cereus* C71 displayed high preference for the (*R*)-enantiomer of 2-arylpropanoate esters (Fig. 1) [55]. The ethyl esters of ibuprofen, ketoprofen and flurbiprofen were hydrolyzed by *B. cereus* C71 lipase with higher rate and excellent enantioselectivity, compared to several commercial lipases. The reaction of flurbiprofen resulted in 47% conversion for 22 h and 97% enantiomeric excess for the (*R*)-acid, corresponding to an *E*-value of 243.6. (*R*)-flurbiprofen was also produced in the presence of whole cells of *Bacillus cereus* C71 with the advantage of reusability of the catalyst – glutaraldehyde-cross-linked cells retained 60% of initial activity after 6 hydrolysis cycles [75]. Sunna et al. tested the enantioselectivity of *Geobacillus* sp. Tp10A.1 lipase in hydrolysis of two substrates containing a chiral centre either in the acyl or nucleophile moiety [84]. Hydrolysis of (*R*,*S*)-1-phenylethyl acetate at 20 °C reached conversion of 49.5% for 24 h with 98.2% ee_p for (*R*)-phenylethanol ($E = 427$). The enzyme also exhibited high enantioselectivity in the second reaction with methyl (*R*,*S*)-2-methyldecanoate, but the rate was much lower – 69 h for 49.4% conversion and 97.8% ee_p for (*R*)-methyldecanoic acid ($E = 347$). Higher temperature (50 °C) decreased conversion rates in both reactions, probably due to enzyme denaturation, and compromised enantioselectivity. It is noteworthy, that the selectivity demonstrated in the second reaction is opposite to nearly all other lipases tested. Lipase A from *B. subtilis* catalyzed the hydrolysis of (*rac*)-*trans*-1,2-diacetoxy-cyclohexane, yielding exclusively (*R*,*R*)-*trans*-cyclohexane-1,2-diol (Fig. 1) [106]. Temperature, higher than 30 °C, and pH, higher than 7.0, of the reaction did not improve enzymatic activity essentially, but lowered enantioselectivity of the process, due to non-selective chemical hydrolysis of the substrates. *B. subtilis* lipase B displayed the same enantioselectivity, but slightly lower activity in this reaction. *G. thermocatenulatus* lipase (BTL2) demonstrated good enantioselectivity in the hydrolysis of 1-phenylethyl acetate and the acylation of 1-phenylethanol and 1-phenylpropanol with vinyl acetate. In both cases enantioselectivity of BTL2 was excellent, $E > 100$. The reactions were slow as the temperature during the test was below the optimum of the enzyme. Changing the organic co-solvent increased hydrolysis rates [126]. *G. thermoleovorans* lipase was selective for the (*R*)-enantiomer in the hydrolysis of 1-phenylethyl acetate. At 20 °C the reaction was slow, but increasing the temperature compromised enantioselectivity ($E > 200$ with $ee_p = 99\%$) [127]. The enzyme hydrolyzed prefer-

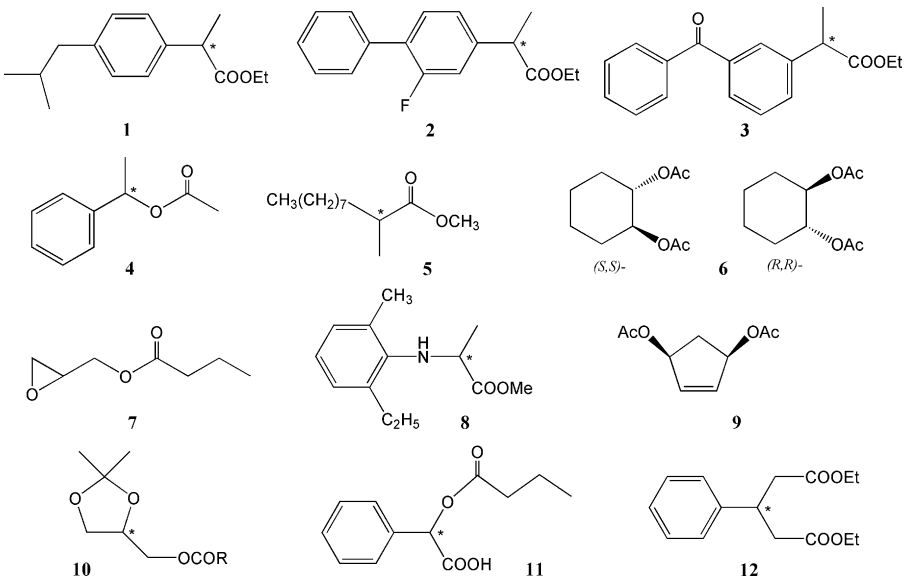

Fig. 1. Substrates for racemic resolution catalyzed by *Bacillus* lipases. **1**, ibuprofen; **2**, ketoprofen; **3**, flurbiprofen; **4**, 1-phenylethyl acetate; **5**, methyl 2-methyldecanoate; **6**, *trans*-1,2-diacetoxycyclohexane; **7**, glycidyl butyrate; **8**, *N*-(2-ethyl-6-methylphenyl)alanine methyl ester; **9**, diacetoxycyclopentene; **10**, 1,2-*O*-isopropyliden-*sn*-glycerol esters; **11**, 2-*O*-butyryl-2-phenylacetic acid; **12**, diethyl phenylglutarate.

ably the (*R*)-enantiomer of methyl 2-methyldecanoate ($E > 200$, $ee_p > 99\%$ after 20 h), which distinguishes it from other reported lipases [127].

Enantioselectivity, as mentioned, is determined primarily by the structure of the biocatalyst. Due to the process of interfacial activation that is specific to lipases, the experimental conditions (solvents, detergents, pH, temperature) can substantially alter the results of the reaction by modifying either the preferred enzyme conformation or the solubility of the reactants. Detergents were shown to increase substrate solubility without compromising the *E*-values [84]. Enantioselectivity of the lipase from *B. subtilis* in the hydrolysis of racemic glycidyl butyrate was improved by the addition of 1,4-dioxane. The increasing volume fractions of 1,4-dioxane (0–30%) decreased the rate of hydrolysis of the (*R*)-enantiomer, but increased lipase activity in the hydrolysis of the (*S*)-enantiomer. As a result, (*R*)-glycidyl butyrate remained unhydrolyzed with $ee_s > 98\%$ at 52% conversion in the presence of 18% (v/v) 1,4-dioxane. Enantioselectivity markedly increased when the reaction mixture was cooled to 5 °C [128]. The lipase from *B. subtilis* liberated preferably the (*S*)-acid in the hydrolysis of *N*-(2-ethyl-6-methylphenyl)alanine methyl ester (Fig. 1). The enantioselectivity of the reaction was improved significantly from $E = 2.7$ to $E = 60.7$ (though not of practical importance) in the presence of 10% (v/v) of diisopropyl ether, compared to aqueous buffer [129].

The feasibility of the genetic approach to modification of enantioselectivity was demonstrated with lipase A from *B. subtilis* [130–133]. The enzyme is a good model for that purpose, since it is one of the few members of *Bacillus* lipases that have been extensively characterized with regard to structure, biochemical properties, and selectivity. BSLA was subjected to saturation mutagenesis aiming to improve its enantioselectivity in the hydrolysis of 1,4-diacetoxy-cyclopentene. A complete library was constructed in which every single amino acid position in BSLA was exchanged for all possible amino acids, so the library contained all theoretically possible variants of BSLA with a single-point mutation. As a result, a mutant was created with inverted and enhanced enantioselectivity from $ee = 38\%$ for the (1*S*,4*R*)-isomer to $ee = 56\%$ for the (1*R*,4*S*)-enantiomer [130]. In another study the amino-acid residues flanking the active site of BSLA were mutated by localized saturation PCR mutagenesis [131,134]. Among all variant proteins the N18I mutant displayed higher preference for the esters of (+)-IPG (1,2-O-isopropylidene-sn-glycerol), opposite to the wild-type lipase [131,134].

5.8. Immobilization

Immobilization is a powerful tool for fine modification of the catalytic properties of the enzymes for industrial purposes. Immobilized lipases have the advantages of enhanced thermal and chemical stability, ease of handling, easy recovery and repeated use as compared with non-immobilized forms. Immobilization can also enhance activity and even reverse selectivity. Several different approaches were applied to the immobilization of *Bacillus* lipases on various supports, providing more evidence of the enormous unexplored potential of this group of biocatalysts.

5.8.1. Covalently bound enzymes

Immobilization of lipases by covalent binding can greatly enhance their thermostability and activity, because the carrier provides an external backbone, which minimizes the negative effect of heat and the solvent on the catalytically active 3D-structure of lipases [135]. And higher reaction temperature means better diffusion of substrates into the active site of the enzyme and of the products away from the enzyme, resulting in higher reaction rate. *B. coagulans* BTS-3 lipase immobilized on glutaraldehyde-activated polyethylene retained 60% activity after 2 h at 70 °C, while the free lipase lost almost half of its activity after 2 h at 55 °C [136]. *Bacillus* sp. lipase immobilized on HP-20 beads by cross-linking with glutaraldehyde had the same pH optimum as the native enzyme, but higher temperature optimum. The half-life of the immobilized lipase at 70 °C was 4 h compared to 85–90 min of the free enzyme [86]. Covalent attachment often involves a multi-step procedure and use of chemicals, that can inactivate part of the protein. The loading of the carrier is lower, compared to immobilization by adsorption, but this is compensated by the multiple use of the enzyme [137].

Covalently bound enzymes are indispensable in hydrolytic reactions, since the enzyme cannot desorb (leach) from the support into the aqueous medium. *B. coagulans* BTS-3 lipase immobilized on glutaraldehyde-activated polyethylene retained more than 50% activity after 10 cycles of hydrolysis of olive oil, while the enzyme on the non-activated carrier was half-inactivated after 8 cycles

[136]. *B. coagulans* BTS-3 lipase immobilized on glutaraldehyde-activated Nylon-6 retained 85% of its initial activity after the 8th cycle of hydrolysis of *p*-nitrophenyl palmitate (pH 8.5, 55 °C, 10 min) [121]. Covalently immobilized preparations of the lipase from *B. coagulans* BTS-3 were applied in the synthesis of several esters of short-chain acids and small alcohols. They demonstrated good performance for about 4 repeated cycles [136,138,139]. *Bacillus* sp. lipase immobilized on silica and HP-20 beads, catalyzed with good activity over 25 cycles in the esterification of oleic acid and methanol [86].

Depending on the orientation of the enzyme molecule on the support and the degree of flexibility and the microenvironment, immobilized lipases can differ significantly in selectivity from the native enzymes. The catalytic properties of *G. thermocatenulatus* lipase were significantly altered by the immobilization protocol [140,141]. While all BTL2 preparations had similar specific activity against *p*-nitrophenyl butyrate, one preparation showed preference for the (*R*)-enantiomer in the resolution of (*R*/*S*)-2-*O*-butyryl-2-phenylacetic acid, and the rest were selective for the (*S*)-isomer. Furthermore, slight changes in the reaction conditions (pH, temperature, co-solvent) affected the performance of the immobilized enzymes differently. For instance, changing the pH of the hydrolysis reaction from 7.0 to 5.0 increased the activity of some preparations and decreased the activity of others and reversed the enantioselectivity of G(9)-BTL2-A. As a result, at pH 5.0 all preparations preferred to hydrolyze the (*S*)-isomer, with two of them showing enantiospecificities higher than 100 [142].

5.8.2. Gel entrapment

Gel entrapment combines lesser structural strain for the enzyme as it is captured in its native form and a specific microenvironment, that can enhance the stability and activity of the lipase. In comparison, in covalent attachment and cross-linking the enzyme molecule can be bound to the carrier or another protein in a way, that hinders the reaction by distorting the active conformation of the enzyme or blocking the access of substrate to the active site. *Bacillus* lipases have been successfully immobilized by gel entrapment in copolymers of methacrylic acid, dodecyl methacrylate and a cross-linker – *N*,*N*-methylene bisacrylamide [143–149], and alginate [150,151]. The immobilized enzymes did not differ substantially in thermostability from the native lipases. The preparations demonstrated good synthetic activity in alkanes (*n*-nonane, *n*-heptane, *n*-octane), and moderate reusability. In some cases metal ions and surfactants increased the activity of the enzyme [143,145,150].

5.8.3. Hydrophobic and ionic adsorption

Physical adsorption is a simple and inexpensive method for immobilization of lipases in particular, as they tend to adsorb on hydrophobic surfaces. It requires minimum preparation steps, milder conditions for the enzyme and compared with covalent binding, gives higher activity yield, i.e., more active molecules per unit surface area [137]. Desorption of the enzyme from the carrier in aqueous medium is a major disadvantage of the method, limiting the repeated use of the biocatalyst. There are, however, remarkable exceptions. *G. thermocatenulatus* lipase immobilized on octadecyl-Sepabeads, displayed high thermostability, comparable to the covalently attached enzyme, and 20% higher activity in the hydrolysis of *p*NP palmitate [102]. *Bacillus* sp. GK8 lipase immobilized via hydrophobic adsorption on phenyl-Sepharose showed excellent reusability in the hydrolysis of *p*NP laurate [135]. Lipase from *G. thermoleovorans* CCR11 was adsorbed on porous polypropylene (Accurel EP-100) in the presence and in the absence of Triton X-100 [152]. The immobilized preparations displayed enhanced thermostability and a 10° increase in the optimum temperature compared with the native lipase, although the lipase immobilized with Triton X-100 was more sensitive to higher temperature than the one without the surfactant. The lipase immobilized with Triton X-100 retained 53% residual activity after 7 cycles, and was totally inactivated after 9 cycles of hydrolysis of *p*NP laurate. The lipase immobilized without Triton X-100 retained 69% after 7 cycles and was inactivated after 12 hydrolysis cycles. The enzyme did not desorb from the support after 12 h in phosphate buffer at room temperature and 250 rpm [152].

Leaching of the enzyme is overcome in organic media, since the solubility of the enzyme in it is limited [70,86,136]. Lipase from *B. coagulans* BTS-3 immobilized on silica achieved 98% conversion in the esterification of ethanol and propionic acid for 12 h at 55 °C in *n*-hexane [153]. Lipases immobilized by adsorption are particularly interesting for solvent-free synthesis at high temperatures. Higher temperatures help maintain lower water activity in the system by rapid evaporation, thus increasing the rate of esterification. *G. stearothermophilus* SB-1 lipase adsorbed on Accurel EP100 catalyzed the synthesis of ascorbyl palmitate without addition of solvent. Initial reaction rates increased directly with temperature and maximal (95%) conversion was achieved after 30 min at 90–100 °C, or after 1 h at 80 °C [154]. Solvent-free synthesis is of particular advantage in food industry where the use of organic solvents is restricted. In this regard thermostable lipases have an immense importance. Physical adsorption significantly improved the thermostability of *Bacillus* lipases. *Bacillus* sp. J33 lipase adsorbed on phenyl-Sepharose retained full activity after 12 h at 60 °C, whereas the native enzyme retained 53% activity after 5 h at 60 °C [116]. The lipase from *B. coagulans* BTS-3 immobilized on silica retained almost 90% activity after 2 h at 65 °C and 70 °C [153]. The lipase from *G. stearothermophilus* MC7 was immobilized by ionic adsorption on DEAE-cellulose. The preparation showed improved thermostability and catalyzed the solvent-free acidolysis of tripalmitin with oleic acid with 60% conversion after 48 h [155,156].

Immobilization via hydrophobic adsorption mostly affects residues close to the active centre. By modulating the nature of the support, the strength of the hydrophobic interactions between the lipase and the carrier can be altered. Thus, the final structure of the enzyme active centre and its catalytic properties can be modified [141,157]. The lipase from *G. thermocatenulatus* was immobilized by interfacial activation on four different hydrophobic supports (hexyl- and butyl-Toyopearl and butyl- and octyl-Agarose) and their properties were compared [157]. The enantioselectivity of the preparations in the hydrolysis of (*R*,*S*)-2-*O*-butyryl-2-phenylacetic acid differed dramatically. For example, BTL2 immobilized on octyl-Agarose was selective for (*S*)-2-*O*-butyryl-2-phenylacetic acid ($E > 100$), whereas when immobilized on hexyl-Toyopearl, the enantiomeric value was only $E = 8$. BTL2 immobilized via interfacial adsorption on octadecyl-Sepabeads catalyzed the hydrolysis of the (*R*)-enantiomer with $ee_p > 99\%$ and $E > 100$ at pH 7 and 4 °C [6]. In another reaction – the asymmetric hydrolysis of phenylglutaric acid diethyl diester, BTL2 immobilized on hexyl-Toyopearl was the most enantioselective catalyst with $ee > 99\%$ in the production of (*S*)-monoester product.

5.8.4. Genetic modification

An interesting approach to the selective immobilization of *G. stearothermophilus* L1 lipase was to fuse a gene fragment corresponding to the cellulose-binding domain (CBD) of *T. hazianum* cellulase into the gene of the lipase [158,159]. The fusion protein was expressed in *S. cerevisiae*. The enzyme was then effectively immobilized by adsorption on Avicel (microcrystalline cellulose), due to preferential binding via its cellulose-binding domain. The specific activity of the fusion lipase increased when it was immobilized on Avicel and was twice as high as the specific activity of the free wild-type enzyme. The preparation did not show any significant loss of activity after 12-h incubation in oil-free 10 mM

phosphate buffer (pH 10) [158] and retained more than 70% of its initial activity after 5 uses in hydrolysis of olive oil [159].

6. Conclusion

Bacillus lipases are a large and diverse family of enzymes, that can be easily produced and demonstrate versatile specificity and stability, great tolerance to solvents, salts and detergents. Therefore, *Bacillus* lipases can potentially be applied in food industry, laundry formulations, paper and leather industry, waste water treatment, etc. However, up to date very few of more than 70 enzymes have been tested in resolution of racemic mixtures in 3–4 test reactions for application in the pharmaceutical industry. Their activity in synthetic transformations (esterification, transesterification, acidolysis) is widely unexplored. Several examples demonstrate the possibilities of the genetic approach for additional modification of the properties of the biocatalyst for a particular application. Few lipases have been immobilized and the preparations displayed enhanced thermostability and activity and even improved and reversed enantioselectivity. These immobilized preparations can be used in a wide range of applications from modification of oils to synthesis of flavors, fine chemicals and biodiesel production.

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