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REVIEW ARTICLE

Cold Active Lipases: Biocatalytic Tools for Greener Technology

Nutan Mhetras[1] · Vidhyashri Mapare[2] · Digambar Gokhale[2]

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Abstract

Lipases are enzymes that catalyze the ester bond hydrolysis in triglycerides with the release of fatty acids, mono- and diglycerides, and glycerol. The microbial lipases account for $400 million market size in 2017 and it is expected to reach $590 million by 2023. Many biotechnological processes are expedited at high temperatures and hence much research is dealt with thermostable enzymes. Cold active lipases are now gaining importance in the detergent, synthesis of chiral intermediates and frail/fragile compounds, and food and pharmaceutical industries. In addition, they consume less energy since they are active at low temperatures. These cold active lipases have not been commercially exploited so far compared to mesophilic and thermophilc lipases. Cold active lipases are distributed in microbes found at low temperatures. Only a few microbes were studied for the production of these enzymes. These cold-adapted enzymes show increased flexibility of their structures in response to freezing effect of the cold habitats. This review presents an update on cold-active lipases from microbial sources along with some structural features justifying high enzyme activity at low temperature. In addition, recent achievements on their use in various industries will also be discussed.

Keywords Cold active lipases · Psychrophilic microbes · Synthesis of chiral molecules · Frail compounds

Introduction

Humans have been continuously searching novel materials from the natural environment and using them for their survival. Organic chemists are probably more familiar with the organo-metallic catalysis because they have no experience with the biocatalysis. Though much

✉ Digambar Gokhale
dv.gokhale@ncl.res.in; digambar_52@yahoo.com

[1] Symbiosis School of Biological Sciences, Symbiosis International (Deemed University) Lavale, Pune, India

[2] NCIM Resource Center, CSIR-National Chemical Laboratory, Pune 411008, India

information is available on chemical catalysis, both chemical catalysts and biocatalysts are required to develop greener technologies [1]. Myriad of products were produced by chemical and allied industries for well-being of our society. Copious amounts of waste generated by chemical processes was not an important issue until 1980s. However, negative effects of many chemicals on human health and natural environment presented a problem. Therefore, it was necessary to look for alternative resource-efficient and cleaner technologies which minimize or eliminate waste. As a result, emphasis gradually shifted to waste prevention as opposed to waste remediation [1]. The US Pollution Prevention act of 1990 focused much attention on waste prevention that eliminated the cost of waste treatment and also reduced the environmental pollution. Waste prevention not only led to efficient use of raw materials but also strengthened the economic competitiveness. This fundamental shift from waste generation to waste prevention led to the emergence of the term "Green Chemistry" at the US Environmental Protection Agency in 1990. The Green Chemistry utilizes renewable resources efficiently, minimizes or eliminates waste generation in the manufacture and application of chemical products. The principle of Green Chemistry is to design the environmentally benign processes for production of chemical products [2]. Biological tools are becoming increasingly important which may replace harsh chemical tools of processing materials that generate copious amounts of waste [3]. Biocatalysis has been integrated in to the mainstream of organic synthesis making the industrial processes greener and sustainable. Modern approaches in molecular biology, system biology, and directed evolution enabled the previously unimaginative insights in to processes with promising industrial applications. The developments in these approaches helped to expand the boundary of biocatalysis in organic chemistry.

Majority of the Earth's crust and atmosphere (85%) experiences perennially cold (< 5°C) environments that harbor psychrophilic microorganisms that include archaea bacteria, yeast, fungi and algae [4]. The ability of these microbes to thrive at such cold environment requires several adaptation strategies that enable them to grow and perform metabolic activity at low temperature. These adaptation strategies developed by psychrophiles along with their underlying mechanisms have been very well reviewed [5, 6]. Adaptation to cold habitats leads to change in membrane fluidity, expression of cold-shock proteins involved in transcription and translational and posttranslational processes. The cell membrane of cold-adapted microbes reveals the presence of unsaturated fatty acids with a greater number of double bonds. The role of these unsaturated fatty acids in cold adaptation is very well understood. The long-chain polyunsaturated fatty acids are presumed to be involved in maintaining membrane fluidity during cold adaptation [6]. Psychrophiles are subjected to freeze-thaw cycles in frozen environments and face harmful crystallization stress leading to cell damage. In response to these freezing-related detrimental challenges, psychrophiles produce novel compounds such as ice-binding proteins, biosurfactants, and extracellular polysaccharides (EPS) [7, 8]. Psychrophilic organisms produce ice-binding proteins (Antifreeze proteins) that bind to ice and prevent ice growth and recrystallization. These AFPs are believed to stabilize the cell membranes and protect the cells from damage [8]. Interestingly, cold-adapted organisms produce biosurfactant [9, 10] but their potential role in cold adaptation is not understood. Hence, further studies are required to elucidate their precise role and mechanism in cold adaptation. Further studies will also clarify whether they constitute part of the cells toolkit to help in cold adaptation. Similarly, pigment production (especially carotenoids) is common in psychrophiles isolated from ice cores and glaciers [11] and high-altitude soils [12]. It is presumed that carotenoids might have some role in modulation of membrane fluidity and especially polar carotenoids are believed to enhance membrane rigidity leading to stabilization of membrane [13]. Cold-adapted bacteria

are known to produce high concentrations of EPS especially at freezing temperatures and their role in cold adaptation is well documented [14, 15].

The cold active enzymes produced by psychrophiles are now being explored for industrial and biotechnological applications. They have a huge market potential due to their inevitable applications in various industries because of their high catalytic activity and increased affinity towards substrates at low temperatures. In addition, the reduced energy consumption and diverse adaptation of these enzymes make them to perform well in unfavorable conditions in industrial processes [16]. Among the group of cold active enzymes, cold active lipases are gaining importance because of their use in fine chemical synthesis, bioremediation, food processing and mainly as detergent supplement. Very few reports are available on the isolation of cold-active lipases as compared to mesophilic and thermophilic lipases [17, 18]. Particularly, not much attention has been paid to the bacterial lipases from extremely cold environments [19]. This review provides, in particular the recent information of cold-active lipases with their potential industrial applications.

Lipases and Their General Structure

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) have emerged as potential enzymes accounting for approximately 30% of the world's enzyme market. The microbial lipase market was $400 million in 2017 and it is expected to reach $590 million by 2023 (https://www.marketsandmarkets.com/Market-Reports/microbial-lipase-market-248464055.html). Lipases play a major role in metabolism of fats and lipids in plants, animals, humans and microorganisms. In humans and animals, the role of lipases is to control the digestion, absorption and reconstitution of fat and lipoprotein metabolism. Plant lipases are involved in metabolism of oil reserves during seed germination. They provide energy required for the synthesis of carbon source (glucose) and nitrogen source (amino acids) necessary for embryonic growth [20]. Lipases catalyze the hydrolysis of esters especially water-insoluble long chain fatty acid esters such as triglycerides.

The three-dimensional structure of lipase of M*ucor miehei* [21] was first revealed using X-ray crystallography followed by the three-dimensional structure of human pancreatic lipase [22]. Very recently, *Rhizomucor meihei*, was known to possess two structures of lipase, mature lipase and its prolipase. In the prolipase structure, the mature domain is in the closed inactive form which is stabilized by a region of the propeptide sitting on the top of the active site which prevents its opening. They also demonstrated that the propeptide inhibits the lipase activity in standard lipase assay [23]. The active site of lipases was shown to possess classical catalytic triad composed of Ser-His-Asp with involvement of nucleophilic Serine for catalytic activity. These enzymes exhibited same structural architecture, α/β hydrolase fold, and were known to be the members of this folding family [24, 25]. The α/β hydrolase fold is made up of central β-sheet of eight parallel β-strands with β2 strand antiparallel with respect to other β-strands. The central β-sheet is connected by α-helices packed on both the sides of β-sheet. The active site located in the β sheet is made up of highly conserved catalytic triad. The catalytic triad consists of serine or cysteine (nucleophilic residue), aspartic or glutamic acid (catalytic acid residue) and histidine residue. In lipases, serine always acts as nucleophile [26]. The serine residue is located in a highly conserved pentapeptide Gly-X-Ser-X-Gly. Glycine in the pentapeptide may sometimes be substituted by alanine, threonine, serine, or valine. The pentapeptide forms a sharp γ-like turn (nucleophilic elbow) between β5 strand and α helix

strand. Aspartic acid situated in the reverse loop after the β7 strand interacts with highly conserved catalytic histidine via hydrogen bond. The catalytic histidine is situated in a loop positioned after β8 strand [27]. Most of the lipases operate at lipid-water interphase enabled by the mobile amphiphilic region called "Lid" that covers the active site and regulates the lipase activity [28, 29]. The lids have been classified in to larger lids and smaller lids based on structures of lid domains. Larger lid domains were observed in thermophilic lipases with two or more helices and smaller lid domains were found in mesophilic lipases in the form of a loop or a helix [29]. Various efforts such as modifications of lid domain using site-directed mutagenesis [30], lid swapping [31], and computational approaches [32, 33] have been employed to modify the activity and thermostability of lipases. Further advancement in the bioinformatics tools will help to predict the accurate function of amino acids present near the lid region of lipases. Protein engineering of lid may provide an opportunity for better understanding of the structural basis of the lipase property. There is a possibility of using these protein engineered thermostable lipases as industrial enzymes at high temperatures.

Cold active lipases (CLPs) are structurally similar to other mesophilic/thermophilic lipases. They also possess α/β hydrolase fold and the catalytic triad at their active site with serine as nucleophilic residue, histidine as basic residue, and aspartic/glutamic acid as acidic residue. CLPs showed high activities at low temperatures and also molecular activities are much increased at 0–10° C. CLPs are structurally modified and show increased molecular flexibility that helps to accommodate the substrates. The studies on psychrophilic *Pseudomonas immobilis* lipase suggest that the high catalytic and molecular activities at lower temperature and its low thermostability were due molecular flexibility of an active site of the enzyme [34]. In addition, the 3D-model of psychrophilic *P. immobilis* lipase revealed several other features which include low proportion of arginine compared to lysine, low content of proline residues, small hydrophobic region and aromatic-aromatic interactions. All these features are typical of cold-adapted enzymes and are responsible for more flexible structure, low activation energy and low thermal stability. The efforts were made to understand molecular basis of cold adaptation by Wintrode et al. [35] in which mesophilic subtilisin-like protease was converted to its psychrophilic counterpart using directed evolution. The identified three variants exhibited improved protease activity compared to wild type at low temperature. These studies particularly suggest that mesophilic to psychrophilic conversion of enzyme was achieved by single point mutation. Such single amino acid substitutions were also reported that confer psychrophilic characteristic to mesophilic enzymes [36].

Interfacial Activation of Lipases

Holwerda et al. [37] observed the phenomenon of interfacial activation for the first time followed by its confirmation by Schonheyder and Volowartz [38]. The natural substrates of lipases are the glycerides that have low solubility in water leading to formation of insoluble droplets. Lipases act on these insoluble droplets using a peculiar mechanism of action, known as interfacial activation which allows the lipases to adsorb on the hydrophobic surface of the glyceride droplets and act at the interface [39, 40]. This interfacial activation is based on the formation of a large hydrophobic pocket surrounding the active center of the enzyme. The lipase with this large hydrophobic pocket has low solubility in water which makes the enzyme unstable. The active site is isolated from aqueous medium by a polypeptide chain called lid which covers the hydrophobic pocket and this is referred as the closed form of the lipase which

is inactive. The internal hydrophobic region of the lid interacts with the hydrophobic regions of the active site while external hydrophilic region interacts with the reaction medium [41, 42]. The huge hydrophobic regions formed by shifting of the lid causes exposure of the active site to the medium which results in open/active form of the lipase. In the active form of lipase, the hydrophilic phase interacts with the protein surface. Both the active and inactive forms of the lipase remain in equilibrium. However, when lipase comes in contact with oil droplets, the open form of the lipase is adsorbed on hydrophobic surface of the droplets shifting the equilibrium towards open form which attacks the glycerides.

Modifications of Lipase Structure for Cold Adaptation

Major studies have been focused on psychrophilic microbes at molecular level particularly on cold active enzymes [43, 44]. These cold-adapted enzymes are characterized by increased turnover and high catalytic efficiency at low temperatures due to their flexile structures, reduced activation enthalpy and negative entropy of activation compared to mesophilic enzymes. However, cold active lipases are heat unstable and rapidly inactivated even at moderate temperatures. The thermal denaturation leads to enzyme inactivation in industrial applications and therefore industrial enzymes need to be thermostable [45]. Many strategies have been proposed to improve the thermostability of the cold active enzymes including lipases which include the use of soluble additives, immobilization, protein engineering, and chemical modification [46, 47]. Recent developments focused on the construction of smaller libraries by combining the rational design and directed evolution procedures [48]. The rational design requires the knowledge of relationships of structure, sequence, function, and catalytic mechanisms of protein. This knowledge is necessary for prediction of amino acid residues needed for mutation. These mutations are introduced at specific site of gene encoding protein by site directed mutagenesis approaches. Directed evolution involves the generation of random mutant library followed by screening of required mutant using a suitable screening system. Smaller libraries are produced by combining rational protein design and directed evolution procedures. These strategies have been used to alter the enzyme structure by changing the one or more amino acids in the protein sequence leading to improvement of enzyme properties. For example, the change in the amino acid sequences of the lid region of lipase led to remarkable improvement in substrate specificity and enantioselectivity [49]. The use of these methods requires information on enzyme structure and target specific regions on them in each revolution cycle. The approaches such as saturation mutagenesis and iterative saturation mutagenesis were introduced as efficient methods for directed evolution of functional enzymes. The goals and various strategies for lipase improvements have been well discussed by Bassegoda et al. [50]. Sharma et al. [51] used error-prone-PCR method for increasing the thermostability of lipase from metagenomic library and the mutant enzyme showed 144-fold enhanced thermostability at 60° C. This *ep*-PCR approach was also employed to enhance the thermostability of cold active lipase from *Pseudomonas fragi* leading to 5-fold increase in its half-life at 42° C [52]. The directed evolution proved to be efficient technique for the engineering *Staphylococcus epidermidis* AT2 cold-adapted lipase in a single round of error-prone PCR. This random mutation replaced non-polar glycine residue with polar cysteine residue in the lid region of the lipase which most probably provided rigidity to the structure thereby increasing the thermostability of lipase [53, 54]. The lid 1 structure of lipase of *Pseudomonas fluorescens* AM8 is rigid and was stabilized by 17 hydrogen bond linkages leading to low hydrophobicity. The

change in Thr-52 and Gly-55 present in the lid 1 region to aromatic tyrosine resulted in achieving higher solvent accessible surface area and longer half-life at 25 to 37°C in 0.5% toluene [55]. Efforts were made to improve thermostability of cold active lipase of *Penicillium cyclopium* by addition of disulfide bridge using software Disulfide (Design version 1.2) and the recombinant lipases were found to show enhanced thermostability and catalytic efficiency [56]. Similar studies were performed to demonstrate that the rationally designed point mutations and introduction of disulfide bonds could effectively reduce the number of screened colonies to enhance the thermostability of *Rhizomucor miehei* lipase [57]. Detection of lipase positive clones/mutants from a library of thousands of transformants requires high-throughput screening. Zhang et al. [58] developed an easy screening method for detection of thermostable lipase activity based on its synthetic activity. They obtained a double mutant (Asn120Lys/Lys131Phe) from *Rhizomucor miehei* lipase saturation mutated library that was based on amino acid residue B factors. The screening method involved the use of pH-indicator in the screening medium on a colony plate which facilitated the high-throughput screening for lipase synthetic activity. The lipase thermostability was further evaluated by heating the colony plate before the addition of synthetic substrate and visualizing the halo zone around the colony which is indicative of lipase synthetic activity. Such high-throughput screening approaches need to be developed for detecting the desired strains from the libraries containing thousands of transformants. Lipases stable in organic solvents are versatile biocatalysts to be employed in their applications in food, pharmaceuticals, and green products manufacturing industries [59]. The inherent structural flexibility of cold active enzymes causes their denaturation by heat and organic solvents and very few reports are available on cold-adapted solvent tolerant lipases [60]. Cold-adapted lipases, PML and LipS showed increased conformation flexibility with increasing concentrations of organic solvent [61]. Majority of the studies employed directed evolution to improve the tolerance of enzymes in organic solvents [62]. *Pseudomonas aeruginosa* lipase, LST-03, has high stability in various organic solvents. The use of directed evolution approach was found to enhance organic solvent stability of LST-03 lipase. The mutation in the Lip9 on the surface of lipase through directed evolution increased stability (9-11-fold) in cyclohexane and n-decane [63].

Sources of Cold Active Lipases

Cold active lipases of microbial origin are being paid due attention for biotechnological applications because of their increasing use in synthesis of chiral intermediates, fine chemicals, biodiesel, and biopolymers [64, 65]. In addition, the high activity at low temperatures favors the production of frail/fragile compounds. Many cold active lipase producing microbes have been isolated but very few bacteria and yeast received attention for commercial exploitation. Most of the psychrophilic bacteria have been isolated from Antarctic and polar regions exhibiting colder habitats (<5° C). A list of various cold active lipase producing bacteria is presented in Table 1. Recently, *B. cereus* isolated from marine habitat was reported to produce highest lipase activity (285 U/mL) at 10° C [67]. Kumar et al [86, 87] reported the existence of psychrophilic bacteria harboring cold active enzymes in Sikkim Himalaya using genome-based predictions. Cold-adapted and broad temperature active alkalophilic lipase was purified from one of the isolates, *Chryseobacterium polytrichastri* ERMR1:04 [88]. Many psychrophilic yeasts have been isolated which produce extracellular or intracellular lipases. However, cold active lipases A and B (CALA and CALB) from *Candida antarctica* have been paid great

Table 1 Bacteria-producing cold active lipases

Organisms	Sources/growth temperature (°C)	References
Acinetobacter sp. XMZ-26	Glaciers in Xinjiang, China /15	[66]
Bacillus cereus	Mediterranean Sea /10	[67]
Pseudomonas sp. MSI057	Marine sponge /30	[68]
Pseudomonas sp. TK3	Dirty and cool tream Water /20	[69]
Pseudomonas sp.	Signy Island, Antarctica /10	[19]
Pseudomonas sp.	Satopanth Glacier, Western Himalaya /20	[70]
Pseudomonas sp. VITCLP4	Tamilnadu coast /20	[71]
Pseudomonas palleroniana	Himalayan environment /25	[72]
Staphylococcus epidermidis	Soil at car service area /NA	[73]
Moritella sp. 2-5-10-1	Antarctic region /5	[74]
Microbacterium luteum	Gangotri glacier, Western Himalaya /15	[75]
Stermotrophomonas maltophila	Chinese General Microbial Culture Collection	[76]
Pseudoalteromonas haloplanktis TAC125	Antarctic seawater /4, 15	[77]
Acinetobacter sp. CR9	Chandra river in sub-alpine region of western Himalaya /5	[78]
Pseudomonas sp. 7323	deep-sea sediment /15	[79]
Psychrobacter sp.	Antarctic habitat /4	[80, 81]
Psychrobacter cryohalolentis K5	Siberian cryopeg /22	[82]
Bacillus sp.	Chandigarh, India /10	[83]
Halomonas sp. BRI 8	Antarctic sea water /15	[84]
Halocynthiibacter arcticus	Arctic marine sediment /21	[85]

attention. CALB was immobilized on a resin Lewatit VP OC1600 via interfacial activation which is commercially available as Novozyme 435. It is most widely used commercial lipase in both academy and industry [89]. Majority of reports on cold active lipases has been focused on bioprospection on bacteria and yeasts from Antarctic or polar region but very few reports are available on fungal cold active lipases. The recent review provides the information on production of cold-adapted enzymes including lipases by fungi from terrestrial and marine Antarctic environments [44]. Table 2 gives the list of various yeast and fungi producing cold active lipases.

Recombinant Expression of Lipases in Heterologous Hosts

Fermentation at low temperatures is advantageous since it prevents and limits the risk of contamination with mesophilic organisms especially in continuous fermentation. However, cold-adapted microbes are not appropriate candidates to be exploited for large-scale fermentations because of requirement of energy consuming cooling systems. The other drawback is the low production level of cold-adapted wild strains. These disadvantages can be overcome by overexpressing genes encoding for cold active enzymes in mesophilic hosts using appropriately designed efficient expression systems which leads to obtaining sufficient yields of enzymes for commercial exploitation. Mesophilic hosts are most commonly used for heterologous expression of genes encoding for cold active enzymes. The problem with use of mesophilic hosts is their optimal growth temperature which is not compatible with the temperature required for proper folding of the cold active enzymes to retain their structure and functional activity [105]. This issue can be circumvented by incubating the host organisms at temperatures lower than their optimal growth temperatures after induction to solve the folding issues for obtaining functional enzymes. However, the growth of mesophilic hosts at

Table 2 Yeast- and fungi-producing cold active lipases

Organisms	Sources/growth temperature (°C)	References
Mariannaea camptospora	koala faeces /25	[90]
Candida parapsilopsis	Not known	[91]
Candida tropicalis	Not known	[91]
Geotrichum sp. SYBC WU-3	Chinese soil / 15	[92]
Candida albicans	GDMCC /15-25	[93]
Candida zeylanoides	Refrigerated poultry meat	[94]
Guehomyces pullulans	Antarctica /25	[95]
Pichia lynferdii NRRL Y-7723	Not known /25	[96]
Cryptococcus sp.Y-32 *Rhodococcus erythropolis* N149	Nella Lake, East Antarctica/15	[97]
Rhodotorulla sp. Y-23	Nella Lake, East Antarctica/15	[98]
Wicherhamomyces psychrolipolyticus	Hokkaido, Japan /4-30	[99]
Penicillium expansum	Antarctic /15	[100]
Penicillium canesens(BPF4)	Not Known /NA	[101]
Penicillium spp.	Indian Himalayan region/25	[102]
Pseudoymnoascus roseus (BPF6)	Not Known	[101]
Truncatella angustata (BPF5)	Not Known	[101]
Rhizomucor endophyticus	Chienes General Microbial Culture Collection	[103]
Geomyces sp.	Antarctic soil	[104]

lower temperatures leads to decreased growth rate and also reduced synthesis of enzymes. Table 3 summarizes the heterologous expression of genes coding for cold active lipases in commonly used mesophilic hosts. Table also provides the information on heterologous mesophilic hosts and vectors used for expression, temperature optimum of recombinant lipase and residual activity at specific lower temperature. Several strategies have been suggested for expression of cold active enzymes including lipases with proper folding in heterologous host to retain structure and functional activity. These strategies include the use of molecular chaperones, cold active promoters, fusion partners, and psychrophilic hosts and these have been well covered in previous reviews [114].

Applications of Cold Active Lipases

Unusual specificities and high activity at low temperature offer great opportunities for cold active lipases to be exploited and applied in versatile industrial fields. Lipases from psychrophilic organisms are promising enzymes that can replace the conventional processes used in biotechnological industries. These enzymes are mainly of interest in organic synthesis of fragile chiral compounds, as additives in food industry (cheese manufacture, bakery), as detergents as well as bioremediations, biomedicines, or molecular biology approaches [115]. The manufacturing of multi-ton scale biotechnological products presently employs fungal lipases from *H. lanuginosa* lipase and *Rhizopus niveus*. These lipases are used especially for the manufacture of detergents and cocobutter substitutes [116]. *C. antarctica* lipase B has been one of the cold active lipases which has seen the commercial success [89, 117]. Number of papers and patents on cold active lipases for their use in various industries are available. However, they have not been exploited much at industrial level due to their high price and low thermostability. Sometimes, low thermostability is desirable property of cold active lipases in biotransformation processes in which biocatalytic reaction can be terminated by heat

Table 3 Cold-adapted lipases that were successfully expressed in heterologous hosts

Organism	Host	Vector	Topt (% residual activity)	References
Psychrobacter sp.	*E.coli* BL21 (DE3)	pColdI + pG-KJE8	35 (30 at 5° C)	[106]
Sorangium cellulosum	*E.coli* BL21 (DE3)	pET22b(+)	30 (35 at 0° C)	[107]
Shewanella frigidimarina NCIMB 400	*E.coli* BL21 (DE3)	pET22b(+)	25 (35 at10° C)	[81]
Psychrobacter sp. *C18*	*E.coli* BL21 (DE3)	pET28a(+)	30 (18 at 0° C)	[108]
Acinetobacter sp. XMZ-26	*E.coli* BL21 (DE3)	pET30a(+)	15 (39 at 0° C)	[66]
Stenotrophomonas maltophilia GS11	*E.coli* BL21 (DE3)	pET30a(+)	35 (55 at 5° C)	[76]
Psychrobacter cryohalolentis K5	*E.coli* BL21 (DE3)	pET32a	25 (60 at 5° C)	[82]
Psychrobacter cryohalolentis K5	*E.coli* BL21 (DE3)	pET32a(+)	25 (80 at 5° C)	[109]
Pseudomonas sp. TK-3	*E.coli* BL21 (DE3)	pET47b	20 (30 at 5° C)	[69]
Candida albicans	*P. pastoris*	pGAPZaA	15 (50 at 5° C)	[93]
Malassezia globose	*P. pastoris X-33*	pGAPZaA	35 (55 at 10° C)	[110]
Bacillus sp.	*E.coli* JM109	pGEM-T	35 (55 at10° C)	[83]
Penicillium expansum	*E. coli Origami B (DE3)*	pMAL-c5E	10 (ND)	[100]
Rhizomucor endophyticus	*P.pastoris* GS115	pPIC9 K	40 (75 at 0° C)	[103]
Candida Parapsilosis	*P.pastoris* GS115	pPIC9K	35 (45 at 5° C)	[91]
Candida tropicalis	*P.pastoris* GS115	pPIC9K	45 (36 at 5° C)	[91]
Staphylococcus epidermidis AT2	*E.coli* (DE3) pLacI	pTrcHis2- TOPO	25 (ND)	[73]
Geomyces sp. *P7*	*S.cerevisiae* (BJ5465)	pYES 2.1	35 (15 at 0° C)	[104]
Yersinia enterocolitica	*E.coli* BL21 (DE3)	pMD-18T	25 (85 at 40° C)	[111]
Pseudomonas sp. LSK 25	*E coli* BL21 (De3)	pET32B (+)	30 (stable at 5-30° C)	[19]
Pseudomonas spp.MG687270	*E coli* BL21(DE3)	pET28YLip	25 (50 at 60° C)	[112]
Aeromicrobium sp. SCSIO 25071	*E. coli* Rosetta (DE3)	pET-28a (+)	30 (35at 0° C)	[113]

inactivation of the enzymes. The following section discusses on the potential applications of cold active lipases in various areas of commercial importance.

Synthesis of Fine Chemicals and Pharmaceuticals Novel synthetic methods are in great demand essentially to produce new classes of organic compounds required for biomedical research. Enzyme-catalyzed transformations for synthesizing new compounds present as alternative and convenient solutions to intractable chemical synthesis [118]. The synthesis of single enantiomer is very important in pharma industry. Though chemical synthesis is one of the approaches to synthesize such enantiomers, biocatalysis route has several advantages over chemical catalysis. Biocatalysis are enzyme-catalyzed reactions that are chemo-selective, stereoselective and region-selective and performed at ambient temperature and pressure [119]. The cold-adapted lipases and their application in fine chemical synthesis have been discussed in many reviews [18, 120].

The 4-(R)-Hydroxycyclopent-2-en-1-(S)-acetate is an important intermediate in the synthesis of cyclopentanoid natural products such as prostaglandins, prostacyclins, thromboxanes and some anti-HIV drugs. *Trichosporon beigelii* NCIM 3326 cells having a pro-R preference were evaluated for the hydrolysis of mesodiacetate; but the enantio-selectivity was poor. Addition of 10% v/v ethanol was found to enhance the enantioselectivity of the enzyme affording 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate with 85% optical purity and 83% yield. Further exploration of inherent consecutive kinetic resolutions to the desymmetrization

afforded 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate of more than 98% optical purity with 74% chemical yield [121, 122]. The chiral product is then converted to 4-(R)-t-butyldimethylsilyloxycyclopent-2-en-1-one which is the precursor for synthesis of cyclopentanoid natural products (Fig. 1). Lavandulol is a constituent of essential oils and also an important additive in perfumery and cosmetic industry. *Y. lipolytica* NCIM 3639 was isolated from refrigerated Tween 80 samples in our laboratory which produced both extracellular and cell bound cold active lipases at 20° C. Cell bound and extracellular cold active lipases preferentially hydrolyzed the racemic lavandulyl acetate at 25° C to corresponding (R)- and (S)- lavandulol respectively confirming that extracellular and cell bound lipases are different (Fig. 2a, b) [123]. Such differences in the enantioselectivity of the extra- and intracellular lipases are not very commonly found in microbes. The (R)-1-Arylallyl alcohols were synthesized with excellent enantioselectivities via kinetic resolution of the corresponding acetates using immobilized *Candida antarctica* lipase B. Very recently, the chitosan immobilized *Candida antarctica* lipase B was used for kinetic resolution of some racemic heteroarylethanols through transesterification with vinyl acetate. The reaction was carried out at 45°C in n-hexane which resulted in 50% conversion in 3-16 h and >96% enantiomeric excess [124]. The immobilized preparation remained active even after 10 recycles proving its potential as promising candidate for getting higher productivities. Ascorbyl fatty acid esters are good antioxidants and surfactants which are prepared by acylation of ascorbic acid (vitamin C) with different acyl donors using lipases. Novozyme 435 and *Pseudomonas stutzeri* lipase immobilized on silica octyl were evaluated for synthesis of ascorbyl palmitate in 2-methyl-2-butanol as solvent. Novozyme 435 was found to give highest yield (81%) of ascorbyl palmitate at 55° C due to its high reactivity and stability in 2-methyl-2-butanol. [125]. The flavor esters

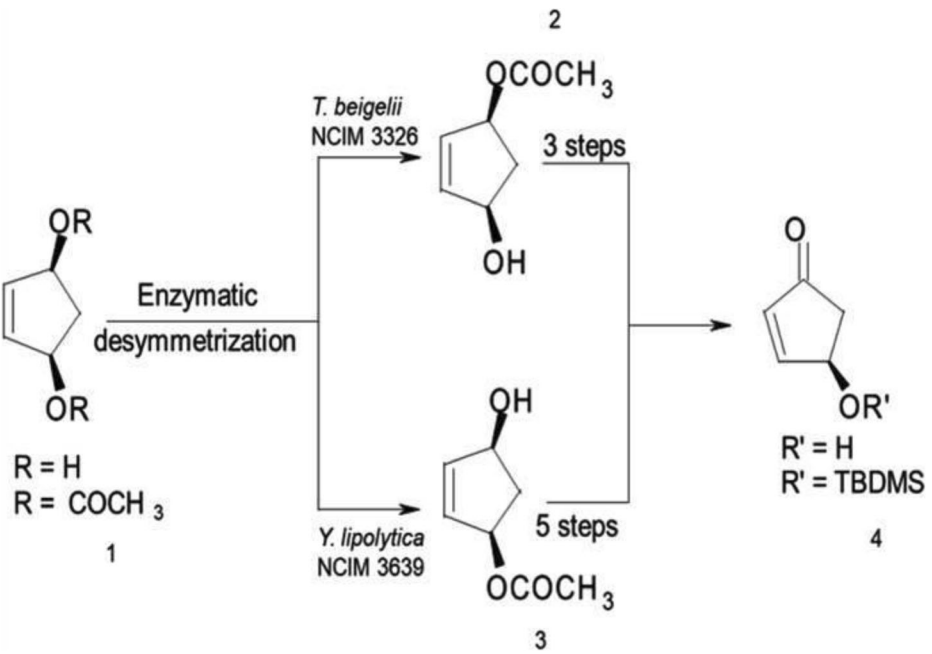

Fig. 1 Lipase-mediated kinetic resolution of meso-cyclopent-2-en-1,4-diacetate (1) to 4-(R)-hydroxycyclopen-2-en-1-(S)-acetate (2) or 4-(S)-hydroxycyclopen-2-en-1-(R)-acetate (3) followed by chemical conversion to 4-(R)-t-butyldimethylsilyloxycyclopent-2-en-1-one (4)

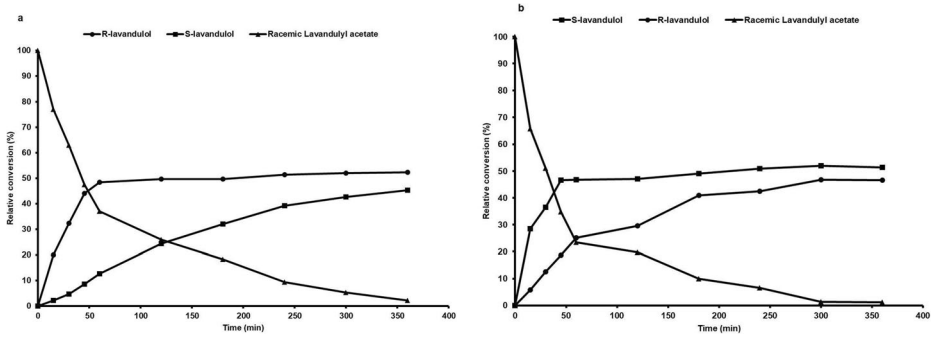

Fig. 2 Reaction course of the hydrolysis of (±)-lavandulyl acetate by *Yarrowia lipolytica* NCIM 3639 lipases. **a** Cell bound. **b** Extracellular

are in high demands especially in food, cosmetic and pharma industries and are produced by commercial lipases (Novozyme 435) using esterification or transesterification reactions [126]. Ethyl hexanoate, an apple-pineapple flavor ester, was synthesized by the esterification of hexanoic acid and ethanol using free and chitosan based immobilized cold active lipase of *Pseudomonas* sp. AMS8 [127]. The reaction was performed at 20° C in toluene as a solvent and the immobilized lipase showed higher conversion of ester (80%) compared with free enzyme (59%). Formate esters, especially phenethyl formate, is a fragrance ester found in cosmetics, perfumes, shampoos and toiletries with its annual requirement of about 1–10 tons and it was recently GRASS cleared for its use as food additive [128]. Phenethyl formate was synthesized by esterification of formic acid and phenethyl alcohol using Novozyme 435 with 96% conversion of formic acid in 1,2-dichloroethane as solvent. However, Novozyme 435 was denaturated in 1,2-dichloroethane. When the reaction was carried out in toluene at 40° C, Novozyme 435 gave 92% conversion and the enzyme was recycled for 20 reactions with the same conversion efficiency [129]. The pure (S)-4-methoxymandelonitrile benzoate was synthesized from 4-anisaldehyde using concurrent bienzymatic cascade. The first enzyme, hydroxynitrile lyase from *Manihot esculenta*, catalyzed the hydrocyanation of 4-anisaldehyde followed by benzoylation of the (S)-cyanohydrin by *Candida antarctica* lipase A in organic solvent [130]. Both hydrocyanation and benzoylation reactions were carried out at 10° C which afforded excellent enantioselectivity with satisfactory reaction rate. The bienzymatic cascade resulted in 81% conversion of (S)-4-anisaldehyde to benzoyl cyanohydrins with 98%ee. The benzoyl cyanohydrins are further converted via single hydrogenation step to (S)-terbamide having antiviral (HIV) and hypoglycemic activity. Acylated cyanohydrins (especially O-acylated) have wide applications in agriculture since pyrethroids insecticides have such structures. Xanthene's are biologically active compounds and derivatives of 2,2'-arylmethylene dicyclohexane-1,3-dione serve as key intermediates in the synthesis of xanthenes. These intermediates are synthesized via the Knoevenagel condensation and Michael addition of aromatic aldehydes with 1,3-cyclohexanedione or 1,3-cyclic diketones using $HClO_4$-SiO_2 as catalyst [131], CsF as catalyst [132] or urea as catalyst [133]. These chemical methods suffer from low yields and harsh reaction conditions. Jiang et al. [134] developed efficient method for synthesis of these derivatives via cascade reactions catalyzed by Amano lipase DF with high yields (94%) fulfilling the requirements of green chemistry and simplified production process. Amano lipase DF is a dietary supplementary grade lipase of *Rhizopus oryzae* manufactured by Amano enzyme, USA.

Applications in Food Industry Food preservation at ambient temperature is necessary to prevent its spoilage and deterioration and also to minimize unwanted changes in the quality of food. The chemical or physical changes in the food usually occur when it is stored at high temperature. From the nutritional point of view, the use of cold active lipases in food and feed are known to prevent spoilage and undesirable changes in the composition of substrates utilized during food processing [135]. The other major requirement of food industry is the improvement in the organoleptic properties of food or the finished products [136]. Flavors and emulsifiers synthesized by lipases improve such organoleptic properties of food products. Short-chain flavor esters include predominantly ethyl acetate, ethyl caproate, ethyl lactate, and butyl butyrate, which are commonly used in food, cosmetic, chemical and pharma industries. They are commonly found in several plant species and the global annual market exceeds $US 22 billion. [137]. Esters produced by action of lipases seem to be the preferred alternative to their synthesis by chemical routes which are not only harsh but require chemical catalysts and high energy consumption resulting in environmentally non-friendly processes [137]. Psychrophilic lipases have been used for synthesis of model esters such as butyl caprylate in non-aqueous media. Cold-adapted lipase from *Pseudomonas* AMS8 exhibited high specific activity and catalytic activity at low temperatures (0–20° C) and immobilized preparation of this enzyme was used for synthesis of ethyl hexanoate by esterification in toluene as solvent [127]. This immobilized preparation could be potentially used as cost–effective lipase in the food industry. Cold active lipase (CALB) was immobilized on to iron magnetic nanoparticles and evaluated for synthesis of methyl and ethyl butyrates using heptane as a solvent system. The optimum conditions for both esters were achieved at 25° C using heptane as solvent which resulted in more than 90% substrate conversion in 8 h of reaction [138]. Sometimes, some organic solvents are not used for safety concerns and hence ester synthesis using solvent-free systems are preferred [139]. A novel lipase from *A. niger* was identified with capability of synthesizing flavor esters in a soybean-oil-solvent free system. The lipase was active at 20° C and showed tolerance to acid and alkaline conditions exhibiting efficient esterification ability for synthesis of ethyl lactate, butyl butyrate, and ethyl caprylate in soybean-oil-solvent system [140]. Phenolic acid esters and alkyl hydroxybenzoates display beneficial effect on health due to their antioxidant property. Such esters have been isolated in small amounts from plants [141]. Though chemical methods for preparation of phenolic esters are available, the enzymatic synthesis of such esters may be advantageous particularly for food use. Long chain alkyl (hydroxyl) benzoates and (hydroxyl) benzyl alkenoates were synthesized by esterification and transesterification reactions using immobilized *Candida antarctica* lipase B [89, 142]. A recombinant *Saccharomyces cerevisiae* was constructed by Han et al. [143] which showed *C. antarctica* lipase B activity on its cell surface. This whole cell recombinant yeast was used to synthesize ethyl hexanoate by esterification of hexanoic acid and ethanol in n-heptane with yield of 98% within 12 h. Polyunsaturated fatty acids (PUFAs) such as omega-3 fatty acid are usually obtained by hydrolysis/alcoholysis of fish oil by lipases [144]. PUFAs such as Omega-3 fatty acid, has great market demand as dietary supplement and nutraceuticals since they have vital role in brain and retina improvement in young people. In addition, they protect the adults and aging people from cardiovascular diseases and improve the muscle function in older women [145, 146]. Although the mesophilic enzymes are employed in food industry, many cold active enzymes have been patented and ready for commercial exploitation [147].

Detergent Industry Normally, the fabrics with lipid stains are washed using hot/warm water to remove lipid stains. The fabrics which cannot withstand higher temperature gets damaged due to wear and tear during washing with hot water. In such cases, cold active lipases offer great advantages in detergent industry since their use lowers the washing temperature, improves the

energy conservation and minimizes the wear and tear [148]. The effectiveness of these enzymes has been proven beyond doubt in cleaning formulations used for laundry and dishwashing purposes. The use of such enzymes reduces environmental pollution and also helps in biodegradation of undesirable chemicals in detergents. Novozymes produced cold active lipase "Lipoclean" which is active at low temperature and was used to remove lipid stains. The detergent formulations blended with such lipases have been applied to porous building materials to clean the surfaces causing no material damages normally caused by the use of other standard formulations [149]. Approximately 1000 tons of lipases make their way into detergents that account for only 5% of the detergent market (https://www.enzymeinnovation.com/lipase-detergent-everything-you-need-know). Cold active lipases in detergents remove oil stains by converting them into more hydrophilic substances without altering the texture and the quality of the fabrics [150]. The most suitable cold active lipases to be used as detergent additives should have the stability at alkaline pH and also should withstand oxidizing and chelating agents [148]. In addition, the enzyme should be effective at low concentration in presence surfactants. Li et al. [151] reported high detergent performance cold-adapted extracellular lipase from *Pseudomonas stutzeri* (isolated from Daing oil fields, China) which was stable in surfactants, solvents and oxidizing agents. These properties of lipase exhibited its potential applications as commercial additive in detergents. Recently, alkaline cold active lipases from *Penicillium canesense* and *Pseudogymnoascus roseus* were found to exhibit 90% stability for 1 h when incubated in commercial detergents at 20° C proving their potential in detergent application [152]. *Fusarium solani* N4-2, isolated from Soda Lake produced alkaline cold active lipase active 10° C with retention of 82% of its original activity [153]. The properties such as stability in surfactants, metal ions, commercial detergents and oxidizing agents makes it most suitable for commercial use as additive in detergent formulations. Very recently, *Chryseobacterium polytrichastri* isolated from Sikkim Himalaya was reported to produce cold-adapted lipase with activity over the temperature range of 5-65° C. Its compatibility with commercial detergents and wide temperature and substrate range renders it a potential candidate for detergent formulations [88]. Such cold active lipases are in high demand and hence considerable research inputs are necessary to look for novel cold active lipases with unique properties from the untapped microbial sources.

Environmental Application Due to the increasing demands for fossil fuels, spillage of petroleum products is causing threat to the environment where the environmental cleaners are required to effectively degrade the pollutants in the cold climate. Cold-adapted microbes can act as environmental cleaners for bioremediation and waste water treatment in cold climates that help in reducing the amounts of toxic compounds earlier considered as non-degradable [154, 155]. Cold-adapted lipase producing organisms play a significant role in bioremediation of fat contaminated wastewater and in lowering of toxic compounds such as hydrocarbons, heavy metals, and diesel oils in the cold environment [18, 156]. Very few reports are available on cold-adapted pyrethroid degrading enzymes with high catalytic activity at low temperatures which can be used for pyrethroid-contaminated vegetables. A novel pyrethroid-hydrolyzing esterase was identified from Mao-tofu metagenome [157]. The esterase was immobilized on a matrix of mesoporous silica to improve its thermostability. This immobilized enzyme was found to efficiently hydrolyze (90%) pyrethroids on contaminated cucumbers in short time. Recently, the use of CAL produced by *B. cereus* HSS was reported to reduce BOD, TSS and oil and grease with highest efficiency proving its potentiality in biological waste water treatment [67]. Alkaline lipase from *Pseudomonas aeruginosa* HFE733 was used in removal of oil in lipid rich food waste water showing its promise for biodegradation of food wastewater treatment [158]. Butyl esters of long chain fatty acids are

widely used as biofuels to reduce environmental pollution. Novozyme 435 is regarded as very efficient biocatalyst for biodiesel production using jatropha oil, sunflower oil, soybean oil and waste cooking oil [89]. Novel lipase isolated from *Halocynthiibacter arcticus* catalyzed the formation of oleic acid esters using methanol, ethanol, and butanol suggesting its possibility to prepare fatty acid methyl esters biodiesels [159].

Conclusions and Future Perspectives

Cold-adapted microbes are regarded as potential sources for lipases exploited for numerous biotechnological applications due to their ability to withstand the permanently cold habitats. The cold active lipases represent versatile extracellular enzymes gaining importance mainly in food, detergent industries due to their features like easy to handle and activity at low temperatures. These enzymes display high catalytic efficiency with low thermal stability differentiating them from their mesophilic and thermophilic counterparts. These enzymes also play an important role in biotechnological applications such as synthesis of biodegradable polymers, synthesis of optically active drugs and drug intermediates and fine chemicals due to their activity in non-aqueous medium and ability to catalyze chemo-, region and enantioselective reactions. In addition, they consume less energy due to low working temperatures. However, their use in industrial exploitation is limited due to instability at moderate temperatures. Hence there is a need to increase the thermostability through immobilization, directed evolution or protein engineering. Enzymes were discovered usually from culturable microorganisms that account for only 1% of microbial diversity. It is now possible to have access to new enzymes by identifying the gene sequences by metagenome mining through metagenomic based activity-screening protocols. Such approaches can help in searching novel cold active lipases performing transformations of molecules that are difficult or impossible to synthesize through chemical transformations to high value fine chemicals leading to greener technologies. In addition, there are daunting demands for green products to be developed through greener technologies using effective cell factories harboring novel biocatalysts. Such rapidly increasing demands compel us to improve the quality and quantity of lipases to develop greener technologies producing products for human consumption.

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Data availability Not applicable

Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable

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