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Cold active lipases – an update

M. Kavitha

School of Biosciences and Technology, VIT University, Vellore, India

ABSTRACT

Cold active lipases (CLPs) are gaining importance nowadays as they are increasingly used in fine chemical synthesis, bioremediation, food processing and as detergent additive. These enzymes exhibit high catalytic activity at low temperatures and flexibility to act at low water medium. Since they are active at low temperatures consume less energy and also stabilize fragile compounds in the reaction medium. CLPs are commonly obtained from psychrophilic microorganisms which thrive in cold habitats. Compared to mesophilic and thermophilic lipases, only a few CLPs were studied and industrially exploited so far. CLPs (*C. antarctica* lipase-A and *C. antarctica* lipase-B) from *Candida antarctica* isolated from Antarctic region are the well studied and industrially employed, and many are being followed up. This review updates the CLPs reported recently and the industrial applications of CLPs.

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Introduction

Microorganisms are capable of growing in unusual environmental conditions, such as high temperatures of volcanic hot springs, low temperatures of polar regions, high pressures of deep seas, very high salt concentrations, and very high and low pH values (Fujiwara 2002). Microbes growing at temperatures below 20°C and above 45°C are classified into extremophiles and categorized as psychrophiles and thermophiles, respectively (Cavicchioli et al. 2002). Psychrophiles are one of the most underutilized resources in the world. In order to thrive at low temperatures, psychrophiles possess enzymes that have a high specific activity at low temperatures and are collectively termed as cold active enzymes. For several industrial applications cold active enzymes provide economic and ecological advantages over their counterpart which operate at higher temperatures (Ohgiya et al. 1999; Marchi et al. 2007).

Lipases (EC 3.1.1.3: Triacylglycerol acyl hydrolases) constitute the third most important category of enzymes, next to carbohydrases and proteases. They are unique in hydrolysing and synthesizing fatty acid esters in aqueous and non-aqueous media. Cold active lipases (CLPs) demonstrate high specific activity in the temperature range of 0−30°C (Feller et al. 1996). These lipases have developed specific structural features which provide them flexibility around the active site. Consequently they display low enthalpy, low affinity towards substrates and high specific activity at low temperatures (Joseph et al. 2008).

In industries, enzymes are steadily replacing chemical reactions since they are greener in approach. Enzymes produce fewer by-products, consume less energy, reduce environmental pollution and add improved value to the products. Consequently, it is not surprising to notice the blooming global enzyme market despite the economic slowdown. According to recent BCC research (a leading market research company) conducted in 2014, the global market for industrial enzymes is expected to reach $7.1 billion by 2018, registering a five-year compound annual growth rate (CAGR) of 8.2% (BCC research 2014, in report BIO030H – Global markets for enzymes in industrial applications). The global market size of lipases in particular is projected to reach $590.5 million by 2020, at a CAGR of 6.5% between 2015 and 2020 (Research and Markets 2015, in report – Lipase market by source, application and geography – Global forecasts to 2020 for the $590.5 million industry).

The research on cold active lipolytic enzymes is gaining importance, as many articles were published

CONTACT M. Kavitha mkavitha1972@gmail.com School of Biosciences and Technology, VIT University, Vellore 632 014, India

recently. Most of these studies were focused on isolation, purification and characterization of the enzymes from various sources; a few were on isolation of lipase gene, cloning and expression in heterologous hosts and some on sequencing of the protein (Joseph et al. 2008). Commercial applications of these lipases are based on their high catalytic activity at low temperatures and they are employed in such industries as detergent, leather, food, pharmaceuticals, fine chemical synthesis and bioremediation (Joseph et al. 2008). One of the key features of these enzymes is consumption of less energy due to low working temperatures.

Several of the cold-adapted lipases studied so far were from psychrotrophic and psychrophilic microorganisms isolated from Antarctic and polar regions, deep-sea environments and refrigerated food samples (Dieckelmann et al. 1998; Xiang et al. 2004; de Maria et al. 2005; Jinwei et al. 2007). But these enzymes are unstable even at moderate temperatures, as they are adapted to act at low temperatures (Joseph et al. 2008). Their thermal stability could be increased through immobilization, directed evolution, protein engineering and chemical modification by adding polysaccharides (Zhang 2003; Siddiqui & Cavicchioli 2005; Lafranconi et al. 2008). CLPs obtained from microbes native of the tropical region exhibit good thermal stability than their counterparts from the alpine region (Cai et al. 2009; Kavitha & Shanthi 2013). Therefore they are better choice for industrial applications.

The steadily growing interest in microbial CLPs is reflected by an increasing number of articles published. This review aimed to update the more recent studies carried out on microbial CLPs.

Structural features and cold adaption

Like all other lipases, CLPs also possess the canonical α/β hydrolase fold (Ollis et al. 1992) (Figure 1). The active site contains the catalytic triad, Ser105 (nucleophile)-His224 (basic residue)-Asp/Glu187 (acidic residue) (Ollis et al. 1992). In almost all lipases, the active site is covered by a lid which opens in the presence of an interface to facilitate contact with substrate (Grochulski et al. 1994; Cygler & Schrag 1997).

CLPs are structurally modified to have high flexibility to accommodate substrates at low temperatures. The correlation between the molecular structure and cold adaption of CLPs is elucidated by comparing with mesophilic and thermophilic lipases using site-directed mutagenesis and crystal studies (Narinx et al. 1997; Wintrode et al. 2000). The molecular structure of lipase from *Pseudomonas immobilis* and *Pseudomonas fragi* IFO 3458 when compared with their mesophilic counterpart revealed the features for cold adaption (Arpigny et al. 1997; Alquati et al. 2002), which are very low content of arginine residues in comparison to lysine residues, low content of proline residues, weak hydrophobic core, very less number of salt bridges and very less number of aromatic–aromatic interactions (Arpigny et al. 1997; Gerday et al. 1997).

Distribution of arginine residues of cold active enzymes is different from that of mesophilic enzymes. A few of them stabilizes at intramolecular salt bridges and a many of them occupy the surface and may confer the conformational flexibility (Alquati et al. 2002; Siddiqui & Cavicchioli 2006; Feller 2013). Additional structural features responsible for cold adaption are

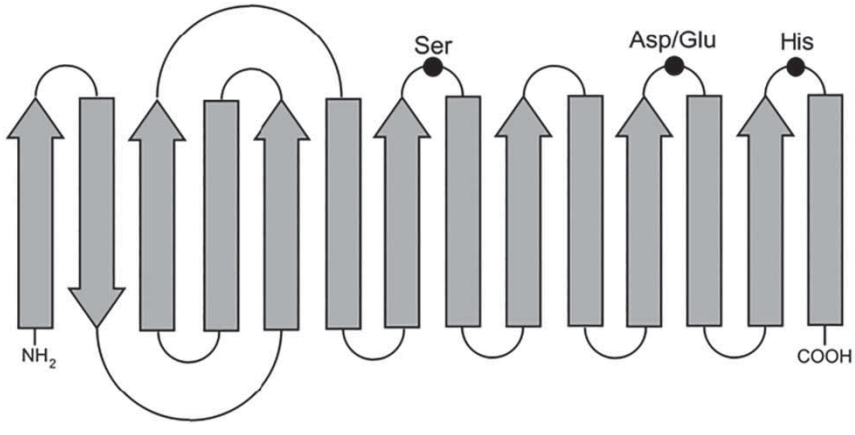

Figure 1. Catalytic Canonical α/β hydrolase fold of lipases.

high content and aggregation of glycine residues (for local mobility), low content of ion pairs and weak charge-dipole interactions in α helices (Georlette et al. 2004; Gomes & Steiner 2004). Increased flexibility of CLPs may be correlated with their ease in accommodating substrates at low temperatures (Joseph et al. 2008; de Pascale et al. 2012) and their ability to catalyze hydrolysis in low water medium (Tutino et al. 2009).

Another feature associated with cold adaption is the production of trehalose and exopolysaccharides which act as a cryoprotectant to prevent precipitation and denaturation of proteins, including the cold active enzymes (Krembs et al. 2002; Ewert & Deming 2013). A shift in the acyl chain length specificity and enhanced thermostability of the enzyme when glycine was substituted with proline was reported (Kulakovaa et al. 2004). A mutation introduced in the lid region of catalytic triad of CLPs from *P. fragi* improved substrate selectivity and thermostability (Santarossa et al. 2005). The catalytic portion of the cold active lipase is observed to possess high plasticity responsible for low activation energy and low thermal stability and the same is conferred by several structural adaptations discussed above.

Recently, modern techniques are increasingly employed to understand the structure–function relationship of CLPs. Gene cloning in heterologous overexpressing hosts (Parra et al. 2008; Xuezheng et al. 2010; Novototskaya-Vlasova et al. 2013), crystal studies (Uppenberg et al. 1995; Juhl et al. 2010; Durmaz et al. 2013) and molecular modelling studies (Maraite et al. 2013; Mohamad Ali et al. 2013) strongly supports the structural attributes of cold-adapted lipases for cold habitation discussed above.

Thermal stability

As the CLPs are structurally modified for cold adaption, they are inherently heat unstable and undergo rapid inactivation at moderate and even low temperatures (Georlette et al. 2004; Siddiqui & Cavicchioli 2006). Thermal denaturation is a common cause of enzyme inactivation in industrial applications; therefore an industrial enzyme necessarily needs to be thermostable (Ruslan et al. 2012). The psychrophilic yeast, *Candida antarctica* expresses two lipases, namely *C.antarctica* lipase-A and *C. antarctica* lipase-B (CAL-A and CAL-B) with different physiochemical properties. The most surprising aspect of CAL-A is its high thermostability. Till date it is considered to be the most thermostable lipase known which is capable of active at >90°C (de Maria et al. 2005). CAL-B is less thermostable and smaller in size than CAL-A (Patkar et al. 1993).

Several strategies have been used to improve the thermostability of CLPs. They are enzymatic or chemical modifications, use of additives, immobilization, directed evolution and protein engineering (Zhang 2003; Eijsink et al. 2004; Siddiqui & Cavicchioli 2005; Lafranconi et al. 2008). Directed evolution with random mutagenesis based on error-prone PCR (ep-PCR) and iterative saturation mutagenesis guided by rational design are more frequently employed nowadays to enhance the thermostability (Bassegoda et al. 2012). The factors commonly considered to increase thermal stability are the hydrophobicity, number of hydrogen bonds, amino acid composition, amino acid distribution and interactions in the protein (Vielle & Zeikus 2001). The structural features of thermophilic and mesophilic lipases are compared with that of CLPs in order to arrive for stabilizing mutations (Suhre & Claverie 2003). But it was very hard to correlate thermostability with specific amino acids interactions (Nawani & Kaur 2007). Therefore directed evolution through random mutagenesis and tedious and time-consuming screening for maximum thermostability had been more effective (Eijsink et al. 2004; Jaeger & Eggert 2004; Reyes-Duarte et al. 2005).

Improved variant of Lip-A from *Bacillus subtilis* after ep-PCR exhibited an increase of 15°C in the melting temperature and 20°C in optimum temperature compared to wild-type lipase (Ahmad et al. 2008). In the case of cold active lipase from *P. fragi*, a variant obtained after two rounds of evolution displayed a fivefold increase in half-life at 42°C and a shift of 10°C in the temperature optimum (Lafranconi et al. 2008). Directed evolution was applied to generate two mutants of CAL-B with >20-fold increase in half-life at 70°C (Zhang et al. 2003).

Saturation mutagenesis combined with B-factor criterion (B-FITTER) to target the amino acid positions to modify (Reetz et al. 2006), several mutants with enhanced thermostability were developed. Two thermostable variants of Lip-A from *B. subtilis* (Reetz & Carballeira 2007), a double mutant from *Rhizomucor miehei* lipase (Zhang et al. 2012) and a mutant of Lip-C

from *Pseudomonas* sp. 42A2 (Cesarini et al. 2012), are a few to specify.

The underlying mechanism of thermostabilization in directed evolution was studied in *Pseudomonas aeruginosa* lipase using circular dichroism spectroscopy which revealed that the secondary structure was retained in mutant up to 70–80°C, whereas the wild-type protein structure was completely distorted above 35°C (Sharma et al. 2012). In another study using circular dichroism, X-ray structure analysis and nuclear magnetic resonance spectroscopy on *B. subtilis* Lip-A, it was observed that mutation of surface residues hinder the tendency of Lip-A to undergo precipitation under thermal stress (Augustyniak et al. 2012).

The protein engineering strategy was adapted to enhance the thermostability where the disulphide and other bonds are modified to decrease the entropy of the unfolded form of proteins or to decrease the unfolding rate of irreversibly denatured proteins (Siadat et al. 2006). CAL-B and *Geobacillus zalihae* T1 lipase were successfully engineered by mutating five amino acid pairs to cysteine and by introducing an ion-pair in the inter-loop (Le et al. 2012; Ruslan et al. 2012).

Sources of CLPs

CLPs are generally present in psychrophillic and psychrotrophic microorganisms capable of surviving at low temperatures close to 5°C. A number of cold active lipase-producing microorganisms are reported so far, but only a few bacteria and yeast were commercially exploited. Most of them were isolated from Antarctic and polar regions that exhibit constant cold habitat at 0 ± 2°C. The bacteria from these regions known to produce cold active lipase include, *Moraxella* sp. TA144 (Feller et al. 1991), *Psychrobacter immobilis* B10 (Arpigny et al. 1997), *Psychrobacter* sp. Ant300 (Kulakovaa et al. 2004), *Psychrobacte*r sp. 7195 (Jinwei et al. 2007) and *Pseudoalteromonas haloplanktis* TAC125 (de Pascale et al. 2008). More recently, *Halomonas* sp. BRI 8 was reported to be isolated from Antarctic seawater sample (Jadhav et al. 2013). Apart from Antarctic and polar regions, the other cold regions such as glaciers and high mountain tops also harbour cold active lipase-producing microorganisms. *Microbacterium luteolum* and *Bacillus sphaericus* MTCC 7526 were isolated from the Gangothri glacier of western Himalayas (Joseph et al. 2012; Joseph & Ramteke 2013). *Acinetobacter* sp. 6 and *Psychrobacter cryohalolentis* K5T were isolated from Siberian tundra soil and cryopeg, respectively (Suzuki et al. 2001; Novototskaya-Vlasova et al. 2013). *Pseudomonas* sp. B11-1 isolated from Alaskan soil was documented to cold active lipase (Choo et al. 1998).

The next major source for cold active lipase-producing bacteria is deep-sea and marine environment. *Aeromonas* sp. LPB4 (Lee et al. 2003), *Pseudoalteromonas* sp. wp27 and *Psychrobacter* sp. wp37 (Zeng et al. 2004) were from deep-sea sediments where temperature is below 3°C. *Aeromonas hydrophila* and *Pseudomonas* sp. MSI057 were isolated from marine environment and marine sponge, respectively (Pemberton et al. 1997; Kiran et al. 2008). Recently, *Janibacter* sp. HTCC2649 isolated from marine environment was described to produce cold active lipase (Yuan et al. 2014). Constant refrigeration of food stuff results in evolution of psychrotolerant microorganisms producing cold active enzymes. *Pseudomonas fluorescens* (Dieckelmann et al. 1998) and *Staphylococcus epidermidis* (Joseph et al. 2006) were isolated from refrigerated milk and fish samples.

Apart from bacteria, psychrophilic fungi and yeast were also reported to produce cold active lipase. *C. antarctica* isolated from Antarctic habitat is a well-known source for two industrially important CLPs, CAL-A and CAL-B (de Maria et al. 2005). Other fungi and yeast reported to produce cold active lipase include *Candida lipolytica*, *Geotrichum candidum* and *Pencillium roqueforti* isolated from frozen food samples (Alford & Pierce 1961).

One of the major advantages of these enzymes is consumption of less energy as they act at low working temperatures. But several of these enzymes are naturally not thermostable to withstand the temperatures of tropical and temperate climates in order to be exploited for industrial applications. Their thermal stability can be increased by immobilization, directed evolution, protein engineering and chemical modifications (Zhang et al. 2003; Siddiqui & Cavicchioli 2005; Joseph et al. 2008; Lafranconi et al. 2008). CLPs obtained from microbes native of tropical region generally exhibit good thermal stability. So far there are only two reports on such organisms. *Geotrichum* sp., a mesophilic yeast and *Pseudomonas* sp. VIT-CLP4, a mesophilic bacterium isolated from subtropical region and tropical seacoast, respectively, were documented to produce CLPs (Cai et al. 2009; Kavitha &

Table 1. Microorganisms producing cold active lipase.

Microorganism	Sources	References
Bacteria		
Acinetobacter sp. 6	Siberian tundra soil	Suzuki et al. (2001)
Acinetobacter baumannii BD5	Mountain water	Park et al. (2009)
Aeromonas sp. LPB4	Sea sediments	Lee et al. (2003)
Aeromonas hydrophila	Food products	Imbert and Gancel (2004)
Bacillus psychrosaccharolyticus	Soil and lowland marshes	Seo et al. (2004)
Bacillus sphaericus MTCC7526	Gangotri glacier (western Himalayas)	Joseph and Ramteke (2013)
Colwellia psychrerythraea 34H	Arctic marine sea	Do et al. (2013)
Desulfotalea psychrophila	Arctic sediments	Rabus et al. (2004)
Halomonas sp. BRI 8	Antarctic habitat	Jadhav et al. (2013)
Janibacter sp. HTCC2649	Marine habitat	Yuan et al. (2014)
Micrococcus roseus	Glacial soil	Joseph et al. (2011)
Microbacterium phyllosphaerae MTCC 7530 and *Corynebacterium paurametabolum* MTCC 6841	Naukuchiatal lake (western Himalayas)	Joshi et al. (2006)
Microbacterium luteolum	Gangotri glacier (western Himalayas)	Joseph et al. (2012)
Moritella sp. 2-5-10-1	Antarctic bacteria	Wang et al. (2013)
Pelagibacterium halotolerans B2T	East China Sea	Wei et al. (2013)
Photobacterium sp. MA1-3	Blood clam	Kim et al. (2012)
Photobacterium aplysiae sp. (GMD509)	Eggs of sea hare	Seo et al. (2005)
Photobacterium lipolyticum M37	Marine habitat	Ryu et al. (2006)
Photobacterium ganghwense sp. FR1311T	Deep sea	Park et al. (2006)
Photobacterium marinum AK15(T) and AK18	Sea sediment	Srinivas et al. (2013)
Pseudoalteromonas sp. wp27	Deep-sea sediments	Zeng et al. (2004)
Pseudoalteromonas sp.	Antarctic marine	Lo Giudice et al. (2006)
Pseudoalteromonas haloplanktis TAC125	Antarctic seawater	de Pascale et al. (2008)
Pseudomonas antarctica sp.	Antarctica	Reddy et al. (2004)
Pseudomonas fluorescens	Refrigerated human placental extracts	Preuss et al. (2001)
Pseudomonas fluorescens	Soil of cold region	Leonov (2010)
Pseudomonas fragi IFO3458	Ns	Alquati et al. (2002)
Pseudomonas putida GR12-2	Arctic plant	Muryoi et al. (2004)
Pseudomonas sp. MSI057	Marine sponge *Dendrilla nigra*	Kiran et al. (2008)
Pseudomonas sp. 42A2	Oil-contaminated water sample	Bofill et al. (2010)
Pseudomonas sp. VITCLP4	Tropical seacoast	Kavitha and Shanthi (2013)
Pseudomonas sp. AMS8	Antarctic soil	Ali et al. (2013)
Psychrobacter sp., *Vibrio* sp. and *Pseudomonas* sp. KB700A	Subterranean environment	Rashid et al. (2001)
Psychrobacter cryohalolentis K5T	Siberian cryopeg	Novototskaya-Vlasova et al. (2013)
Psychrobacter okhotskensis	Sea coast	Yumoto et al. (2003)
Psychrobacter sp. Ant300	Antarctic habitat	Kulakovaa et al. (2004)
Psychrobacter sp. wp37	Deep-sea sediments	Zeng et al. (2004)
Psychrobacter sp. 7195	Antarctic habitat	Jinwei et al. (2007)
Psychrobacter sp.	Antarctic habitat	Parra et al. (2008)
Psychrobacter sp. G	Antarctic seawater	Xuezheng et al. (2010)
Psychrobacter sp. TA144	Antarctic seawater	De Santi et al. (2010)
Psychrobacter sp. C18	Deep-sea sediments	Chen et al. (2010)
Serratia marcescens	Raw milk	Abdou (2003)
Shewanella sp. SIB1	Water deposits in oil reservoir	Suzuki et al. (2004)
Staphylococcus epidermidis	Frozen fish samples	Joseph et al. (2006)
Stenotrophomonas maltophilia CGMCC 4254	Oil soil	Li et al. (2013)
Vibrio ruber sp. Nov VR1T	Seawater	Shieh et al. (2003)
Fungi/Yeast		
Aspergillus nidulans	Ns	Mayordomo et al. (2000)
Candida antarctica	Antarctic habitat	Patkar et al. (1993); Uppenberg et al. (1994b); Uppenberg et al. (1994a); Zhang et al. (2003); Siddiqui and Cavicchioli (2005)
C. albicans ATCC 10231	Ns	Lan et al. (2011)
Geomyces sp. P7	Antarctic habitat	Florczaka et al. (2013)
Geotrichum sp. SYBC WU-3	Subtropical	Cai et al. (2009)
Pichia lynferdii NRRL Y-7723	Ns	Kim et al. (2010)

Note: NS, not specified.

Shanthi 2013). A comprehensive list of various cold active lipase-producing microorganisms reported recently is presented in Table 1.

As previously mentioned, the major setback for industrial exploitation of CLPs is their thermoinstability. Therefore attempts have been made to screen mesophilic organisms for CLPs since they are expected to be inherently stable at moderate to high temperatures. *Geotrichum* sp., mesophilic yeast isolated from subtropical region was reported to produce two CLPs stable at room temperature (Cai et al. 2009).

Industrial applications of CLPs

CLPs are structurally modified to accommodate substrates at low temperatures. One of the key features of these enzymes is consumption of less energy due to low working temperatures. They have true enzyme potentialities for various industrial applications, such as leather processing, medical and pharmaceutical preparations, fine chemical synthesis, detergent additive, food processing, environmental bioremediation, biotransformation, preparation of cosmetics and gene expression in heterologous hosts to block inclusion bodies (Feller et al. 1996). A list of industrial applications of CLPs is presented in Table 2. CLPs are advantageous because they are active under low water conditions due to inherent greater flexibility, whereas mesophilic and thermophilic enzymes show higher rigidity. The other advantages include low cost in production, wide variety, stability to organic solvents, specificity in action, mild reaction condition and low energy consumption.

Applications in the detergent industry

The detergent industry is the largest market for industrial enzymes (Ahuja et al. 2004). Lipases improve the washing capability of detergents towards the fatty food stains from fabrics which are difficult to go off during normal washing conditions (Andree et al. 1980). An ideal detergent enzyme should be stable at alkaline pH and active in the presence of surfactants (Jurado et al. 2007). It should withstand oxidizing and chelating agents, which are used in detergents as active oxygen bleach and builder (Wang et al. 1995). The enzyme also needs to be effective at lower concentration and have broad substrate specificity (Wang et al. 1995).

Traditionally cloths are washed in hot and warm water. Increasing use of synthetic fibres which cannot tolerate temperatures above 50—60°C and the energy crisis has changed the washing habits towards lower washing temperatures of 30—40°C (Nielsen et al. 1981). The use of cold-adapted lipase in detergents would be of great advantage since they specifically

Table 2. Industrial applications of cold active lipases.

Application	Purpose	Source of lipase	References
Detergent industry	Detergent additive	CAL-B from *Candida antarctica*	Uppenberg et al. (1994a)
		Microbacterium phyllosphaerae and *Bacillus sphaericus*	Joseph and Ramteke (2013)
Medical and pharmaceutical applications	Synthesis of optically active amines	*Candida antarctica* and *Pseudomonas* sp	Smidt et al. (1996)
	Synthesis of aryl aliphatic glycolipids	Geotrichum sp. F0401B	Otto et al. (2000)
	Formation of citronellol laurate	CAL-B from *Candida antarctica*	Ganapati and Piyush (2004)
	Synthesis of single isomer chiral drugs	CAL-B from *Candida antarctica*	Gotor-Fernandez et al. (2006a)
	Synthesis of nitrogenated compounds	CAL-B from *Candida antarctica*	Gotor-Fernandez et al. (2006b)
Fine chemical synthesis	Synthesis of optically active esters	CAL-B from *Candida antarctica*	Anderson et al. (1998)
	Synthesis optically active alcohols	CAL-B from *Candida antarctica*	Rotticci et al. (2001)
	Synthesis of ethyl docosahexaenoate	CAL-B from *Candida antarctica*	Shimada et al. (2001)
	Ester synthesis	CAL-B from *Candida antarctica*	Zhang et al. (2003)
	Formation of butyl lactate	CAL-B from *Candida antarctica*	Pirozzi and Greco (2004)
	Asymmetric synthesis of amino acids/amino esters	CAL-A from *Candida antarctica*	de Maria et al. (2005)
	Enantioselective esterification of (R)-ketoprofen	CAL-B from *Candida antarctica*	Ong et al. (2006)
	Organic synthesis of chiral intermediates	CAL-B from *Candida antarctica*	Gotor-Fernandez et al. (2006a)
Food industry	Formation of butyl caprylate as flavour compound	*Pseudomonas fluorescens* P38	Tan et al. (1996)
Environmental applications	Bioremediation and bioaugumentation	*Acinetobacter* sp. 6	Suzuki et al. (2001)
Leather industry	Degreasing using alkaline and acid lipases	Lipases of commercial sources	Afsar and Cetinkaya (2008)
Other applications	Biodiesel synthesis from degummed soybean oil	CAL-B from *Candida antarctica*	Watanabe et al. (2002)
	Lipase-catalysed biodiesel	CAL-B from *Candida antarctica*	Chang et al. (2005)

allow washing under cold conditions which in turn decrease energy utilization and wear and tear of cloth fibres (Feller & Gerday 2003). The other advantages include reduced environmental load of detergent products, reduced use of chemicals in detergents, biodegradable, no negative impact on disposal of domestic waste and no risk for aquatic organisms.

Recombinant cold active lipase from *C. antarctica* is successfully used in detergent formulation (Uppenberg et al. 1994b). CLPs from *Microbacterium phyllosphaerae* and *B. sphaericus* were reported to be efficient in the removal of lipid stains in the presence of commercial detergents from fabrics (Joseph & Ramteke 2013). Lipomax and Lumafest (Genencor International) contain lipase from *Pseudomonas* sp. (Jaeger & Reetz 1998). Incorporation of enzymes in detergent formulations makes them eco-friendly and also reduces laundering temperatures to warm and cold, saving energy (Hemachander & Puvanakrishnan 2000).

Medical and pharmaceutical applications

Cold-adapted lipases are used in the manufacture of single-isomer chiral drugs (Gotor-Fernandez et al. 2006a). Cold active lipase from *C. antarctica* or *Pseudomonas* sp. is used to act on stereospecific N-acylamines to form optically active amines as intermediates in the preparation of pharmaceuticals (Smidt et al. 1996). CAL-B is widely employed in the manufacture and segregation of a large number of nitrogenated compounds intended to be used in the synthesis of pharmaceuticals (Gotor-Fernandez et al. 2006b).

Fine chemical synthesis

Some fine chemicals synthesized from fats and oils by chemical processes can be synthesized using CLPs with good specificity in milder reaction conditions (Sih & Wu 1989; Vulfson 1994). Kinetics of acyl transfer reactions in organic media catalysed by CAL-B was reported by Martinelle and Hult (1995). Anderson et al. (1998) demonstrated the applications of CAL-B in organic synthesis. CAL-B was also used in the synthesis of optically active alcohols (Rotticci et al. 2001). Gustavsson et al. (2004) applied CAL-B to catalyse ring opening polymerization of epsilon-caprolactone in proximity to cellulose fibre surface. Ong et al. (2006) demonstrated the improved performance of free CAL-B in a mixed solvent system for enantioselective esterification of (R)-ketoprofen, leaving the target product (S)-ketoprofen in the unreacted state.

Highly thermostable CAL-A exhibits chemo selectivity for amine groups; it was used in the asymmetric synthesis of amino acids/amino esters (de Maria et al. 2005). CAL-B was successfully used in the ethyl esterification of docosahexaenoic acid to form ethyl docosahexaenoate in an organic solvent-free system (Shimada et al. 2001). Tan et al. (1996) reported the use of cold active lipase from *P. fluorescens* P38 in the synthesis of butyl caprylate in *n*-heptane at low temperatures.

Applications in the food industry

In order to preserve the heat-sensitive micro- and macronutrients present in food ingredients, food industries prefer reactions that occur at low temperatures. Thus cold active enzymes are widely employed in food industries in place of traditional chemical processes. Tan et al. (1996) reported the use of cold active lipase from *P. fluorescens* P38 in the synthesis of the flavouring compound, butyl caprylate in *n*-heptane at low temperatures. Lipases from *C. antarctica* (CAL-B), *Candida cylindracea* AY30, *Hansinuela lanuginosa*, *Pseudomonas* sp. and *G. candidum* were used in the esterification of functionalized phenols to form antioxidants in order to be used in sunflower oil (Buisman et al. 1998; Pandey et al. 1999).

Environmental applications

Biodegradation of petroleum hydrocarbons in cold environments including Alpine soils is due to indigenous cold-adapted microorganisms which are able to degrade these contaminants by producing cold active enzymes. CLPs have great potential in the field of wastewater treatment, bioremediation in oil-contaminated cold environment and active compounds synthesis in the cold condition (Buchon et al. 2000). Suzuki et al. (2001) reported a cold active lipase from a psychrotroph, *Acinetobacter* sp. six efficiently hydrolysed triglycerides in soybean oil at 4°C and has the potential for *in situ* bioremediation or bioaugmentation of oil-contaminated cold environments. Belousova and Shkidchenko (2004) reported

the isolation of 30 strains capable of oil degradation at 4–6°C.

Applications in the leather industry

Among the end users of technical enzymes, the leather industry is on top (Sarrouh et al. 2012). This is due to environmental pollution by the chemicals used and implementation of global environmental regulations. Pre-tanning and tanning steps contribute to 80–90% of the total pollution (Thanikaivelan et al. 2004). With respect to biological oxygen demand, chemical oxygen demand and total dissolved solids (TDS), approximately 70% of the pollutants are generated from pre-tanning operations (Ramasami et al. 1999). Dehairing and chrome-tanning steps result in the release of hydrogen sulphide and chromium plus sulphate ions, respectively (Rao et al. 1997; Marsal et al. 1999). Degreasing leads to discharge of solvents and surfactants (Christner 1992).

In leather processing, lipases are used in the removal of natural fat present in animal skin. A separate degreasing step is required for animal skins with high fat content. Fat is present in sebaceous gland, hair follicle, between collagen fibres and connective tissue fibres in subcutaneous layer of the skin. The level of fat in skin varies with such factors as breed, age and sex. It is approximately 2−4% in cattle, 12−15% in goat and 30% in sheep skin and mainly comprises 56% triglycerol, 23% glycerol, 6% phospholipid, 5% cholesterol and 10% fatty acids (Christner 1992).

Insufficient removal of natural fat during processing prevents the chemicals from penetrating into the leather which leads to negative impacts on the quality of finished leather, such as hardness without sufficient internal softness, fatty spew formation, stained appearance due to chrome soap formation, weak bonding of the finishing layer and bad odour. Traditionally excess fat was removed using solvents and emulsifiers but they add to pollution. Alternatively, lipases of microbial origin can be used in degreasing to reduce pollution. Research on the use of lipase for degreasing dated a few decades back. Use of acid lipase of fungal origin in degreasing on pickled pelts was reported in 1978 (Yeshodha et al. 1978). Acid lipase from *Rhizopus nodosus* along with commercial degreaser was used in degreasing in 1982 (Muthukumaran & Dhar 1982). Recently, with the availability of commercial lipases, the effectiveness of acid and alkaline lipases of commercial origin in degreasing at various stages of leather processing was also reported (Afsar & Cetinkaya 2008).

Conclusions and future prospects

Cold active enzymes are now available for various commercial exploitations, such as organic synthesis, bioremediation, leather processing, biotransformation and biocatalysis. The advantage of CLPs is that they are active under low water conditions due to inherent greater flexibility, whereas mesophilic and thermophilic enzymes show higher rigidity, low cost in production, wide variety, stability to organic solvents, specificity in action, mild reaction condition and low energy consumption. Therefore CLPs are very promising enzymes to replace the conventional enzyme processes of the biotechnological industries. But the specific effort needs to be taken to overcome certain aspects, such as low stability and low biodiversity. With the availability of newer techniques, such as site-directed mutagenesis and metagenomics, it is highly possible to come up with many tailor-made microbial CLPs from diverse sources for several industrial and biotechnological applications.

Disclosure statement

No potential conflict of interest was reported by the author.

ORCiD

M. Kavitha http://orcid.org/0000-0002-1862-8037

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