Current Research in Microbiology

Chapter 2

Microbial Thermo-Tolerant and Solvent-Tolerant Lipases and their Applications

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Abstract

Lipases are the preeminent option(s) for organic chemists because of some common issues of interest such as simplicity of handling, broad substrate acceptance, high stability towards temperatures as well as organic solvents and convenient commercial availability. Moreover, easy amenability of lipases to many matrices for their immobilization has added to their extensive attractiveness among organic chemists. Thermo-tolerant lipases that remain folded and functional at high temperatures besides other environmental conditions such as hydration state, pH or ion concentration have natural advantages to be employed in stringent thermal and solvent conditions for biotransformation reactions and organic syntheses. Lipases show immense flexibility of their catalytic behaviour but the need persists for suitable non-aqueous and safe solvents for use in the reactions. The adaptation displayed by microbial thermophilic/thermo-tolerant and/or organic solvent tolerant lipases can be exploited in many fields, including pharmaceutical, medicine, environment, food, agriculture and biochemical synthesis.

Keywords: thermo-tolerant; adaptations; rigidity; organic solvents; applications

1. Introduction

Lipases (EC 3.1.1.3) dwell in a place of prominence among biocatalysts in a fast surging biotechnological and pharmaceutical market owing to their narrative and multifold applications in resolution of racemic mixtures, synthesis of fine chemicals, oleo-chemistry

and pharmaceuticals. As the industrial applications may necessitate precise properties of the biocatalysts, however there is still a curiosity in finding innovative lipases that could create novel commercial applications. Principally the alkaline and thermophilic lipases showing tolerance to organic solvents are generally appropriate for biocatalytic synthetic reactions [1-3]. This is because of the reality that the majority of the synthetic reactions at lab as well as at industrial scale cannot be catalyzed efficiently in aqueous media. However, now a day's non-aqueous enzymology is an area of prominence and attention. There is a great need to isolate and screen the microbes for enzyme(s) with desired activity, stability in organic solvents and thermostability.

An accurate lipase is an enzyme that hydrolyzes preferentially long chains of fatty acids higher than pH 8 (*i.e*. an alkaline) pH [4-5]. The active site of lipases possesses a catalytic triad which includes a serine positioned within the conserved region Gly-X-Ser-X-Gly, as well as a histidine, which interacts with an aspartate or glutamate residue [6]. Lipases catalyze the hydrolysis of ester bonds at lipid/water interface(s). Lipases are used in the food, beverages, chemical, leather, medical, waste-water treatment and detergent industries [7-9]. In the pharmaceutical industry, lipases are frequently used to encourage the digestion of oil and fat in foods, substitute as digestive enzyme, used in diagnostic kits for blood triacylglycerol assays and for elimination of lipids in cosmetics [9-12]. Due to the peculiarity of existing environments, organic solvent-tolerant extremophiles have recently become research spotlight for the isolation of organic solvent-stable lipases and thermophiles which can exhibit the genetic as well as cellular constituents to flourish in harsh and adverse circumstances. Such extremophiles could also fabricate the protein and enzymes (lipases) in transformed environment conditions.

2. Basis of Thermo-Stability of Microbial Lipases

Thermo-stability of a liapse on the whole favoured asset for bulk of industrially applied biocatalysts. Lipases of thermophilic origin regularly show higher resistance to chemical denaturation and they often become more stable in the presence of diverse organic solvents thus making them ideal tools for use at industrial scale where exposure to relatively high reaction temperatures and/or organic solvents are needed [10,13]. The enzymes that have been isolated from extremophiles have shown some exclusive features, such as extreme thermostability and frequently they are also resistant to diverse chemical denaturants such as detergents, chaotropic agents, organic solvents and extremes of pH [10,14-15]. The lipase-producing thermophilic bacteria include *B. thermoamylovorans* BHK 67 [9], *B. cereus* MTCC 8314 [16], *P. menodocina, B. pumilus, Achromobacter* spp., *P. fluorescens, P. cepacia* and *P. glumae* [6,17]. Technical applications of lipases in the industry are promising only if the enzymes are stabilized against temperature, pH extremes, presence of salts, alkalis and surfactants. The high reaction temperature in biotransformation reactions provides higher conversion rate, negligible

threat of microbial contamination, higher solubility of the substrates and lesser viscosity of the reaction medium which eventually favor the mass transfer. Thermo-stability is also dependent on the structural characteristics of the enzyme such as loop stability, compression, secondary structure, core packing [18], the environment (solvent), pH, the occurrence of additives (substrate and ions) and immobilization [9]. *Bacillus* spp. are well adapted for growth at high temperature, thus majority of lipases produced by *Bacillus* especially *Geobacillus* spp. were reported to be thermostable and have their temperature optima around 45–60^{o}C. A lipase purified from *Bacillus* spp. H1 showed maximal activity at 70^{o}C and conserved almost 80% of it after 20 h incubation at 60^{o}C (pH 7.0) [19]. The lipases sourced from *G. stearothermophilus* MC7 and *G. thermoleovorans* ID1 were reported to be optimally dynamic at 75^{o}C [20-21].

Thermostable lipases with broad substrate selectivity are applied in the processing of paper & pulp and for the removal of pitch (a hydrophobic component of wood composed mainly of triglycerides and waxes). Major applications of thermophilic and solvent-tolerant lipases at high temperature (60 to 70^{o}C) include pharmaceutical textile, biofuel production and several biotransformation reaction systems among others. Thermophilic proteins are thought to be less flexible than mesophilic proteins at mesophilic temperatures [22]. Nevertheless, it is also anticipated that both thermophilic and mesophilic proteins accomplish an analogous degree of flexibility at their respective optimum temperatures [10,23]. On the other hand, it could be possible that hyper-thermophilic enzymes cannot achieve the degree of flexibility of their mesophilic counterparts and their catalytic efficiency is accordingly reduced. A lipase from *Bacillus* sp. A30-1 was reported to preserves 50% of its preliminary activity after 8 h heating even in the absence of substrate [24]. A hyperthermostable lipase reported from *G. stearothermophilus* SB-1 had a half-life of 15 min at 100 ^{0}C (pH 6.0) [25]. Evolutionary biology has long been solicited to invent the robust mechanism of nucleic acids molecules under high growth temperatures. Extensive dissimilarity in the guanine (G) and cytosine (C) content exists among the microorganisms. The causes of variation in the base compositions have remained the matter of apprehension and concern for selectionists. Thermal adaptation hypothesis proposed by Bernardi gives an association between GC content and temperature if there is substantial variation of G and C content between species [26]. G: C pairs are thermally extra stable than A: T pairs because G: C pairs are being linked by three hydrogen bonds as A: T pairs by two hydrogen bonds [27].

Earlier, a positive correlation between genomic G+C content and optimal growth temperature existed in most of the bacterial families was studied [10]. It has been established that there is a considerable correlation between G+C content of structural RNAs and growth temperature [28-29]. A predilection towards C- and G-ending codons for duet amino acids in thermophiles has been noticed. The major question that arises in front of microbial

physiologists and biotechnologists is; what are the differences at molecular level with respect to the mesophilic microbial species that make the extreme conditions feasible for their survival? As formerly reported, natural transformation in thermophiles is a distinctive aspect of *Thermus thermophilus*. The function of natural transformation in genome evolution and bacterial adaptation appears very diverse, habitually extreme environments are very imperative and also DNA transporters of extremophilic bacteria play important roles for their adaptation to extreme environments. In a previous study, an extracellular lipase gene (ln1) was cloned from a thermophilic fungus *Thermomyces lanuginosus* HSAUP0380006. Its coding sequence predicted a 292 residues protein with a 17 amino acids signal peptide. The deduced amino acids showed 78.4% resemblance to another lipase from *T. lanuginosus* while it shared low resemblance with previously reported fungal lipases. Higher frequencies of hydrophobic amino acids were interrelated to lipase thermal stability, such as Ala, Val, Leu and Gly were observed in this lipase [30].

We must ideally comprehend the mechanism of adaptations, types of proteins involved, amino acid composition and conformational changes in proteins which facilitate thermophilic organisms to stay alive in the harsh environments. At morphological level the major features involves altered cell wall and cell membrane structure, cell membrane lipids and their structure, altered molecular biology, chemosynthetic mechanisms, changes in lipid fluidity, and metabolic pathways. Thermophiles have need of proteins that stay folded and functional at high temperatures on a diversity of protein structures in addition to other environmental conditions like pH or ion concentration, hydration state; temperature exerts an intense pressure on the reasonable interaction of structural flexibility and rigidity. The mechanical strains created by protein motions in stress conditions persuade restricted unfolding in chaperones and functional conformations of enzymes. An adequate structural rigidity preserving the specific changes in the inimitable fold of a protein structure is as imperative as enough internal conformational flexibility is obligatory for appropriate enzymatic activity.

3. Stabilizing Factor of Thermostable Microbial Lipases

The association among the habitat changes and molecular interactions in protein conformation needs to be thoroughly explored. A fundamental structural modulation in proteins that would combine with conformational change is local unfolding, has a direct impact on protein function by affecting structures and flexibility. It relies on the nature of protein involved and kind of environmental condition. The free energy of stabilization of a protein (the ΔGstab) is the difference between the free energies of the folded and the unfolded states of that protein which directly measures the thermodynamic stability of the folded protein [31]. The stabilization enthalpy and the stabilization entropy fluctuate almost linearly with temperature in the range of the activities of most enzymes. Not many proteins have been premeditated to find out the free energies of stabilization. Such studies are stuck by the fact that the thermal

denaturation of the majority of the proteins is irreversible and the complete denaturation is frequently followed by aggregation and precipitation. Consequently, most stabilization entropy data are for small monomeric proteins [32].

Since the denaturation of hyperthermophilic proteins transitions takes place outside the temperature range of most calorimeters, so stabilization entropy calculations become made even more difficult [33-34]. To conquer this obscurity, most thermodynamic studies of hyperthermophilic protein stability are performed at pH outside the physiological conditions or in the presence of guanidinium hydrochloride [35-36]. As an outcome of which, the ΔGstab *Vs* T curve of the hyperthermophilic protein will be different from that of its mesophilic counterpart. The three hypothetical traditions by which increased protein thermodynamic stability (**Figure 1**) can be considered [10]; (Ta) the ΔGstab *Vs* T curve of a hyperthermophilic protein can be shifted toward higher ΔGstab values, and (Tb) it can be shifted toward higher temperatures or (Tc) it can be flattened (due to a smaller difference in partial molar heat capacity between the protein's folded and unfolded states). These situations permit the temperature of the denaturation transition to become reachable to physical measurement and in some cases they allow the enzyme to unfold reversibly. Therefore, a majority of thermophilic and hyperthermophilic proteins (lipases) may use combinations of these mechanisms to reach their advanced thermodynamic stabilities [10].

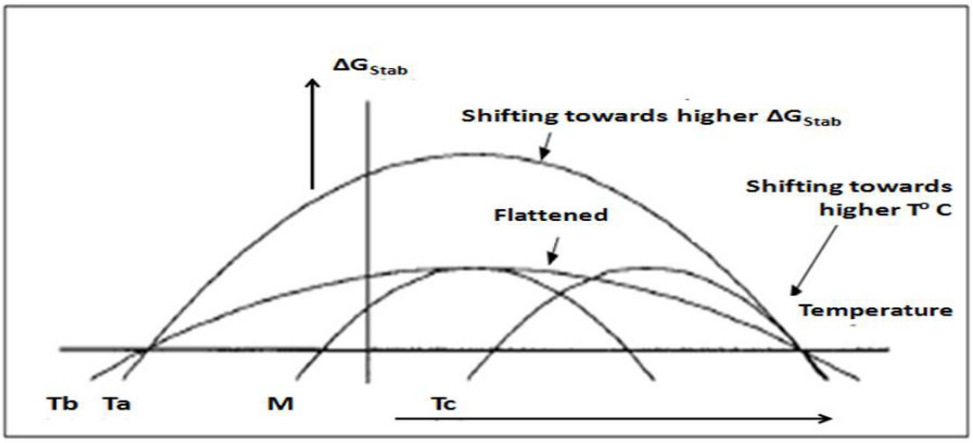

Figure 1: Evaluation of theoretical Gstab Vs T curves for hyperthermophilic (Ta, Tb and Tc) as well as mesophilic (M) enzymes [33].

4. Organic Solvent-Tolerant Microbial Lipase

Lipases are diverse in their sensitivity towards different organic solvents and these enzymes are extra destabilized in polar water miscible solvents than in water immiscible solvents [3,11,37]. Hence, to screen the enzymes that are naturally stable with high activity in different types of toxic organic solvents, diligent efforts are needed and consequently comprise great prospective in extending the expedient catalysis system. An organic solvent tolerant lipase isolated from *Pseudomonas stutzeri* LC2-8 was purified by acetone precipitation and anion exchange chromatography with molecular mass of 32 kDa by SDS-PAGE [38]. Lipase purified from *Brevibacillus agri* 52 was found to be stable in various solvents such as up to 90% diethylenglycol, glycerol and 1, 2 propanediol at 37 °C for at least 1 h and it's stability reduced

only just about 20% after 12 h incubation, However, in 40% dimethylsulfoxide (DMSO), lipase was stable merely for 1 h. An arsenic resistant *Bacillus* sp [39]. ORAs2 which was competent to grow in the presence of toluene and benzene was isolated from contaminated sediments [40]. A toluene-tolerant *Bacillus cereus* R1 was isolated in an atmosphere saturated with toluene [41]. Gram-positive organic solvent-tolerant bacteria were also isolated by incubation of an appropriate agar medium with benzene for numerous days. The major bacterial genera isolated by this method are *Arthrobacter* ST-1, *Bacillus* sp. DS-994 and *Bacillus* sp. DS-1906 [42-44]. Another novel strain of *Staphylococcus xylosus* was isolated *via* tributyrin or olive oil emulsion and it's lipase purified from the same strain contained the activity 6300 U/mg at pH 8.5 and 55 ◦C. It showed the maximum overall uniqueness (98.74%) with *S. xylosus* lipase (SXL1) by N-terminal sequencing [45]. Some strains of Gram positive bacteria were modified to solvent loaded environment consecutively by increasing the concentration of solvents in the culture medium. Islam et al., 2009 [46] has made an effort to obtain solvent resistant strain by consecutively transferring the culture into medium containing increasing concentrations of ethanol. This stepwise alteration ultimately gave rise to cells that tolerated up to 8% (w/v) ethanol and also the enzyme we also produced by the same culture. Some lipases were noticed to be extremely stable in toxic organic solvents even at extremely high concentration. *P. aeruginosa* LST-03 lipase has been purified [47] which exhibited high activity in *n*-decane, *n*-octane, DMSO or N, N dimethylformamide (DMF) and feeble hydrolysis in chloroform, 1-hexanol, 1-pentanol, 1-heptanol, 1-decanol, 1-octanol, 1-butanol, and benzene. The reduced activity in *p*-xylene, methanol, toluene or ethanol is not due to denaturation since the half-life of the enzyme in these solvents was more than 7 days. For that reason, these organic solvents exclusively decrease the hydrolytic activity of the LST-03 lipase. In the last decade some organic solvent-tolerant lipases (esterases) have been isolated from a variety of microorganisms such as *Bacillus* spp., *Burkholderia cepacia* strains, *Pseudomonas* spp., thermophilic archaea, yeast and fungi. The prominent examples of organic solvent-tolerant bacteria are *B. thermoamylovorans* [9, 11], *B. pumilus* [12], *P. aeruginosa* LST-03, *Bacillus sphaericus* 205 [48] and *Staphylococcus saprophyticus* M36 [49].

There has been a great attention diverted towards the development of rules to envisage the possessions of various solvents on the biocatalyst [50]. A good quality association was found between the ester mole fraction at equilibrium and *log* P of the solvent (partition coefficient between *n*-octanol and water). The equilibrium constant for esterification correlates well with solubility of water in the organic solvents. The catalyst activity, measured as the initial rate of the esterification reaction, is best correlated as a function of *n*-octanol-water partition (*log* P) coefficient; electron pair acceptance index or the polarizability [51]. When *log* P < 2, deformation of water structure occurs; if 2 < *log* P < 4, the consequence of solvent is unpredictable and if *log* P > 4, water structure is intact. Even though the equilibrium point for lipase-catalysed esterification reactions is free of the enzyme, it is attractive to note that

it is not independent of solvent [52]. The hydrophobic solvents often yielded higher reaction rates than the hydrophilic ones even at a steady water activity; nevertheless, improved enantio-selectivity was observed [53]. Enzymatic methods of ester synthesis are more valuable when performed in non-aqueous media [54].

Enzymatic reactions in organic media are in reality separated into two systems; reactions performed in organic solvent systems and in solvent-free systems. The solvent-free system, *i.e.* the reaction mixture comprising simply liquid organic substrates without any organic solvent, if it is possible, has high volumetric performance and inexpensive advantages over the organic solvent system principally for large scale production. Conventional biocatalysis is carried out in aqueous media, and it is not surprising that most of the methods developed to study enzymes performance are water based. Restriction of the lipases to the aqueous phase effectively immobilises the lipases and allows its straight forward separation, using phase separators developed for the established chemical process industry, from product-containing organic phase. The main asset of these systems, however, is their ability to shift the thermodynamic equilibria of the reactions. Although lipases do not change the equilibrium constants (K_{eq}) of reactions but changes in the physical conditions (*i.e.* temperature, pH and pressure) do affect the K_{eq} of a reaction, usually this effect is relatively slight over the physical range allowed by stability of the biocatalysts. Use of a biphasic aqueous-organic system, however, may result in substantial changes in the practically useful 'apparent' K_{eq}. The use of lipases within organic solvents normally results in a two phase system as all water-soluble enzymes possess a significant amount of strongly bound water even when they exist in apparently dry state (**Figure 2**)**.** One phase is almost-anhydrous lipase suspended in the organic solvent. Lipase (L) is surrounded by a thin interphase consisting of water or water plus immobilisation support. The other phase is that in which enzyme dissolved in a reversed micellar medium. The micelles are formed by the surfactant molecules with assistance from the cosurfactant (*e.g.* butanol, hexanol and octanol) added to vary the properties of this interphase, is generally less polar and more soluble in the organic continuous phase. Both preparations provide optically transparent solutions.

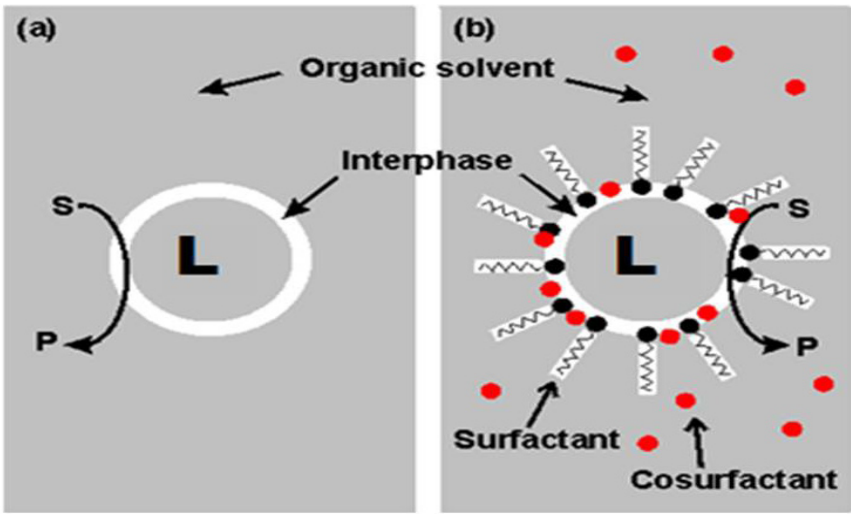

Figure 2 (a-b): Schematic diagram showing two configurations for an enzyme (Lipase) with in an organic solvent (a) anhydrous enzyme suspended in the organic solvent. (b) Enzyme dissolved in a reversed micellar medium.

5. Advantages of Biocatalysis by Microbial Lipases in Organic Solvents

Enzymatic reactions in organic solvents offer remarkable industrial advantages, such as increased solubility of non-polar substrates, reversal of the thermodynamic equilibrium of hydrolytic reactions, suppression of water-dependent side reactions, alternation of substrate specificity, enantioselectivity and abolition (**Figure 3**) of microbial contamination [3,55]. Excellent stability in polar solvents is also correlated with substrate inhibition in the synthesis of flavor esters where one of the reactants is usually methanol or ethanol. Beside bacterial lipases, the *Penicillium expansum* fungal lipase has received great attention from researchers in catalyzing biodiesel production from corn oil [56] and waste oil [57] in organic media. In a study, the catalytic activity of this enzyme in both ionic liquid and organic solvent systems has been explored in catalyzing the methanolysis of corn oil in the ionic liquids [58].

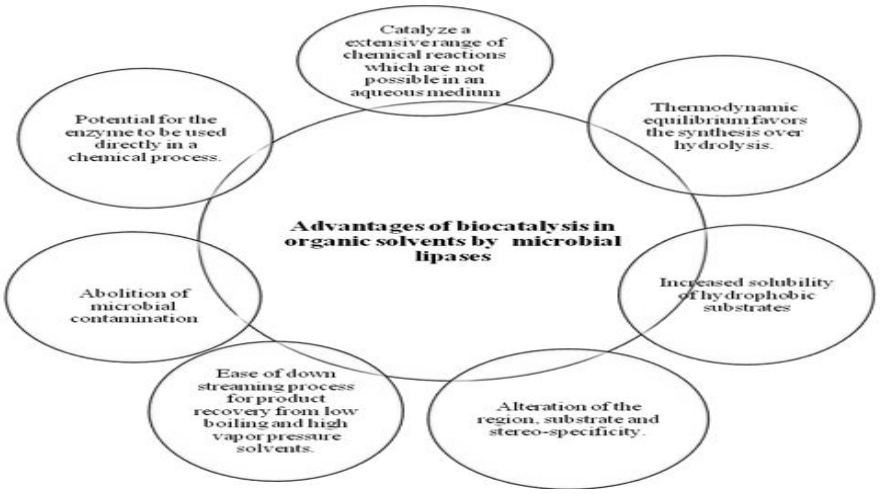

Figure 3: Advantages of biocatalysis by microbial lipases in organic solvents.

6. Loss of Lipase Activity in Organic Media

Numerous factors come into view to unfavorably affect the catalytic function of enzyme in organic solvents. Nevertheless, the circumstances are not discouraging as enormous bulk of techniques have been developed to conquer the poor enzyme activity in non-aqueous media. As far as the sensitivity of lipase towards different solvents is concerned, lipases are diverse in their sensitivity to solvents; there is a tendency for water-miscible solvents (polar) to cause more significant enzyme inactivation than water-immiscible solvents (non polar). In contrast, the enzymes from *Pseudomonas* sp. S5 [59], *B. sphaericus* 205y and *Arthrobacter nitroguajacolicus* Ru61 [60] were inactivated by the addition of a highly hydrophobic solvent such as hexadecane. This might be due to the comparatively high stickiness of the solvents, which slowed down well-organized communication between the enzymes and substrates [48]. On the other hand, note worthy enzyme activation by the addition of organic solvents was observed in the cases of lipases from *P. aeruginosa* LST-03, *Pseudomonas* sp. S5, *B. sphaericus* 205y and *Bacillus megaterium* [61]. The activation of lipase in the presence of some water-miscible organic solvents, such as 2-propanol can be explained probably by the disturbance of aggregates formed between the enzyme and lipids of the fermentation medium or between the

enzyme molecules themselves [62]. An extra work has been performed to gain a deeper insight into unfavorable consequence of organic solvents in enzyme inactivation (**Figure 4**).

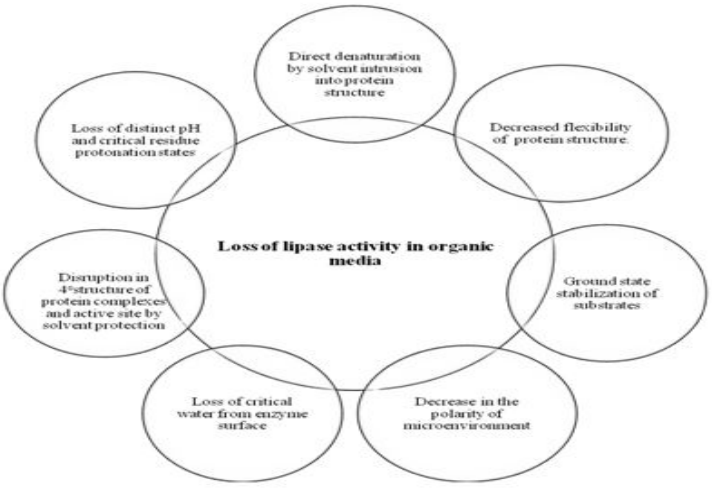

Figure 4: Loss of microbial lipase activity in organic media.

7. Applications of Microbial Thermo-Tolerant and Solvent-Tolerant Lipases

Lipases are very attractive class of enzymes amenable for biotechnology industries as they catalyse a variety of reactions mainly esterification, amidation and transesterification by accepting a wide range of acyl donors and nucleophiles besides their usual substrates. They also show high tolerance to diverse physico-chemical factors like broad range of pH and temperature and organic solvents. This group has the ability to meet diverse needs for a broad range of applications **(Figure 5)**, because it comprises enzymes of wide or rather narrow selectivity toward the type and the position of the fatty acids in the ester as well as enantioselectivity [63]. The establishment of enzyme technology represents an important revolution in the biotechnology industry with the global usage of enzymes being nearly U.S. $ 1.5 billion in year 2000 [13,64-65].

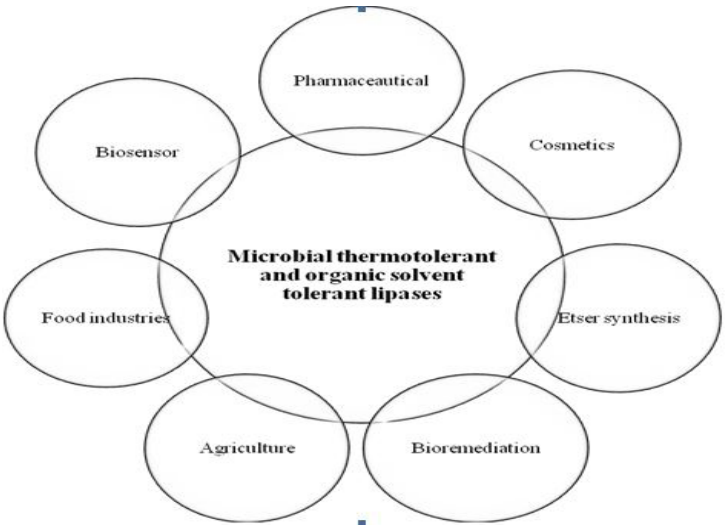

Figure 5: Diverse applications of microbial thermo-tolerant and solvent-tolerant lipases.

In modern times, lipases have appeared as key enzymes in incessantly growing biotechnology based-industries due to their properties such as activity over a wide temperature and pH range, diverse substrate range, substrate specificity and enantioselectivity. Their importance is increasing day by day in industries especially dealing in foods, detergents, chemicals, pharmaceuticals *etc* [13,66]. Lipases enantio-selective and regio-selective nature have been exploit for the resolution of chiral drugs, biofuels, fat modification [3,12], syntheses of personal-care products and flavour enhancers [55]. Although lipases have been found to exist in many species of animals, plants, bacteria, yeast and fungi, yet the enzymes from micro-organisms are most fascinating as their possible uses in diverse industries such as food, diary and pharmaceuticals, detergents textile, improved bio-diesel synthesis, cosmetic industry, synthesis of fine chemicals, agrochemical and new polymeric materials [7-12,67]. Over the last couple of years, there has an been increasing surge in the use of enzymes for the biosynthesis of molecules in organic media [9,13,65,68]. In the presence of trace of water, the lipase reverses the reaction and leads to esterification and formation of glycerides from the fatty acids and glycerol [69-70]. A significant practical example is the use of lipases and esterases to catalyze esterification reactions in organic solvents such as isopropyl acetate, ethyl ferulate, isopropyl ferulate and butyl ferulate [71-72].

Lipases have the distinctive feature to act at an interface between aqueous and non-aqueous (*i.e*. organic) phase and this feature differentiates them from esterases. In organic solvents lipase catalyzes the reverse reaction of synthesis or transesterification and show high broad range of temperature, pH and tolerance to different organic solvents. Thermophilic lipases attracted more attention in pharmaceuticals as well as biotechnology industry as they remain unaffected to thermal and chemical denaturation [10-11]. Foremost lipase producing organisms are *B. prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which represent today's best studied lipase producing bacteria now named *Serratia marcescens*, *P. aeruginosa* and *P. fluorescens*, respectively [3,13,16,55]. Esterification, transesterification, alcoholysis and other hydrolytic reactions occur in mild conditions. The lipase-catalyzed reactions are usually very enantio- and region-selective and are thus highly appreciated in fine chemicals synthesis. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified and generally used at commercial scale [10-12,73]. The distinguishable catalytic features of lipases have enabled significant applications in processes generally considered as pollution-prone. Lipases can recognize a broad range of organic substrates and they work excellently in organic solvents, too.

8. Conclusion

Lipases are amongst the most startling class of biocatalysts that carry out novel catalytic reactions in both aqueous and non-aqueous media. Lipases have the extraordinary ability to carry out an extensive variety of chemo-, regio- and enantio-selective transformations.

Nowadays, lipases find enormous applications in diverse areas of industrial microbiology and biotechnology. Rational design of lipases remains largely unexplored but such an approach has the potential for enhancing the enzyme thermostability, improving its solvent tolerance, increasing its specificity and heightening its activity. The unique interfacial activation of lipases has always fascinated enzymologists and the biophysicists. On other hand, the crystallographers have made advancement in understanding the structure–function relationships of these enzymes. However, comprehensive understanding of the lipase molecule requires greater contribution of research effort(s). Utilizing lipases will greatly boost many biotechnology-based industries in India as well as in other countries. Hence, industrial applications of thermotolerant as well as organic solvent tolerant microbial lipases will continue to provide an imperative function in maintaining and enhancing the eminence of life we enjoy today while protecting the environment for generations to come.

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