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Thermostable Lipases: an Overview of Production, Purification and Characterization

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Thermostable lipases occupy a prominent position in aqueous and non-aqueous biocatalysis. Isolation of wild strains with novel properties has been on-going and recombinant strains are being constructed so as to meet the biotechnological applications of lipases. Different purification methods prove effective and the purity of the enzyme is dependent on the intended applications. Unlike medical and pharmaceutical applications where highly pure preparations are needed, partial purification can be sufficient for general applications. Thus, characterization associated with enzyme activity, specificity, thermostability, enatioselectivity and tolerance to various solvent systems are the pre-requisite for thermostable lipase selection which make them highly demanding in lipase catalyzed reactions. This article intends to give an insight on thermostable lipases so as to stimulate researchers to explore other unique properties from wild and recombinant strains that could be of benefit for wider industrial applications.

Key words: Thermostable lipases, purification, application, characterization

Lipases (triacylglycerol ester hydrolases (EC 3.1.1.3) have the ability to catalyze several reactions of biochemical importance including hydrolysis of triglycerides, esterification, transesterification, interesterification, acidolysis, aminolysis, alcoholysis, acylation and resolution of racemates[1,2].

Lipases that are thermostable have attracted attention to be utilized in production of biofuels, organic synthetic compounds, detergents, perfumes, cosmetics, leather, enantiopure pharmaceuticals, medical diagnostics, foods and feeds[3,4].

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Thus, lipase specificity and stability under different conditions are the key for its selection for a particular application. Microbial sources from fungi, bacteria and archaea produce lipases with unique properties that can be used for biotechnological applications. As such microbial lipases cannot be comparable with plant and animal lipases in terms of activity, yield, ease of purification and molecular modifications, continuous production which is independent of season and stability[5]. Lipase producing microorganisms isolated from thermal environments produce thermostable lipases with other unique properties including solvent tolerance and resistance to chemical inactivation[6].

Some of the thermostable lipases reported in the literature include *Bacillus* sp. RSJ-1[7], *Bacillus thermoleovorans* ID-1[8], *Burkholderia*

ambifaria YCJ01[9], *Aneurinibacillus thermoaerophilus* strain HZ[4], *Penicillium simplicissimum*[2], among others. However, most wild microbial strains produce lipases that hardly meet the required characteristics; as such molecular techniques that can alter the pre-existing genes prove effective in developing new enzyme systems with desired properties[10]. Several reports on cloning of lipase gene into a suitable host results in high level of expression of the lipases; using RT-PCR and RACE amplification, *Thermomyces lanuginosus* cDNA was cloned in *E. coli* DH5± and expressed in *Pichia pastoris*. The recombinant lipase showed high alkali tolerance with excellent thermostability[11]. The lipase gene of *Candida thermophila* SRY-09 was cloned and expressed in *Saccharomyces cerevisiae* and *Pichia pastoris* respectively. The recombinant lipase was stable with ability to catalyze various reactions at 55°C[12]. Vieille and Zeikus[13] showed that thermostable lipases showed high rate of catalysis, which is related to high diffusion coefficient associated with low viscosity of the substrates. Thus, the type and the number of amino acid residues participating in secondary interactions, hydrogen bond formation and hydrophobicity profile contribute significantly in enhancing the thermal stability of most enzymes.

This article presents an overview of thermostable lipases based on the fact that industrial applications where lipases are required occur mostly at elevated temperatures. Thus, wild strains with this novel property are always isolated and produced; while genetically engineered organisms for producing lipases with unique properties are being constructed. Purification and characterization aid in proper placement of the enzyme in terms of its biotechnological applications.

Thermostable lipases

Thermostable lipases are needed in several biocatalytic reactions and some of the advantages of catalysis at higher temperatures include high rate of product formation with minimal diffusional restriction, high dissolution of hydrophobic substrates, high conversion efficiency, increased kinetic energy of reactants and limited chance of contamination[14,15]. Additionally, noncovalent interactions result in the formation of salt-bridge which contributes in stabilization of enzymes at elevated temperatures by entropically restricting the flexibility of enzyme active site[16]. Thus, activity and specificity tend to be temperature dependent as seen in most lipases.

Naturally occurring lipases

Different microorganisms living in hot springs, soils, waste dumpsites, industrial effluents and other extreme environments have been found to produce thermostable lipases. The environments where organisms are isolated or produced confer some characteristics to the enzymes. The search is always on for thermostable lipases with unique properties through isolation and production.

Groundnut rhizosphere was used for isolating a thermostable lipolytic strain. *Staphylococcus aureus* produces a novel lipase with good characteristics including thermostability and alkaline tolerance[17]. Soils from El Hamma thermal station in Tunisia were collected by Romdhane *et al.*[18] for isolation of thermostable and lipase producing microorganisms. *Talaromyces thermophilus* produces lipase which has an optimum temperature of 50°C and was found to be thermostable at 55°C and 60°C retaining more than 90% and 60% of its activity for 1 hr.

Burkholderia cepacia S31 isolated from lipid containing soils produce thermostable lipase, whose optimum temperature was found to be 70°C. Thermostability studies revealed that the lipase retained more than 80% of its activity after 1 hr incubation at 70 °C. While at 50°C and 55°C, more than 96% and 85% of enzyme activity retention were respectively recorded after 12 hr of incubation[19].

Bradoo *et al.*[20] carried out extensive isolation of thermostable and lipolytic microorganisms using compost heaps, soils and oil industry effluents in India. The identified strains were *Bacillus stearothermophilus* SB-1 and *Bacillus licheniformis* SB-3 whose lipase activities were optimum at 50°C. Stability studies at 100°C indicated the half lives of lipase from *B. stearothermophilus* SB-1 as 15 min and 25 min at pH 6 and pH 3 respectively. Similarly, *B. thermoleovorans* CCR11 lipase showed optimum activity at 60°C with 100% stability when incubated for 1 hr at <50°C but at 50–60°C under the same condition 75% activity was retained[21].

Thermophilic *Bacillus* sp. was found to

produce lipase whose optimum activity was found at 60–65°C, with full retention of its activity within this range after incubating for 1 hr. The half life of this enzyme was found to be 170 hr at 60°C, pH 8; but when the temperature was increased to 70°C, the half life drastically reduced to 45 min[22]. Also, purified lipase from *Amycolatopsis mediterranei* DSM 43304 was stable between 50 – 60°C, retaining its full activity after 3 hr of incubation[23].

Two thermostable lipases (Lip A and LipB) from *Thermosyntropha lipolytica* DSM 11003 have optimum temperature of 96°C. The enzymes were found to have excellent stability at alkaline pH retaining 50% activity after incubating for 6 hr (LipA) and 2 hr (LipB) at 100°C. Thus, assessing a 24 hr half life of the enzymes, temperatures of 74.1°C (LipA) and 76.5°C (LipB) were recorded[24].

The optimum temperature of lipase obtained from thermophilic *Bacillus* strain THL027 isolated from oil-contaminated soil was 70°C. When the enzyme stability study was carried out between 60 – 75°C, deactivation velocity of < 2 U h^{1} was recorded, which indicates more than 80% retention of enzyme activity after 1 hr incubation within the selected temperature range[25].

Production of thermostable lipase from *B. stearothermophilus* MC 7 was achieved in a medium containing 0.5% soybean flour and 0.1% olive oil at 55°C, air flow rate of 0.5 vvm, impeller speed of 300 rpm using the initial pH of 8.5 and inoculum concentration of 1% in bioreactor. The produced lipase showed optimum activity at 75–80°C. The crude lipase was more stable with a half life of 3 hr at 70°C, while the purified lipase by gel filtration and ion exchange chromatographic method had a half life of 30 min at the same temperature[26]. In another development, lipase from *Rhizopus homothallicus* was found to have different properties in terms of thermostability when produced using submerged fermentation (SmF) or solid state fermentation (SSF). The half lives of ~26 min and ~43 min at 50°C were recorded for lipases from SmF and SSF respectively. Likewise the temperature optimum was higher in lipases produced via SSF by 10 units[27].

Other lipases obtained from different organisms that are thermostable are presented in

Table 1. Properties of some naturally occurring thermostable lipases

Source	T_{max} (°C)	pH optimum	Stability	Reference
Bacillus sp. RSJ-1	50°C	8 – 9	$t_{1/2}$ = 150, 90, 55 and 45 min at 60°C, 65°C, 70°C and 75°C, respectively	7
Pseudomonas sp	90°C	11	$t_{1/2}$ = 13 hr at 90°C	28
Bacillus thermoleovorans ID-1	70–75°C	7.5	$t_{1/2}$ = 30 min at 70°C and 1 hr at 60°C	8
Bacillus strain A30-1	60°C	6 – 9	$t_{1/2}$ = 8 hr at 75°C	29
Aneurinibacillus thermoaerophilus strain HZ	65°C	7	$t_{1/2}$ = 3 hr 10 min and 1 hr 20 min at 65°C and 70°C, respectively	4
Burkholderia multivorans V2	45° – 50°C	8	$t_{1/2}$ = 50 and 10 min at 50°C and 60°C, respectively	30
Burkholderia sp.	60°C	8.5	$t_{1/2}$ = 2 and 0.5 hr at 50°C and 60°C, respectively	31
B. subtilis NS 8	60°C	7	$t_{1/2}$ = 4 hr 33 min at 60°C, 51 min at 70°C and ~42 min at 80°C	32
B. coagulans MTCC-6375	45°C	8.5	$t_{1/2}$ = 20 min at 55°C.	33
Geobacillus thermodenitrificans IBRL-nra	65°C	7	$t_{1/2}$ = 8 hr at 60°C, 16 hr at 65°C – 75°C	34

Table 1. Thus, demand for thermostable lipases has motivated researchers to explore different samples for isolation and to develop medium for production of lipases with desirable properties. This is based on the fact that most industrial applications require lipases that are thermostable in addition to other characteristics.

Recombinant lipases

The key features required in lipases for industrial usage include thermostability, reaction specificity and properties associated with organic solvent tolerance; most of these features are often limited in lipase producing wild strains.

Cloning and expression of several thermostable lipase genes into appropriate vectors and expression systems have been achieved. Protein engineering techniques associated with mutagenesis and directed evolution prove effective in enhancing the properties of enzymes[35,36]. Table 2 shows some of the recombinant thermostable lipases reported in the literature.

Fragmentation of LipA and LipB genes was achieved using different restriction enzymes and the fragments were cloned into pBBR1Tp vector; this was then followed by multistep parental conjugation of pBBR-*lip*AB into *B. cepacia* strain G63 aided by pRK2013. Large scale production was achieved using 10 L capacity bioreactor and the recombinant *B. cepacia* G63 (pBBR-*lip*AB) lipase following its purification to homogeneity showed an optimum temperature of 70°C, with good stability retaining more than 96.2% and 86.1% of its activity after 2 hr and 10 hr of incubations respectively[44].

Cloning and expression of lipase gene from from hot mineral spring isolate identified as *B. subtilis* DR8806 were achieved in pET-28a(+) expression system and *E. coli* BL21 respectively. The recombinant lipase had an optimum temperature of 70°C with ability to retain 90% and 87.5% of its activity after 1 hr incubation at 75°C and 80°C respectively. The enzyme was found to

Table 2. Some of the recombinant thermostable lipases

Source	T_{max} (°C)	pH optimum	Stability	Reference
B. stearothermophilus P1	55°C	8.5	$t_{1/2}$ = 7.6 hr and 2 hr at 55°C and 65°C respectively	37
B. thermoleovorans ID-1	75°C	7-8	$t_{1/2}$ = 1 hr at 60°C	38
Bacillus sp. strain 42	70°C	8	$t_{1/2}$ = 5 hr 15 min at 60°C, 2 hr 5 min at 65°C and 45 min at 70°C.	39
Geobacillus sp. strain ARM	65°C	8	$t_{1/2}$ = 2 hr 30 min (pH 6 -7), 2 hr 10 min (pH 8), 1 hr 50 min (pH 9) at 50°C	40
Lip M 1 (Excellent homology to *Geobacillus stearothermophilus*)	40°C	9	$t_{1/2}$ = 12 hr at 60°C, 1 hr 15 min at 65°C, 45 min at 70°C	10
Bacillus sp. strain L2	70°C	8	$t_{1/2}$ = 0.5 hr at 70°C, 1.77 hr at 65°C, 3.45 hr at 60°C	41
Geobacillus sp. T1	70°C	9	$t_{1/2}$ = 12 hr at 60°C, 5 hr 15 min at 60°C, 1 hr 10 min at 70°C.	42
LipS and LipT (Metagenomic derived lipases)	70°C (LipS), 75°C (LipT)	8	$t_{1/2}$ = 48 hr at 70°C (LipS), 3 hr at 70°C (LipT)	43

be stable with half life at 70°C and 80°C of 2.42 hr and 1.53 hr respectively[45]. The lipase encoding gene of *Geobacillus* sp. SBS-4S was cloned and expressed in *E. coli* BL21 (DE3). The optimum temperature of the recombinant lipase was found to be 60°C with ability to retain more than 95% of its activity after incubating for 80 min at 50°C. However, the enzyme half life at the optimum temperature was only 20 min[35]. Also, recombinant *G. thermoleovorans* YN lipase was reported by Soliman *et al*.[46] to be highly active at 60 – 65°C, with 30 min stability when incubated at 70°C and the activity began to decrease slowly beyond this time. Rapid decrease in activity was observed at temperatures ≥ 75°C. In case of recombinant lipase from *G. thermoleovorans* Toshki constructed by Abdel-Fattah and Gaballa[47], 65°C was the optimum temperature with ability to retain its full activity between 40°C – 60°C. Following incubation for 1 hr at 70°C, 80°C and 100°C, the recombinant lipase showed 80%, 60% and 30% residual activity which indicated its good thermostability.

The effect of temperature on recombinant thermostable lipase from *Fervidobacterium nodosum* Rt17-B1 was studied, and highest activity was found at 70°C. The stability of the enzyme was found to be excellent at temperatures lower than optimum as the activity remained unaffected even after 50 hr of incubation at 60°C. Its half life at optimum temperature was recorded to be 8 hr and rapid decrease in activity was observed at 75°C where the enzyme lost about 70% of its activity after 10 min incubation[6].

Error prone mutagenesis which causes one to two mutations per 1000 bases was reported by Kumar *et al*.[36] in order to improve the catalytic efficiency and thermostability. The developed mutagenic library was used for selection of thermostable lipase gene which was cloned into pQE-30UA expression vector and transformed in *E. coli* M15 cells (LipR3). The thermostability of LipR3 was compared with that of wild strain (LipR1). The optimum temperature of LipR3 was found to be lower than that of LipR1 but in terms of thermostability, at 55°C and 60°C LipR3 was found to have about 17 and 90 folds higher half lives respectively compared to LipR1. Complete loss of activity was observed in LipR1 at 65°C and 75°C; while LipR3 showed 32 min and 15 min half lives respectively. In addition to these, organic solvent tolerance was further enhanced in LipR3. Sequence analysis and homology modelling showed that a single mutation of Arg with Cys at 214 position resulted in stable hydrophobic and hydrogen bond interaction of loop with side chains from lid helix and helix 7 which impart LipR3 with additional characteristics in terms of thermostability and organic solvent tolerance.

Similarly, thermostable Lip M1 mutant generated from mutagenic library showed that substitution of Asn with Lys in the mutant lipase clone led to hydrogen bond formation between Lys355 and Glu284 which causes loop modification, making the Lip M1 to have excellent thermostability. Thus, about 144 fold higher stability was observed at 60°C in Lip M1 as compared to wild type[10].

Site-directed mutagenesis was used in producing mutants (D311E and K344R) by introducing ion pair interaction between and within the loop, so as to study the thermostability of T1 lipase obtained from *Geobacillus zalihae*. Mutant D311E was found to have higher stability than K344R and T1 wild lipase with a temperature optimum of 70°C. The half life of D311E mutant lipase was found to be 110 min and 12 hr at 60°C and 70°C, respectively compared to 30 min and 10 hr for T1 wild strain under the same conditions[48]. Thus, the change of Asp with Glu residue at 311 position resulted in additional non covalent interactions associated with ion pair and hydrogen bond formation.

Purification of Lipases

In order to study the nature and to improve the specificity, stability and tolerance as well as specific activity of lipases, the enzyme has to undergo a series of different purification methods. The distinct industrial applications of lipases make purification process necessary as it aids in resolving its amino sequence as well as its three dimensional structure. Some of the methods used by different researchers in purifying thermostable lipases to homogeneity have been reviewed here and Table 3 further shows the summary of the steps as well as yields of different purification procedures.

Mathew and Juang[52] grouped the purification of enzymes into three; which include low purity with high yield (cell lysis, precipitation and extraction procedures, ultrafiltration); high purity with low yield (ultracentrifugation,

Table 3. Summary of the steps and yields of different thermostable lipase purification procedures

Source	Purification steps	Purification fold	Yield (%)	Molecular mass (kDa)	Reference
B. coagulans BTS-3	Ammonium sulphate precipitation and DEAE–Sepharose column chromatography	40	2.5	31	49
Haloarcula sp. G41	Ammonium sulphate precipitation, DEAE-cellulose ion-exchange chromatography and Sephacryl S-100 gel filtration chromatography	7.7	18.6	45	50
B. stearothermophilus P1	Ultrafiltration (10 kDa cut off) and Q HyperD column anion-exchange chromatography	18	71	43	37
Bacillus sp	Hydrophobic interaction (Phenyl Sepharose), gel filtration and *Q-sepharose* ion exchange chromatography	4	0.38	60	22
Thermosyntropha lipolytica (Isoforms)	Ammonium sulphate precipitation and Octyl Sepharose hydrophobic interaction chromatography	140 (LipA) 134 (LipB)	23 13.4	50 57	24
Rhizopus oryzae	Ammonium sulphate precipitation, DEAE-Sephacel ion-exchange chromatography and Sephacryl S-200 gel filtration chromatography	1260	22	32	51
Amycolatopsis mediterranei DSM 43304	Ammonium sulphate, Q Sepharose anion exchange chromatography and Toyopearl Phenyl-650M hydrophobic interaction chromatography	398	36	33	23
B. subtilis NS 8	Ultrafiltration, DEAE-Toyopearl 650M 650M hydrophobic interaction chromatography and Sephadex G-75 gel filtration	500	16	45	32
B.stearothermophilus MC 7	Ultrafiltration, Sephadex G-200 gel filtration and DEAE-cellulose ion exchange chromatography	19.25	10.2	62.5	26
Rhizopus homothallicus	Butyl-Sepharose hydrophobic interaction chromatography and Superdex G-200 gel filtration chromatography	77	15	29.5	27
B. coagulans	DEAE-cellulose ion	76.4	7.3	103	33

MTCC-6375	exchange chromatography and Octyl-Sepharose hydrophobic interaction chromatography				
Geobacillus sp. T1	Two affinity chromatography (glutathione-Sepharose HP column, HiTrap Glutathione-Sepharose 4FF)	4.6	51.5	43	42
G. zalihae Strain T1 (mutant)	Glutathione Sepharose 4 FF affinity chromatography and sets of affinity columns packed with Glutathione Sepharose 4 FF, GSTrap and Hi-Trap Benzamidine attached in series	10.56	15.71	66	48

electrophoresis, affinity separation techniques) and finally high purity with high yield techniques associated with membrane based processes and fluidized bed technology.

Several purification strategies are available in the literature. Saxena *et al*.[53] showed that the major drawbacks of conventional purification methods are associated with low yields and time intensiveness, as such industries prefer purification processes that are less capital intensive, high yielding, rapid and robust with easy upscaling procedure. Based on this, moderate level of lipase purity can be sufficient for lipase utilization as industrial applications can be carried out with less homogenous preparations.

Joshi and Khare[54] reported that lipase obtained from *P. aeruginosa* can be purified to homogeneity by using ultrafiltration as a concentration and a prepurification step followed by gel filtration technique using Sephadex G-100. Interestingly, these two steps resulted in purifying the lipase as single band with 60 kDa was obtained after SDS-PAGE. The yield and purification fold were found to be 48% and 33.5 indicating the effectiveness of the steps in achieving the purity of the enzyme. Similarly, Peng *et al*.[55] showed that ultrafiltration and cold acetone saturation as prepurification steps followed by ion exchange chromatography by DEAE–Sephadex A50 led to 25.5 purification fold with 45.5% recovery of *P. aeruginosa* CS-2 lipase. SDS-PAGE and gel filtration revealed a molecular mass of 33.9 kDa and 36 kDa respectively.

However, low purification fold of 7.1% and recovery of 10.1% were obtained following ammonium sulphate precipitation and DEAE-cellulose column chromatography of thermostable lipase from *B. pumilus*. The purified lipase showed high specific activity of 5,173 U/mg with molecular weight of 55 kDa. The amino acid composition of *B. pumilus* lipase deduced by HPLC indicated the presence of 53.5 % and 45.9 % polar and non-polar amino acid residues respectively[56].

Hydrophobic interaction chromatography (HIC) using phenyl sepharose CL-4B column was first applied for purification of alkali tolerant and thermostable lipase from *S. aureus* followed by Superose-12 gel filtration chromatography. The lipase was found to have a molecular weight of 49 kDa based on purification fold of 6.76 with a yield of 20%[17]. Also, *Acinetobacter* sp. EH28 lipase was partially purified by ammonium sulphate precipitation and HIC with 47% recovery (24.2 purification fold) and specific activity of 57.1 U/mg[57].

Sharma *et al*.[7] reported multistep purification of thermophilic *Bacillus* sp. RSJ-1 lipase to homogeneity by ultrafiltration, ammonium sulphate precipitation, Q-Sepharose ion exchange and Sephacryl S-200 SF gel permeation. The lipase obtained using these protocols was purified 201-fold with a yield of 19.7% and 37 kDa as molecular mass deduced by SDS-PAGE. Two purification steps were reported to be sufficient for lipase from *A. thermoaerophilus*. The purified lipase by Q-Sepharose ion exchange chromatography and Sephadex-G75 gel filtration has specific activity of 43.5 U/mg with 19.7% and 15.6 recovery and purification fold respectively. The apparent molecular mass of this thermostable and solvent tolerant lipase was found to be 50 kDa[4].

Ultrafiltered lipase from *G.*

thermodenitrificans IBRL-nra was further purified using HiTrap Heparin affinity column and Sephadex G-100. This resulted in complete purification of the enzyme to homogeneity with purification fold of 34 and recovery of 9% with apparent molecular mass of 30 kDa[34]. However, Sifour *et al*.[58] reported a lower purification fold of 22.6 with 8.8% yield but higher molecular weight of 61 kDa when lipase from *G. stearothermophilus* was purified by ultrafiltration, Q-Sepharose ion exchange, Sephadex G-100, and adsorption on hydroxyapatite.

His-tagged recombinant lipase from *B. subtilis* DR8806 was purified to homogeneity using one step Ni-NTA affinity chromatography. The purified lipase showed an apparent molecular weight of 16.8 kDa with specific activity of 1364 U/mg. This method resulted in 57% recovery with 3 fold purity[45]. In case of recombinant lipase from *Geobacillus* sp. strain ARM, Ebrahimpour *et al*.[40] showed that immobilized metal affinity chromatography (IMAC) containing ProBond™ Nickel-Chelating Resin was used for purifying this His-tagged recombinant lipase with 63.2% and 14.6 yield and purification fold respectively. High specific activity of 7092 U/mg was obtained with a molecular weight of 44 kDa. Also, a homogenous L2 lipase of molecular mass 45 kDa deduced by SDS–PAGE was obtained following its purification by IMAC. The recombinant L2 lipase had specific activity of 458.1 U/mg with recovery and purification fold of 63.2% and 1.8 respectively[41].

Reverse micellar extraction (RME) technique showed potential to be used as a single extraction process for enzyme purification. This involves the use surfactants dissolved in isooctane followed by interaction with suitable stripping solution containing NaCl and isopropanol. Lipase from *Pseudomonas* sp. CSD3 was subjected to this technology and the results obtained were compared with conventional purification procedure involving ammonium sulphate precipitation and gel filtration. RME method led to 80% yield of the enzyme with 15-fold purification compared with 52% yield obtained by conventional procedure. Amazingly, the time required for RME process was lower by 40 – 50 times as only 45 min was found to be sufficient for the whole process against 30 – 40 hr in the conventional method[59].

Purification and separation of various proteins using membrane chromatography proved effective where specific ligands are embedded on the membrane surface which bind and interact with the protein of interest during its flow through the membrane. The reversible interaction of the protein of interest with the ligands facilitates the purification process by affinity chromatographic techniques[60]. Also, ultrafiltration as the commonly used method for protein separation and purification has been electrified to improve its efficiency; this was tested on five different enzymes (two each from amylase and protease and one lipase which were obtained from Novozymes). About 3 – 7 fold increment in flux was observed compared to the conventional method, suggesting the practicability of applying an electric field across the membrane for enhanced purification; although more pronounced effect was seen in amylases compared to proteases and lipase[61].

Generally, the target of most purification methods is to obtain an enzyme solution free from any impurities that can interfere with its catalytic functions. Depending on the industrial applications, partial purification may be sufficient in processes associated with feed formulation, waste water treatment, pulp and paper processing, biodiesel, among others. While highly purified lipases are needed in resolution of racemates as well as medical and pharmaceutical applications.

Characterization of Lipases

Enzymes that are of biotechnological importance are characterized so as to determine their suitability in various applications. This can be carried out using crude and purified enzymes. In case of thermostable lipases, factors associated with their stability, activity and specificity are determined; these include temperature and pH optimum and stability, solvent tolerance (organic solvents), effects of divalent cations, surfactants or detergents, protease inhibitors, kinetic properties (such as K_m, V_{max}, K_{cat}), etc.

In order to determine the effectiveness of using thermostable lipase from *B. pumilus* for waste water treatment. Saranya *et al*.[56] determined the properties of the produced enzyme based on the heterogeneity of the intended application. The lipase was found to be inhibited by most cations (K^+, Cu^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}) with the exception of Ca^{2+} which not only stabilize but stimulated the enzyme activity by 121%. Thus, Calcium ion was

thought to have inductive effect on lipases which was attributed to a form of interaction associated with active site and regulatory site resulting in stabilization of tertiary structure of lipases[62]. Also, EDTA was found to be stimulatory (108%), and non-ionic surfactants of Tween series markedly decreased the enzyme activity while Triton-X at 0.1% concentration increased the lipase activity by 121%. Among all the organic solvent tested, DMSO was the only one with stimulatory effect. Kinetic parameters of *B. pumilus* lipase in the presence of palm oil revealed a V_{max} of 111.11 mM/min and K_m of 152.32 mM, while changing the substrate to olive oil resulted in V_{max} and K_m of 35.34 mM/min and 312.98 mM, respectively[56].

The purified lipase from *B. cepacia* S31 was activated by Ca^{2+}, Mn^{2+}, K^+, Na^+ and Mg^{2+}. Complete inhibition of enzyme activity in the presence of EDTA indicated that the enzyme is a metalloprotein. The lipase showed non-positional specificity in hydrolyzing ester bonds of triglycerides with good stability in the presence of organic solvents including methanol, glycerol, n-hexane, n-butanol, toluene and ethyl acetate. This showed the potential of the produced lipase in non-aqueous biocatalysis especially in biodiesel production because of its high tolerance to methanol and glycerol which often affect the lipase catalyzed transesterification reactions[19]. Similarly, lipase from *B. coagulans* MTCC-6375 reported by Kanwar *et al*.[33] was stimulated by divalent cations including Mg^{2+}, Cu^{2+}, Ca^{2+}, Hg^{2+}, Al^{3+}, and Fe^{3+} at 1mM; and inhibitory effect was seen in Zn^{2+} and Co^{2+} ions. Inhibition of the enzyme by EDTA as a metal chelator was restored by 10 mM of Hg^{2+} or Al^{3+}. Kinetic parameters showed the specificity of *B. coagulans* lipase to *p*-nitrophenyl esters of caprylate (pNPC) and palmitate (pNPP). K_m of 28 mM and V_{max} of 0.44 mM $mg^{1}min^{1}$ were obtained for hydrolysis of pNPP while 32 mM and 0.7 mM $mg^{1}min^{1}$ were found to be K_m and V_{max} for hydrolysis of pNPC, respectively.

Lipase from *Euphorbia peplus* L. purified 12.57-fold by ammonium sulphate precipitation and anion exchange chromatography using DEAE-Cellulose column showed pH and temperature optima of 8 and 40°C with good stability at 50°C; specific activities of 249 ± 12.45 and 161.4 ± 8.07 U/mg were obtained in the presence of tributyrin and olive oil respectively. Decreased in enzyme activity was observed after 10 min incubation in the presence of divalent cations (Fe^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+}) while Triton X-100 and Tween-80 increased the enzyme activity after 30 min incubation. In case of organic solvents at higher concentration of about 90%, 100 % retention of lipase activity was observed for hexane, 77 ± 3.85 % for acetone, 61 ± 3.05 % for butanol and 53 ± 2.65 % for methanol following 1 hr incubation. This stability under various conditions indicated the potential of *E. peplus* lipase for several industrial applications[63]. *Haloarcula* sp. produced lipase which showed optimum activity and stability at 70°C, pH 8.0, and 15 % NaCl with excellent stability over wide ranges of temperature (30–80 °C), pH (6.0–11.0) and NaCl concentration (10–25%). Complete inhibition of enzyme activity was observed in the presence of EDTA (metal chelator), serine protease inhibitor (PMSF) and PAO (cysteine protease inhibitor) which indicated the metal ion, serine and cysteine residues dependence of *Haloarcula* sp. lipase for its activity respectively. Organic solvents with log $P \leq 2$ not only stabilized but enhanced the lipase activity[50].

Recombinant thermostable lipase from *B. subtilis* DR8806 was characterized following its one-step purification by Emtenani *et al*.[45]. Several divalent cations tested at various concentration showed that Hg^{2+} and Cu^{2+} have strong inhibitory effects which could be associated with inhibition of enzyme active site. Minimal reduction in enzyme activity was observed in the presence of Pb^{2+}, Zn^{2+} and Ba^{2+} ions. The lipase also exhibited good stability toward various oxidizing agents (ammonium persulfate and potassium iodide), reducing agents (ascorbic acid and ^{2}-mercaptoethanol), surfactants (CTAB, Triton-X and SDS), inhibitors (urea, DMF, DTT, DNTB, PMSF) as well as chelating agents. In case of organic solvents, the recombinant lipase had excellent stability in various organic solvents at 10 – 20% concentrations including isoamyl alcohol, methanol, ethanol, n-hexane, n-heptane, chloroform, butanol and diethyl alcohol. This tolerance to organic solvents motivated the Emtenani *et al*.[45] for studying its compatibility with ionic liquids (ILs). Among the ILs considered, 1-Butyl-3-methylimidazolium chloride ([BMIM][Cl]) showed the highest stimulatory effect. This compatibility is of great interest especially in non-

aqueous biocatalysis as ILs have been found useful as pure solvents, as co-solvents in aqueous systems and in biphasic systems associated with different catalytic reactions[64].

Tayyab *et al*.[35] showed that recombinat lipase from *Geobacillus* sp. SBS-4S showed 2.5 fold increments in enzyme activity at 1 mM Ca^{2+} ion. Highest enzyme specific activity of 2034 Umg ^{1} was obtained at 10 mM Ca^{2+}. Other divalent cations with no inhibitory effects include Mn^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+} and to a lesser extent Co^{2+}. Complete inhibition of lipase activity was found in the presence of EDTA which indicated the metal ion dependence of the enzyme. Inhibitors specifically serine proteases at 1 mM had no effect on the enzyme activity. Kinetic parameters using p-nitrophenyl acetate as a substrate at 0 to 15 mM by double reciprocal plot resulted in K_m and V_{max} values of 3.8 mM and 2273 ¼mol min"^{1} mg"^{1} respectively. In addition, the recombinat lipase exhibited high hydrolytic potential on various oils of medium to long chain fatty acids.

The purified *F. nodosum* Rt17-B1 lipase with a yield of 52.5% following ion exchange chromatographic procedure was activated by 9 – 56% in the presence of hydrophilic organic solvents such as DMSO, propanol, acetone and DMF. Metal ions enhanced the activity of the enzyme by 6 – 24% and the notable ones include Mg^{2+}, Ca^{2+}, Na^{+}, Ni^{2+} and Co^{2+}; however increasing the concentration of the metal ions to 10 mM showed no inhibitory effect except in Ni^{2+} where the residual activity decreased to 76%. EDTA (a metal chelator) up to 10 mM did not cause any inhibitory effect indicating that metal ions are not essential for its overall activity. The purified lipase was found to be serine hydrolase based on the significant inhibition observed in the presence of PMSF. Substrate specificity studies showed the preference of the enzyme towards C_8–C_{12} fatty acids and the best substrate was found to be C_{10} fatty acid with 180-fold higher catalytic efficiency than C_2[6].

Sabri *et al*.[41] carried out extensive studies involving the cloning of the gene for thermostable lipase from *Bacillus* sp. L2 and its expression in *P. pastoris*. The recombinant lipase showed high dependence on Ca^{2+} with significant increase in activity from 300–500% at 1 – 10 mM. High enzyme activity in the presence of Ca^{2+} was suggested to be as a result of precipitation of Ca-salts associated with fatty acids during hydrolysis which prevent product inhibition. In addition, Ca^{2+} confers structural stability to enzymes against denaturation by heat and proteolysis[65]. Other metal ions including Na^{+}, K^{+}, Mn^{2+}, Fe^{2+} and Cu^{2+} were not inhibitory to recombinant lipase from *Bacillus* sp. L2. However, PMSF, pepstatin A and EDTA inhibited the enzyme activity which indicated that serine, aspartic acid residues and metal ions respectively are essential for its catalysis. Fatty acids of C_{10}–C_{16} chain lengths appeared to be the preferred substrates with higher degree of hydrolysis in unsaturated fatty acids[41].

Enzyme characterization is important in predicting the potential applications that the enzyme in question can be best suited for. Thus, activity, specificity, thermostability, enatioselectivity and tolerance to various solvent systems are the pre-requisite for lipase selection which make them highly demanding in aqueous and non-aqueous biocatalysis.

CONCLUSION

Different industrial processes require stable lipase preparations for catalyzing the reactions of interest. Most thermostable lipases have other properties which make them unique in the reactions they catalyze. Properties including organic solvent tolerance and substrate specificity are always assessed so that these novel biocatalysts can adequately fit with numerous applications. Depending on the intended applications, thermostable lipases (wild and recombinant) are suitable to be utilized in partially purified form. Any purification process that results in adequate yield and high specific activity could be considered appropriate. Thus, a scope of thermostable lipase studies is challenging and needs careful consideration of the enzyme production, purification and characterization.

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