CHAPTER 2

LITERATURE REVIEW

Lipase (triacylglycerol acylhydrolase, EC. 3.1.1.3) is an important hydrolytic enzyme and find applications in various industries. Lipids are major macromolecules which play a significant physiological role and lipolytic enzymes are required for their turnover. Lipolytic enzymes include esterases commonly called carboxyesterases (EC. 3.1.1.1) and lipases originally called true lipases. The fundamental difference between esterases and lipases is that the former hydrolysis short chain triglycerides which are soluble in water. Lipases, however, act on lipids which form aggregates in water and require a water-lipid interface for the reaction. Lipases can hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acids (Angkawidjaja and Kanaya 2006). Bacterial lipases cannot be distinguished as lipolytic and esterolytic enzymes despite many attempts by researchers to classify them (Rosenstein and Gotz, 2000).

Lipases are ubiquitous enzymes which are widely distributed in plant, animal and microbial world (Dutta and Ray 2009). Lipases have gained special attention over few decades owing to their ability to act in micro-aqueous environment and catalyse esterification, trans-esterification, aminolysis and acidolysis reactions. Many industrial applications of lipases focusses on its regio-and enantio-selectivity (He et al 2010). Also, lipases do not require cofactors to catalyse hydrolysis reactions and are active in the presence of organic solvents. Lipase have become a prime focus of
enzymologists and extensive reviews are available on its classification, assay
and detection methods, purification strategies and industrial applications.

Lipases have been isolated from a large number of plant, animal
and microbial sources. The ease, with which enzymes could be isolated from
microbes, has made both bacteria and fungi as predominant sources of lipase.

2.1 BIOCHEMICAL CHARACTERISTICS OF BACTERIAL
LIPASES

The biochemical characterization of any enzyme becomes
indispensable for understanding its function. The characteristics that are
usually studied are the optimal pH and temperature, influence of the presence
of cofactors, inhibitors and enhancers on catalytic activity, tolerance of the
enzyme to organic solvents and proteases for its function.

2.1.1 Acidic and Alkaline Lipases

Bacterial lipases are mostly alkaline in nature and alkaline lipases
are the promising catalysts for many industrial processes (Ahmed et al 2009).
Despite many reports on lipases and lipase producing microbes, the studies on
acidic lipases are few. There are scant reports on acidic lipases from fungi,
especially Aspergillus niger, except for a study by Ramani et al (2010) who
investigated the production of acidic lipase by P.gessardii.

2.1.2 Thermophilic and Psychrophilic Lipases

Thermal stability is a desirable feature of an industrial enzyme
which is employed in processes that require temperatures greater than equal to
60°C. Lipases are most preferred if they are thermostable. This is mainly
because of the high melting point of the lipidic substrates undergoing the
hydrolysis reactions. Thermostable lipases from many Pseudomonas and
Bacillus sp. have been isolated and studied (Ahmed et al 2009; Dutta and Ray 2009). The potential of cold adapted lipases has a wide range of biotechnological applications. These enzymes exhibit high catalytic activity at temperatures between 0 and 30°C and are generally produced by psychrophilic microorganisms which survive at temperatures around 5°C. Cai et al (2009) have reported cold-adapted lipases from a mesophilic Geotrichum sp.

2.1.3 Effect of Surfactants

Surfactants increase the interfacial tension at lipid-water and thus enhance the rate of lipolysis. However, this does not hold true for all surfactants. Moreover, the effect of surfactants is concentration dependent. For instance, high concentrations of Tween-80 (1%) inhibited lipase production by B. pumilus while at 0.5% concentration Tween-80 assisted maximum lipase production (Zhang et al 2009). In some cases, Dutta and Ray (2009) have been observed that SDS exhibited stimulatory effect while Triton and Tween inhibited lipase activity.

2.1.4 Dependence on Metal Ions/ Cofactors

Certain metal ions enhance the catalytic activity of enzymes and also confer thermostability to them (Chakraborty and Paulraj 2008). Many enzymes require the presence of metal ions for the integrity of their active structures. Different lipases show different response to these metal ions and those ions which function as activators for certain lipases inhibit the activity of few others. The commonly studied metal ions as activators and inhibitors of lipases are Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}. Many metal-dependent/metallo lipases have been reported and Ca^{2+} has been found to exhibit a stimulatory effect in all those enzymes (Chakraborty and Paulraj 2008; Ahmed et al 2009; Zhang et al 2009). This effect could be attributed
to the structural alterations imposed by the binding of Ca\textsuperscript{2+} to the enzyme. The folded enzyme harbours a region of negatively charged amino acid residues which try to move apart to reduce the electrostatic repulsions and this proves to be detrimental to the stability of the enzyme. The metal ion, however, binds to the enzyme and forms a bridge that cross-links the polypeptide chain (similar to a disulphide bond) and the enzyme-metal ion complex becomes stable and rigid. On the contrary, lipases which exhibit Ca\textsuperscript{2+} independent thermostability and activity also have been reported (Kim et al 2002). Calcium ion-independent lipases can function effectively in the presence of chelating agents like EDTA.

2.1.5 Tolerance to Organic Solvents

Organic solvents are advantageous as compared to water when used in reaction systems involving biocatalysts. They help in increasing the solubility of substrates, easy recovery of products and assist in shifting the equilibrium in the forward direction in synthetic reactions (Zhang et al 2009). Organic solvent tolerant-lipases act as effective catalysts in the synthesis of biopolymers, transesterification reactions and production of biodiesel (Singh et al 2010). The stability and activity of lipase is usually carried out in the presence of organic solvents like 2-propanol, methanol, ethanol, acetone, glycerol, n-hexane, n-heptane, n-octane, n-decane, benzene, toluene, xylene, styrene, benzene, ethylbenzene, cyclohexane, dimethylsulfoxide, tetrahydrofuran, chloroform and acetic acid (Zhang et al 2009). The sensitivity of lipases to solvents varies depending on their polarity, with polar solvents being more destabilizing than non-polar solvents (Ahmed et al 2010). Lipases from different sources exhibit different extents of tolerance to various organic solvents and thus commercial exploitation of lipase demands a thorough analysis of the interaction of the enzymes with these solvents.
2.1.6 Tolerance to Proteases

Lipases have a wide range of applications of which few employ lipase in combination with other enzymes like amylase and protease. Proteases are hydrolytic enzymes which are capable of auto-digestion and also digest other enzymes produced simultaneously (Aguilar et al 2002). A thorough study of literature reveals several reports which state that the production of lipases and proteases are inter-related. These studies have shown that when the production of protease is affected either due to the influence of production parameters or due to genetic alterations, the production of lipase is enhanced. Westers et al (2005) have investigated on the susceptibility of a lipase produced by *B. subtilis* to degradation by extracytoplasmic proteases located in the cell wall or in the growth medium. There are also few documents on proteolysis-resistant lipases. A lipase from *Streptomyces fradiae* and *B. cereus* respectively which were resistant to commercial neutral and alkaline proteases has been produced (Dutta and Ray 2009).

Lipases produced by *P.aeruginosa, B.pumilus* and *B.licheniformis* were found to be resistant to co-produced native proteases (Sangeetha et al 2010).

2.2 PRODUCTION OF LIPASES

2.2.1 Screening of Microorganisms for Lipase Production

Several methods have been proposed for the screening of organisms for lipase production. These methods either directly use the microorganism under study or measure lipolytic activity in the crude or purified culture preparations (Singh et al 2010). The plate detection methods use agar plates containing lipid substrate and lipolysis was observed as clear halos or opaque zones around the well containing culture or enzyme preparation (Hun et al 2003). Plates containing chromogenic substrates with pH indicators like
phenol red, Victoria blue etc are also used. A decrease in pH due to release of fatty acids due to lipolysis causes change in the colour of the indicators is measured. Rhodamine B plates which indicate lipolysis by the formation of fluorescent orange halos are widely used (Kim et al 2001). The colorimetric methods are based on measuring the complexes formed between the released free fatty acids and a divalent metal ion, usually Cu$^{2+}$. The widely adopted spectrophotometric methods use designed substrates usually 4-nitrophenyl esters of fatty acids and the lipolytic activity is assayed by measuring the amount of 4-nitrophenol released after to the process (Wang et al 2009). The titrimetric method employs the neutralisation of the free fatty acids that is released after lipolysis and the volume of the base consumed indicates the extent of lipolysis. The other least commonly used methods like chromatographic, turbidimetric, fluorimetric, immunological and radioactive assays are best elaborated in a review on detection methods by Hasan et al (2009).

2.2.2 Production Media

Bacterial lipases are produced by both submerged and solid-state fermentation. Lipase production requires carbon and nitrogen sources as required for any fermentation process. Most of the lipase production studies do not use simple sugars as carbon sources but rather use lipid substrates as sole carbon sources (Zhang et al 2009). However, there are few studies that utilize sugars like glucose as carbon sources while lipid substrates are considered as inducers for lipase production. Lipase production is rarely constitutive and the quantity of the extracellular lipase produced is meagre. Hence inducers like vegetable oils, Tween 20/80, hexadecane and synthetic triglycerides like tributyrin and tripalmitin are used. High concentrations of free fatty acid or vegetable oil repress lipase synthesis. Hence, many lipase production studies use Tween 80 as a sole carbon source. Moderate and
sustained release of oleic acid from Tween 80 does not cause repression (Li et al 2004). Nitrogen sources have varied effect on lipase production and sources like peptone have been reported to augment lipase production (Gunasekaran et al 2006). Unlike carbon sources, nitrogen sources have not been reported to repress lipase production.

The presence of surfactant is an important pre-requisite for maximum lipase production. Catalytic activity of lipases is governed by interfacial activation, a property observed when the lipid substrate starts to form an emulsion thereby presenting an interface for the enzyme to act. Addition of a surfactant decreases the surface tension between the organic and aqueous phase present in the reaction mixture and enhances the rate of emulsification. The commonly used surfactants are Tween, Triton-X 100 (Pogaku et al 2010).

2.3 PURIFICATION OF LIPASES

Downstream processing is fundamental for any fermentation process and involves isolation and purification sequences to obtain a pure and homogenous product. Since commercial lipases are generally extracellular, the purification processes are easy though extensive. The cell-free supernatant which is considered as the crude enzyme source is subjected to preliminary purification steps like ultrafiltration, spray-drying using milk powder or gum arabic, ammonium sulphate fractionation (Kim et al 2002) and/or precipitation using ice-cold organic solvents like ethanol, ether and acetone. This initial preparative purification is followed by purification using a combination of chromatographic techniques. A single chromatographic purification step will usually not be sufficient to get an enzyme of high purity. The commonly reported chromatographic methods to purify lipase are Ion Exchange (IEC), Gel Filtration (GFC) and Hydrophobic Interaction Chromatography (HIC). The IEC is the most commonly employed
chromatographic method and the frequently used ion exchangers are Q-Sepharose, DEAE-Sepharose and CM-cellulose. GFC, the next best adopted method uses matrices like Sephacryl, Sephadex. HIC using octyl or phenyl adsorbents is adopted for lipase purification (Saxena et al 2003). Affinity chromatography using Ni\(^{2+}\)-chelated nitriloacetic acid (Ni-NTA) agarose column is also used by many researchers (Zhang et al 2009; Akbari et al 2010).

Pauwels and Gelder (2008) devised a new strategy to purify lipase and this method exploits the principle behind lipase secretion mechanism. Bacterial lipases, particularly those belonging to the family I are secreted in an unfolded state in to the periplasm where they are folded and conferred the native conformation by an inner membrane bound protein called lipase-specific foldase (Lif). Lif has high affinity for lipase and exhibits high specificity (Pauwels and Gelder 2008). Purification of lipase was performed using immobilised His-tagged Lif. The purified lipase-Lif complex was homogenous and was devoid of the lipopolysaccharides which are usually found as a contaminant in lipase preparations obtained by conventional purification procedures. Da Silva (Padilha et al 2009) purified an alkaline lipase from \textit{P.cepacia} using Expanded Bed Absorption (EBA) on Amberlite ion-exchange resin. EBA is a novel technique which can purify crude enzyme preparations without subjecting them to initial preliminary purifications processes. Aqueous Two-Phase System (ATPS) extraction is another principal downstream processing method which promises purification of lipase in less time than other conventional methods. ATPS employs two phases which are immiscible beyond a particular concentration and may be polymer/polymer or polymer/salt or organic solvent/salt systems.
2.4 IMMOBILIZED LIPASES

Use of enzymes as industrial catalysts is beneficial if the whole process is economical and the cost of the process includes the production of the biocatalyst also. Hence recovery of the catalysts for repeated use becomes necessary. Free enzymes are labile and vulnerable to degradation during the process of recovery after use. Also, most lipases exhibit low stability and activity in organic media. These disadvantages could be overcome by the use of immobilized enzymes. Immobilization improves the stability of enzyme under the reaction conditions, enhances enzyme activity, thus makes the repeated use of the enzyme feasible, permits the use of enzyme for diverse applications and lowers production costs (Liu et al 2009). Immobilisation provides a better environment for the enzyme to act and also offers better product recovery.

Lipases may be immobilized on different kinds of hydrophobic or hydrophilic supports. Various support materials like ion-exchange resins (Amberlite IRC-50), inorganic materials (sand, silica), biopolymers namely sodium alginate, synthetic polymers like polypropylene, polyethylene, polymethacrylate, celite, ceramic, complexes like polypropylene imine-agarose are used to immobilize lipase. Hydrophobic silicates derived from a mixture of TMOS and alkyl trimethoxysilanes such as propyl trimethoxysilane (PTMS) and \( n \)-butyl trimethoxysilane (BTMS) have been identified as best supports for lipase. Mesoporous silica materials with high surface area and ordered pore structures have been used to immobilize lipase and the immobilized enzyme was found to be more efficient and stable than the free enzyme.

Mesoporous materials meet all the criteria for a perfect immobilization support; water insolubility, high surface area, mechanical strength, chemical stability, thermo tolerance and non-toxic. Ramani et al
(2010) have reported the use of mesoporous activated carbon derived from rice husk as carrier for immobilizing lipase. Protein-Coated Micro Crystals (PCMC) has been extensively for enzyme immobilization in the recent years. PCMC are produced by mixing the enzyme with an excipient and the mixture when added to a water-immiscible solvent precipitates and microcrystals of the excipient forms on whose surface the enzyme gets immobilized. Ruchi et al (2008) have prepared PCMC of lipase from P. aeruginosa and found to be stable and efficient. Devi et al (2009) have immobilized lipases as cross-linked enzyme aggregates using precipitants and cross-linkers. These enzyme aggregates do not require pre-existing carriers and can function as effective catalysts under both aqueous and non-aqueous conditions.

2.5 Acinetobacter Lipases

Acinetobacter is a strictly aerobic, Gram-negative coccobacillus that is ubiquitous in geographical distribution. The genus is best known for its capacity for bioremediation of alkanes and aromatic hydrocarbons, as well as production of high molecular weight heteropolysaccharides that act as powerful emulsifiers, many with high commercial potential (Kaplan N et al 1982).

Acinetobacter strains have also been identified as causative agents of nosocomial infections. They are easily isolated and many of them have been found to secrete esterolytic enzymes. Interest in Acinetobacter lipases has increased recently, with the growth of the enzyme industry and the concomitant widening search for novel enzymes and applications.
2.5.1 Occurrence of Lipolytic Strains

Lipolytic strains of *Acinetobacter* have been isolated from a variety of substrates, including human skin, dairy and other food products, in addition to diverse soil and water habitats, both pristine and highly polluted. These strains are also commonly isolated, along with *Pseudomonads*, from waste water effluents and sewage, where they may be continually exposed to petroleum pollutants and xenobiotic compounds (Abdel-El-Haleem D 2003).

2.5.2 Biochemical Properties

Following the classification proposed by Arpigny and Jaeger, lipases produced by *Acinetobacter* sp. are true lipases belonging to subfamily I.1. They share many biochemical properties with other *Pseudomonas/Burkholderia* group of lipases that have been described, including *Pseudomonas aeruginosa* (Gilbert et al 1991), *Proteus vulgaris* (Kim et al 1996), and *Burkholderia cepacia* (Jorgensen S et al 1991).

Many *Acinetobacter* lipases show stability and maximum activity at alkaline pH, a useful characteristic in application that requires detergents. High pH optima reported in studies of lipase A1 from *A. radioresistens* stimulated exploration of technologies designed to enhance production and yield that would be appropriate for large-scale production required in such applications (Chen et al 1999). Activity at acidic pH (ca. pH 5.0) is minimal (Snellman et al 2002), presumably due to protonation of the active site histidine. Incubation at lower pH results in inactivation, which Lang et al. (1996) attributes to loss of Ca$^{2+}$ due to protonation of its coordinating residues. In addition, these lipases demonstrate broad substrate specificity typical of microbial lipases that have been shown to be useful in attacking mixed fat stains.
A near universal property of *Acinetobacter* lipases is the positive effect of Ca\(^{2+}\) on enzyme stabilization and activity, most probably a function of the Ca\(^{2+}\)-binding pocket (Sullivan ER et al. 1999), leading to correct active-site configuration. Calcium ion binding has been demonstrated in crystallized *P. aeruginosa* lipase, the subfamily prototype enzyme (Nardini M et al. 2000). Furthermore, analyses of structural data from the closely related family I.2 clearly show a commonality of the Ca\(^{2+}\)-binding and strongly suggest its conserved nature throughout the family. The effect of Ca\(^{2+}\) may also be attributed to interaction with the assay medium, precipitation of free fatty acids, or otherwise increased enzyme access to the substrate.

The effect of metals and inhibitors has been studied with respect to suitability of lipases for industrial applications. Generally, incubation in the presence of metal ions has little effect on lipase activity, which most likely depends on specific reaction conditions, rather than general properties of the enzyme. Incubation in the presence of EDTA demonstrated a variable dependence on Ca\(^{2+}\) for activity, i.e., 70% loss in activity occurred 8 h post incubation with the chelator (Bompensieri S et al. 1996). Addition of Ca\(^{2+}\) after exposure to EDTA resulted in reactivation of Lip009, presumably by stimulating refolding of the enzyme. However, other investigators have found no “rescue effect” of Ca\(^{2+}\) incubation post exposure to EDTA (Snellman EA 2002). These seemingly contradictory findings suggest that Ca\(^{2+}\) may be required for prolipase folding, in addition to activity of the mature lipase. Addition of phenylmethylsulfonylfluoride (PMSF) to lipase preparations also yielded mixed results.

Dharmshiti et al. (1998) hypothesized that the deeply recessed nature of the active site serine imparts resistance to a serine hydrolase inhibitor. Incubation of purified enzymes in the presence of reducing agents [2-mercaptoethanol, dithiothreitol (DTT)] resulted in activity that was not
dependent upon intact disulfide linkage. Since the presence of disulfide bridges has been confirmed in related crystallized proteins, these results suggest a more important role in the interaction with their cognate lipase-specific foldase, or Lif, during folding and export. It is quite plausible that, during purification, lipases aggregate with various cellular materials, i.e., emulsifying agents, lipopolysaccharides (LPS), or other materials that confer protection from potential inhibitors [Breuil C et al 1975].

Stability in the presence of organic solvents is a requisite property of enzymes used in organic synthesis in non-aqueous systems. *Acinetobacter* lipases appear to be ideally suited for such syntheses since many lipolytic strains have been isolated from petroleum- polluted environments. Incubation of purified A1 lipase in either 40% (v/v) dimethylsulfoxide (DMSO) or 20% (v/v) dimethylformamide (DMF) greatly increased activity (140% in DMSO) (Hong M et al 1988), leading to the hypothesis that solvents act to decrease enzyme aggregation, modify the substrate-water interface, or convey positive conformational changes. However, storage of Lip009 in solvents for 24 h (4°C) resulted in significant deactivation (Dharmshtiti S et al 1998). In contrast, lyophilized RAG-1 preparations retained significant enzyme activity in non-polar solvents, presumably because of the increased rigidity of the molecule (Snellman EA et al 2002).

### 2.5.3 Potential for industrial applications

Lipases are supposed to be the fastest growing enzyme class, fuelled by new applications in organic synthesis and pharmaceutical production, and by expanded penetration into the detergent industry. The potential for many new lipase applications has driven a wide ranging search for novel enzymes. Studies of the structural basis of enantioselectivity, engineering enzyme specificity through directed evolution, and improving
technology to enhance production and yield are also being pursued vigorously. The primary focus is on bacterial and fungal lipases because they are easier to produce, can be modified by recombinant DNA technology, and are amenable for manufacturing applications. In this regard, lipases produced by *Pseudomonas* spp. are well described and play a dominant role in industry. However, lipolytic strains of *Acinetobacter* have received increased attention as the search within the enzyme industry as a source of novel biocatalysts. *Acinetobacter* sp. is also known for production of other potentially important commercial products, notably bioemulsifiers (Desai JD et al 1997) and enzymes for bioremediation of hazardous wastes (Abdel-El-Haleem D 2003).

### 2.6 APPLICATIONS OF BACTERIAL LIPASES

Lipases obtained from fungal sources were thought to be the ideal for commercial applications until bacterial lipases were explored. Many microbial lipases have been commercialized by popular enzyme producers in the world like Novozyme (Denmark), Amano Enzyme Inc (Japan), Biocatalysts (UK), Unilever (Netherlands) and Genencor (USA). Bacterial lipases produced form the genera *Burkholderia* and *Pseudomonas* are commercially available. Lipase PS isolated from *Burkholderia cepacia* and Lipase AK isolated *P. fluorescens* are supplied by Amano and Lipase SL and Lipase TL isolated from *B. cepacia* and *P. stutzeri* are supplied by Meito Sangyo (Japan).

Lipases are valuable biocatalysts with diverse applications. Though lipases share only 5% of the industrial enzyme market, they have gained focus as valuable enzymes in biotechnology. They play vital roles in food, detergent and pharmaceutical industries.
Lipases catalyse the hydrolysis of lipids to fatty acids and glycerol. The reversal of this reaction takes place in an environment with low water content or rather in the presence of organic solvents. Such reactions include esterification, transesterification and interesterification. The term interesterification has been used in a broader sense throughout the literature and refers to exchange of acyl group between an ester and a carboxylic functional group (acidolysis), an ester and a hydroxyl functional group (alcoholysis) or between two esters (transesterification). All these reactions serve to alter the physical properties of the oil or fat and produce new esters. These reactions provide a platform for lipase to act as a potential industrial enzyme.

2.6.1 Food Industry

2.6.1.1 Hydrolysis of oils

Hydrolysis of oils and fats are usually performed to concentrate the fatty acids present in the oil. The production of a number of high-value products like adhesives, cosmetics and other personal care products, lubricants and coatings require fatty acids. Thus production of fatty acids by the hydrolysis of oils and fats becomes indispensable for them to be exploited by various industries. Ramani et al. (2010) used an immobilized acidic lipase from P. gessardii for olive oil hydrolysis.

The Poly Unsaturated Fatty Acids (PUFAs) are simple lipids with two or more double bonds and play diverse physiological roles which contribute to the normal healthy life of human beings. PUFA’s belong to two major families namely n-3 (ω3) and n-6 (ω6). The nutritionally important ω3 fatty acids include α-linolenic Acid (ALA), eicosatrienoic Acid (ETA), eicosapentaenoic Acid (EPA) and docosahexaenoic acid (DHA). Linoleic acid and arachidonic acid are the most common ω6 fatty acids. These are currently
in high demand as they are formulated in nutraceutical and pharmaceutical products. The demand is met by concentrating these PUFA’s from alga, fish oil, fish by-products and edible oil (Chakraborty et al 2010). Purification and concentration of PUFAs can be best done using lipases and many bacterial lipases have been studied for their efficiency in enhancing PUFA content.

Kojima et al (2006) used two lipases (AK-lipase and HU-lipase) produced by *P. fluorescens* to selectively concentrate EPA and DHA from fish oils. This method is economical for concentrating PUFAs when compared to the methods which involve distillation, chromatography and fluid extraction which are cumbersome and costly. The hydrolysis of cuttle-fish oil using AK-lipase enhanced the DHA composition from 16.3 to 44.6%. The composition of EPA in cod-oil was increased from 12 to 43% using HU-lipase. Byun et al (2007) used a *Pseudomonas* lipase to hydrolyse sardine oil in the presence of emulsifiers and observed a decrease in the level of saturated fatty acids and increase in the levels of mono and poly unsaturated fatty acids after hydrolysis. Yamauchi et al (2005) purified arachidonic acid from *Mortierella* oil using lipases from *Alcaligenes* and *B. cepacia*. The process involved a nonselective hydrolysis of the oil using *Alcaligenes* lipase followed by a selective elimination of saturated fatty acids using urea adduct fractionation and finally a selective enrichment by esterification using *B. cepacia* lipase.

2.6.1.2 Modification of Fat and Oil

The nutritional benefit of fats and oils depends largely on the distribution pattern of the fatty acid present in them. Tailored vegetable oils with structured triglycerides popularly known as structured lipids are desirable as they contribute to benefits in health. Many vegetable oils like sunflower, coconut, olive, corn and rice bran oil are rich in o6 fatty acids and fish, linseed oil, walnuts and milk are rich in o3 fatty acids. Though both
these poly unsaturated fatty acids play a predominant role in contributing to the health of an individual, the ingested ratio of $\omega 6/\omega 3$ needs to be monitored. This ratio is significant and should be balanced between 1 and 4; a high $\omega 6/\omega 3$ ratio may pose hazards to health.

The use of vegetable oils particularly those enriched with $\omega 6$ fatty acids results in high $\omega 6/\omega 3$ ratio in the diet. The best strategy to improve the $\omega 6/\omega 3$ ratio is the enzymatic modification method which uses lipase-catalyzed interesterification (Mitra et al 2010). Oleoylpalmitoyl glycerol (OPO) is a structured triglyceride produced by acidolysis of tripalmitin with oleic acid. Guncheva et al (2008) evaluated the efficiency of a lipase from *B. stearothermophilus* MC7 in producing OPO. Synthesis of a structured lipid requires high temperatures for better homogenization during the process and presence of organic solvents to assist the lipase to resist high temperatures. The presence of organic solvents may sometimes prove to be disadvantageous as undesired isomeric products form. Lipase MC7 could operate in non-solvent high temperature reaction systems and thus averted the side reactions of acidolysis.

### 2.6.1.3 Glycerolysis

Monoacyl Glycerols (MAGs) and Diacyl Glycerols (DAGs) are surface-active molecules that are widely used as emulsifiers in food, pharmaceutical and personal-care products. They possess excellent emulsifying properties and are traditionally produced by chemical glycerolysis. DAGs have also gained attention due to their positive impact on health unlike triglycerides (Cheirsilp et al 2009). Enzymatic glycerolysis averts the negative side effects of chemical process like products with undesirable color and flavor (Cheirsilp et al 2009).
2.6.1.4 Synthesis of flavor esters

Flavour compounds that are extracted from plants are too expensive and hence are replaced by flavor esters synthesized using catalysts. Flavor or fragrance materials which include various aliphatic and aromatic compounds share a major market of food additives throughout the world. Flavor esters are low molecular weight compounds synthesized by the esterification of fatty acids, preferably by microbial lipases. These compounds are classified as natural, despite being synthesized and hence are a subject of intensive research. Some of the esters synthesized by esterification reactions catalysed by bacterial lipases are ethyl acetate, ethyl butyrate, ethyl methyl butyrate, ethyl valerate and ethyl caprylate (Ahmed et al 2010).

2.6.1.5 Lipolysed milk fat

The Lipolysed Milk Fat (LMF) is prepared from condensed milk or butter oil using lipases which release free fatty acids and give it a cheesy aroma. LMF is used in chocolate coatings, artificial flavor additives, margarine etc. The bacterial lipases used to prepare LMF include those obtained from Achromobacter and Pseudomonas sp.

2.6.1.6 Cheese

Lipases from Lactobacillus play a prominent role in manufacture of bacterial ripened cheese like Parmesan and Grana Padano cheese. Mandrich et al (2006) have investigated the role of lipase/esterase from Alicyclobacillus acidocaldarius in milk and cheese. They observed that the recombinant enzyme was more efficient than the native enzyme and could be used in dairy industry to impart flavor or enhance cheese ripening.
Enzyme Modified Cheese (EMC) is a concentrated cheese flavour food ingredient produced by treating cheese curd with enzymes. It is used as cheese powders, in soups, salads, sauces and coatings. EMC has more intense flavour than naturally ripened cheese and small chain fatty acids (C2-C6) contributes primarily to this flavour. Esterification produces new esters like ethyl butanoate, ethyl hexanoate which confer characteristic flavours to cheese.

2.6.1.7 Bread making

Emulsifiers are additives and are required to improve the quality of foodstuffs. As an example, addition of emulsifiers improves bread volume and texture and dough stability. These emulsifiers are detectable in the final baked and marketed loaf and thus find a place in the label. Enzymes on the other hand get denatured during baking and thus provide bread improving functions without appearing on the label. Lipase from *B. subtilis* has been proved to play a role in bread making in a study by Sanchez et al (2002). These enzymes can completely or partially replace the traditional volume improving agents.

2.6.2 Detergents

The most noteworthy application of hydrolytic lipases is their use in house-hold and laundry detergents. Lipases were developed as detergent enzymes after the successful introduction of proteases in powder and liquid detergents. Lipases should meet the following criteria to serve as a detergent additive: stability at alkaline pH, solubility in water, tolerance to detergent proteases and surfactants and low substrate specificity (Rahman et al 2006). Genencor International introduced commercial bacterial lipases Lipomax from *P. alcaligenes* and Lumafast from *P. mendocina* which could be used as detergent enzymes in the year 1995 (Rahman et al 2006). Though fungal
lipase was the first introduced detergent enzyme, bacterial lipases captivated the detergent market. This was due to the acidophilic nature of fungal enzymes that make them incompatible with the alkaline wash conditions. During laundering, the lipase adsorbs on to the fabric surface to form a stable fabric-lipase complex which then acts on the oil stains and hydrolyses them. The complex is resistant to the harsh wash conditions and is retained on the fabric during laundering.

A detergent stable lipase was isolated from B. cepacia by Rathi et al (2001). The enzyme was found to meet all the criteria necessary for a detergent additive and exhibited better stability than Lipolase, a detergent stable lipase marketed by Novo Nordisk and Denmark. Suzuki (2001) has patented an alkaline Pseudomonas lipase which remains active at low temperatures and has improved wash performance. The patent also pertains to a detergent composition containing such lipase. Wang et al (2009) and Zhang et al (2009a, b) have isolated lipases from B. cepacia and P. fluorescens and the lipases have proved to be suitable for detergent industry. A lipase isolated from B. licheniformis was not stable and lost its activity in the presence of commercial detergents but its activity was restored by the addition of calcium chloride to the enzyme-detergent complex. Such lipases lose their activities in the presence of a chelating agent, if any, in the detergent (Bayoumi et al 2007).

2.6.3 Tannery

Leather processing operations are classified into three groups: pre-tanning which cleans the hides or skins, tanning which stabilizes them and finally post-tanning which adds aesthetic value. All these stages use many chemicals and enzymes. Enzymatic leather processing gained importance in 1980s and industrial scale operations are now routinely carried out using enzymes. The different stages of leather processing are curing, soaking,
liming, dehairing, bating, pickling, degreasing and tanning. Lipases are
employed in soaking, bating and degreasing stages. Soaking is the first stage
of leather processing and is performed to rehydrate the skin or hide.
Conventional processes use soda ash or sodium tetrasulphide in the presence
of surfactant while enzymatic processes use lipase along with protease
(Choudary et al 2004). Forezym SK is a commercial bioproduct comprising of
a mixture of bacterial protease and lipase marketed La ForestalTanica, Spain.

Dehairing is a process of removing hair from skin and hides. Loosening of the hair is performed by lime and sulphide in conventional
processes while enzymatic processes depend on proteases. Dorezym LM is
also a mixture of bacterial protease and lipase marketed by La Forestal Tanica
despite the role of lipase in dehairing being unclear. Degreasing is performed
to the remove the residual grease left out after liming process. Large
quantities of natural fat present in the skin, particularly sheep skin cannot be
removed by the liming operation and hence requires a subsequent degreasing
process. Degreasing initially performed with lipase alone was not satisfactory
and hence a combination of protease and lipase was evaluated. The
degreasing process involves breaking down of the protein membrane of the
fat sac, removal of the fat and its emulsification in water or solvent. Hence a
combination of protease and lipase and an enzyme-compatible surfactant
becomes necessary for effective degreasing (Thanikaivelan et al 2004). Both
acid and neutral lipase has been used for degreasing. La Forestal Tanica has
marketed Forezym WG-L, Forezym DG as degreasing bacterial lipases.

2.6.4 Textile Industry

Processing of garments promises a refined and polished look to the
finished fabric. Desizing is such a process and is required to remove the size
material which has been impregnated from the fabric prior to weaving.
Traditional desizing uses acid or oxidizing agents which damages the
cellulose material in the fabric. Enzymatic desizing has multiple advantages over the traditional process and uses enzymes like cellulase, amylase, protease and lipase depending on the sizing agent. Bacterial lipases can be used if the size material is a synthetic sizing agent like polyesters. Lund (2001) has patented a process for combined desizing and stone-washing of denim. Lipolytic enzymes from P. cepacia, P. fluorescens, P. fragi and P. stutzeri were evaluated and the lipase of P. cepacia was preferred.

Polyethylene Terephthalate (PET) fabrics are known for their strength and wrinkle resistance. The lack of ability for dyes to bond to its surface is an undesirable feature which is because of its hydrophobic character. Any attempt to increase its moisture regaining nature will make the PET fabrics more fashionable. Kim and Song (2006) have used lipases from P. cepacia and P. fluorescens in an eco-friendly method to improve the moisture regain of PET fabrics and found it to be more effective than the alkaline method.

2.6.4.1 Synthesis of Polymers

Polylactate (PLA) fibres are made of lactic acid obtained by fermentation of natural sugars. It is an eco-friendly material which has low energy requirements for synthesis and is highly bio-degradable. It is used in textile fabrics, packaging material, bio-medicine and as bio-plastics. PLA fibre is hydrophobic and thus the affinity of dyes and chemicals to it is less. This necessitates the modification of the surface of the fibres which makes them more wettable. Since ester bonds are present in poly-lactate, some of the ester bonds can be hydrolysed at the surface enzymatically using a lipase. Thus the presence of carboxyl groups at the surface increases the hydrophilicity of the PLA.
PLA has mechanical properties similar to those of polystyrene and polyethylene terephthalate and can be obtained from different isomers of lactic acid. Poly-L-Lactic Acid (PLLA) is semi-crystalline in nature and is more potential than amorphous PLA (Lim et al 2008).

2.6.5 Pharmaceutical Applications

Miyazaki and Fujikawa (2009) patented methods to treat skin, scalp disease and hair-loss using compositions that contain a protease, lipase and a metallic salt. The lipase was obtained from a fungal or bacterial source, preferably from *Pseudomonas*. Sani (2006) proposed that bacterial lipases can replace pancreatic lipases used to treat cystic fibrosis and pancreatitis. Pancreatic lipase is used as a digestive aid in lipid mal absorption disorders but its application is limited as it loses its efficacy at low pH and in the presence of a protease. Many bacterial lipases are hence promising alternatives to pancreatic lipase.

Polyphenols are natural antioxidants which lose their activity in stabilizing oils. This disadvantage is due to their hydrophilic nature. Solubility of phenolics in a fatty region becomes necessary for it to exhibit its action and thus production of lipophilic phenols will make the antioxidant properties of phenols more efficient. Biocatalytic synthesis of cinnamoyl esters using *Pseudomonas* lipase has been reported (Buisman et al 1998) and cinnamoyl esters have higher free radical scavenging activity than cinnamic acid.

An enantioselective lipase from *Acinetobacter* hydrolysed cis-(±)-2-(bromomethyl)-2-(2,4-dichlorophenyl)-1,3-dioxolane-4-methyl acetate. This is a racemic intermediate in the synthesis of itraconazole, an antifungal agent. Diltiazem is a calcium channel blocker and is used to treat angina pectoris, hypertension and other vascular disorders. Synthesis of Diltiazem hydrochloride requires an optically pure (−) trans-methoxylphenylglycidic
acid methyl ester. A lipase produced from *Serratia marescens* was efficient to
catalyse the bioresolution of (±) MPGM (Hu et al 2009). A *Serratia
marescens* lipase was used for the bioresolution of racemic ketoprofen, a
non-steroidal anti-inflammatory drug.

Hydrocinnamate esters are precursors for the synthesis of 1,3,4,9-
tetrahydropyrano [3,4-b]indole-1-acetic acid which is used as a analgesic,
antipyretic and anti-inflammatory agent. These esters also act as inhibitors of
HIV-protease. These esters have been synthesized using *P. cepacia* lipase
by Priya and Chadha (2003).

### 2.6.6 Biosensors

Qualitative and quantitative determination of lipids and
lipid-binding proteins is possible with the help of biosensors which may be of
chemical or biochemical in nature. Bacterial lipases have been used as
biosensors and this exploits immobilized lipases. Such biosensors are used to
detect triglycerides in food and clinical samples, pollution analysis
like pesticide contamination and pharmaceutical industry. Setzu et al
(2007) fabricated a potentiometric biosensor using a lipase immobilized on a
mesoporous silica matrix.

### 2.6.7 Agrochemical Industry

Lipases are used to synthesize intermediates involved in the
production of pesticides, insecticides and other agrochemically useful
compounds. Transesterification and resolution by *Pseudomonas* lipases was
studied to produce insecticides, fungicides and secondary alcohols like
(R, S)-4-hydroxy-3-methyl-2-(2′-propenyl)-2-cyclopenten-1-one. (Xu et al
2005).
2.6.8 Cosmetics and Personal Care Products

Production of flavors by transesterification and resolution of racemic intermediates by lipases boosts the economy of the cosmetic and perfume industry. Lipases produced by *P. cepacia* have been used to resolve the racemic rose oxides produced by the bromomethoxylation of citronellol.

Esters of aliphatic and aromatic acids, alcohols including terpene alcohols, aldehydes, phenols are commonly present in the flavor materials used in perfumes and other personal care products. Priya and Chadha (2003) studied the synthesis of hydrocinnamic acid esters by *P. cepacia*. The esters of hydrocinnamic acid are used in perfumes and sunscreens. Mouth washes and shaving creams contain menthol to provide a peppermint flavor and a cooling sensation. Menthol can be artificially produced by the esterification process when there is a dearth for the natural menthol. Chaplin et al (2006) have patented a process to produce menthol esters and similar compounds and this process utilizes lipases of *P. fluorescens* and *P. cepacia*.

2.6.9 Biodiesel Production

Biodiesel is an alternative fuel for petroleum-based diesel and is biodegradable, renewable, non-inflammable and non-toxic. Biodiesel is defined as monoalkyl esters of long chain fatty acids usually a methyl ester of fatty acid and denoted commonly as FAME (fatty acid methyl ester). Biodiesel is used for diesel engines and heating systems and its demand has increased due to the soaring petroleum prices. Biodiesel is produced from vegetable oils such as soybean oil, rice bran oil, sunflower oil, palm oil, cotton seed oil and jatropha oil, animal fat, algae, waste edible oil and industrial acid oil.
Biodiesel is synthesized by chemocatalytic, thermocatalytic and biocatalytic approaches where, the latter employs lipases as biocatalysts. The lipase catalyzed transesterification reaction takes place between a lipid and a short chain alcohol to produce an ester and glycerol (Raita et al 2010). The most commonly employed bacterial lipase for biodiesel synthesis is from *P. cepacia*. The production of biodiesel requires a micro-aqueous environment because the presence of water does not promote transesterification rather favours the hydrolysis of oil. Hence immobilized lipases are employed to function in a micro-aqueous of solvent-free system (Xu et al 2009). Methanol competitively inhibits lipase and also denatures it and thus decreases biodiesel production. A solute like ectoine decreases the affinity of the lipase for methanol but increases its affinity for the triglyceride and this strategy improved the yield of biodiesel.

Glycerol, the by-product of biodiesel production is converted to 1,3propanediol which is a monomer for the synthesis of novel polymers like polymethylene terephthalate. Bacterial species like *Klebsiella*, *Citrobacter*, *Clostridium*, *Enterobacter* and *Lactobacillus* can convert glycerol to 1,3 propanediol (Xu et al 2009).

### 2.6.10 Environment Studies and Management

Biogeochemical studies include the analysis of the relationship between Dissolved Organic Matter (DOM) and bacterial dynamics, the response of bacteria to environmental conditions. These studies utilize various parameters for analysis and bacterial activities are one among them. Bacteria use the readily available pool of DOM for their growth and breakdown major molecules enzymatically when the small molecules deplete. Thus the analysis of bacterial enzyme activities like peptide hydrolysis and polysaccharide degradation *in situ* was studied as indicators of DOM dynamics. Recently Bourguet et al (2009) studied the relationship between the levels of
lipids and in situ lipase activities during different environmental conditions like spring and summer.

Waste-water treatment processes remove lipidic residues by air-floatation and discard it in a sanitary landfill dumping yard. These pose a threat to the waterbeds and ground water by decreasing the oxygen transfer rate and hence bioremediation is adopted to control pollution (Mongkolthanaruk and Dharmsthiti 2002). Oil spills in the soil and water during rigging and refining can be handled using lipases. The most common method of treating waste water is by cultivating pure cultures which produce lipase and mixed cultures which produce lipase and other enzymes. Effluents emanating from food processing, tannery, automobile industries and restaurant and fast-food outlets can be treated with by cultivating lipase producing bacteria (Nelson and Rawson 2010). The commonly used bacterial genera are *Pseudomonas, Bacillus* and *Acinetobacter* (Mongkolthanaruk and Dharmsthiti 2002).