2.0 LITERATURE REVIEW

2.1 INTRODUCTION TO LIPASE

In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them to soluble products. Lipases have traditionally been obtained from animal pancreas and are used as a digestive aid for human consumption either in crude mixture with other hydrolases (pancreatin) or as a purified grade. Initial interest in microbial lipases was generated because of a shortage of pancreas and difficulties in collecting available material (Hasan et al., 2006).

Lipases are valued biocatalysts because they act under mild conditions, are highly stable in organic solvents, show broad substrate specificity, and usually show high regio- and/or stereo selectivity in catalysis. Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The physiologic role of lipases is to hydrolyse triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their natural function of hydrolyzing carboxylic ester bonds, lipases can catalyse esterification, interesterification, and transesterification reactions in non-aqueous media (Snellman et al., 2002).

2.2 PROPERTIES OF LIPASE

Besides the conventional ability of lipases to catalyze hydrolytic reactions, they can catalyze synthetic reactions such as esterification and transesterification in form of acidolysis, alcoholysis and interesterification in the presence of small amount of water.
Unlike other enzymes, oil-water or air-water interfaces activate the lipases (Shimada et al., 2005; Villeneuve et al., 2000).

Divakar and Manohar (2007) grouped the reactions catalyzed by lipases into three important types:

I. Hydrolysis: This occurs in aqueous media, when there is high amount of water, cleavage of ester bonds is the dominant reaction. This technology is currently employed in the production of fatty acids, diglycerides, monoglycerides, flavouring agents for dairy products and detergents for laundry and household uses.

II. Esterification: This reaction occurs under low water conditions such as in nearly anhydrous solvents; a high yield of the esterified products is obtained under controlled conditions. Production of oleic acid esters of primary and secondary aliphatic and terpenic alcohols is among the commonest example. Others are the formation of geranyl and menthyl esters from butyric acid, geranol or lauric acid and menthol (Marlot et al., 1985).

III. Transesterification: this involves the exchange of acid moiety between two or more compounds (if the acyl donor is a free acid the reaction is called acidolysis, whereas the reaction is called interesterification if the acyl donor is an ester; in alcoholysis, the nucleophile alcohol acts as an acyl acceptor) (Macrae, 1985).
2.2.1 Thermostable Lipases

Thermostable lipases are commonly found in bacteria, fungi, and yeasts. Several Bacillus lipases were thermostable functioning at over 60 °C. Lipase from Bacillus thermoleovorans and R. oryzae can function well at 70–75 °C. On the other hand, lipases from Archaea Pyrococcus horikoshii were found to be stable at 95 °C (Haki and Rakshit, 2003). Interestingly, at least in certain instances, the phenomenon of thermostability and alkalinity appear to go hand-in-hand, and it is an industrially desirable trait. A moderately thermostable lipase from Streptomyces thermocarboxydus ME168 with optimum activity at 50°C and a half-life of 3h at 65°C was immobilized on celite and was successfully employed in synthesis of sugar esters (Kittikun et al., 2012).

Chakravorty et al., (2011) were analyzed a detailed Insilico analysis of various structural features that contribute to thermostability of lipases. The outcome of the analysis revealed that each thermostable lipase adopts its own unique strategy to display enhanced stability at high temperatures in relation to the 3D arrangement of amino acids. Certain general strategies include (1) increasing titratable amino acids near the active serine and (2) increasing charged residue at surface and decreasing beta-branched residues at helices. For example, Bacillus lipases display enhanced thermostability by increasing percentage of Gly residues in loops, decreasing percentage of free Cys residues, increasing polyAla residues in the lid, and replacing thermolabile residues with amino acids that display high helix propensity. Lastly, but importantly, increment of inverse gamma turn near carboxy and amino end of helixes
were reported for the first time to contribute to thermostability. In general, strategies adapted by bacterial lipases differ from that employed by thermostable fungal lipases.

### 2.2.2 Cold Active Lipases

Cold active lipases function efficiently at lower temperatures, where mesophilic enzymes display little or no activity. Cold active enzymes are potentially employed as additives in detergents under cold wash conditions, in food industry, and in bioremediation (Jaeger and Eggert, 2002; Joseph et al., 2008; Suzuki et al., 2001). Antarctic polar regions and deep sea are a good source of cold active lipase producing microbes. Among bacteria, cold active genera include *Aeromonas, Pseudoalteromonas, Moraxella, Acinetobacter, Staphylococcus, Serratia, Psychrobacter,* and *Pseudomonas* (Joseph et al., 2008). Within fungi, *Candida antarctica, C. lipolytica, G. candidum, Aspergillus nidulans,* and *Penicillium roqueforti* secrete cold active lipases. Cold active enzymes are generally thermolabile (Rashid et al., 2001).

A recent review has exhaustively summarized structural determinants for cold activity, sources, production, purification, protein engineering, and application of cold active lipases. Cold active lipases exhibit certain specific structural features like high level of flexibility around the active site, which enable them to easily accommodate the substrates and thereby display low substrate affinity and retain high specific activity at lower temperatures (Joseph et al., 2008). Cold adapted lipases probably are structurally modified by an increasing flexibility of the polypeptide chain enabling an easier accommodation of substrates at low temperature.
The fundamental issues concerning molecular basis of cold activity and the interplay between flexibility and catalytic efficiency are of important in the study of structure-function relationships in enzymes. Such issues are often approached through comparison with the mesophilic or thermophilic counterparts, by site directed mutagenesis and 3D crystal structures (Narinx et al., 1997; Wintrode et al., 2000). The molecular modeling of Pseudomonas immobilis lipase revealed several features of cold-adapted lipases (Arpigny et al., 1997). A very low proportion of arginine residues as compared to lysine, a low content of proline residues, a small hydrophobic core, a very small number of salt bridges and of aromatic-aromatic interactions are the possible features of lipase for cold adaptation. Similarly the weakening of hydrophobic clusters, the dramatic decrease (40%) of the proline content and of the ratio Arg/Arg+Lys makes lipases active at low temperature (Gerday et al., 1997).

2.2.3 Alkali Stable Lipases

Alkaline lipases are widely preferred as an additive in laundry detergent formulations. In many instances, alkali stable lipases were also found to exhibit thermal stability. Among bacteria, alkaline lipases have been reported from low temperature active A. johnsonii (Qi et al., 2011), thermophilic Bacillus sp. (Sidhu et al., 1998), Alcaligenes sp. (Masahiro et al., 2009), Burkholderia multivorans (Gupta at al., 2007), Pseudomonas (Lin et al., 1996), and even from Corynebacterium (Joshi et al., 2006), S. xylosus (Bouaziz et al., 2011), and Streptomyces (Mander et al., 2012). Among fungi, Penicillium cyclopium (Dai, and Xia, 2005), Fusarium solani (HueyMin et al., 2009), and edible basidiomycete A. cinnamomea (Lin and Ko, 2005) were reported to secrete alkali stable lipase. F. solani was reported to secrete a low temperature active alkaline
lipase with potential to be used as a detergent additive (HueyMin et al., 2009). A mesophilic Acinetobacter sp. EH 28 was found to secrete a thermostable alkaline lipase that had a pH optimum of 10.0 at 50 °C, and was observed to be stable in the presence of organic solvents, and hence, it was ideally suited for the synthesis of flavor ester ethyl caprylate (Ahmed et al., 2010).

In a recent study, Staphylococcus sp. isolated from soil was observed to produce a thermostable alkaline lipase that displayed a pH optimum of 12.0 and temperature optimum of 60 °C. This enzyme was also shown to possess remarkable stability in the presence of nonionic and anionic detergents and various commercial detergents, highlighting its suitability for application as an additive in laundry detergent formulations (Cherif et al., 2011). Ralstonia sp. CS274 lipase displayed maximal lipase activity at pH 8.0 - 9.5, temperature of 50–55 °C, and was found to be highly effective for biodiesel production at pH8.0, using 5 % methanol and 20 % water content (Yoo et al., 2011).

Thermophilic anaerobe Thermosyntropha lipolytica secreted two thermostable alkaline lipases that displayed maximal lipolytic activity at 95 °C and at a pH of 9.4 and 9.6. This is the maximal temperature at which optimal lipase activity has been ever reported (Salameh and Wiegel, 2010). A newly isolated fungi Talaromyces thermophilus was shown to secrete extracellular lipase that showed optimum activity at pH9.5 and an optimum temperature of 50 °C. It was found to exhibit resistance to interfacial denaturation, hence could be potentially employed as an additive in detergent formulations (Romdhane et al., 2012).
2.2.4 Acidic Lipases

Reports on acidic lipases from microbial sources are scarce. An *A. niger* NCIM 1207 strain produced an extremely acid stable lipase on SSF containing wheat bran, olive oil, and retained enzyme activity at pH 1.5. This lipase displayed optimum activity at pH 2.5 and temperature of 45 °C. Upon incubation, at pH 2.5 for 24 h, the enzyme retained 63 % of its activity. Interestingly, the lipase also exhibited thermal stability by retaining 63 % of activity at 70 °C for 5 h (Mahadik *et al.*, 2002). Another recent study also reported production of acidic lipase by *A. niger* at pH 3.0 in submerged culture using olive oil as the inducer with potential use in pharmaceutical industry (Colin *et al.*, 2010).

2.2.5 Organic Solvent-Tolerant Lipases

Most of the industrial reactions like esterification, transesterification, biodiesel production, and racemic resolution require that the lipase used should retain activity in the presence of organic solvents (Jaeger and Eggert, 2002). Organic solvents generally inactivate enzymes by decreasing conformational flexibility, stripping of crucial water, exposing hydrophobic residues, interfacial inactivation, etc. Since lipases are interfacial enzymes, when compared with other enzymes, lipases in general retain stability in the presence of organic solvents (Doukyu and Ogino, 2010). Hence, numerous reports on organic solvent-tolerant lipases from various sources exist; these include *P. aeruginosa*, *Pseudomonas sp.*, *Bacillus sphaericus*, *Bacillus megaterium*, *B. thermoleovorans*, *Burkholderia cepacia*, *Staphylococcus saprophyticus*, and among fungi, *Penicillium chrysogenum*, *R. oryzae*, and *Cryptococcus sp.*
Recently, methanol-tolerant *Streptomyces* sp. CS133 was shown to successfully produce biodiesel by enzymatic transesterification of a mixture of soybean oil and olive oil in methanol (Mander *et al.*, 2012). In another study, an alkaline thermostable mesophilic lipase from *Acinetobacter* sp. was found to display remarkable stability in organic solvents and was employed for successful synthesis of ethyl caprylate in cyclohexane medium (Ahmed *et al.*, 2010). A whole cell lipase from *Burkholderia* sp. ZYB002 was found to exhibit both thermal stability and tolerance to wide variety of organic solvents (Shu *et al.*, 2012). Lipase from *S. marcescens* by virtue of its stability in both water-miscible and immiscible organic solvents was found to be highly useful in enantiomeric resolution of glycidyl butyrate, naproxen methyl ester, etc. (Xu *et al.*, 2008). These studies highlight the importance of organic solvent-tolerant property of lipases for various industrial applications.

### 2.3 STRUCTURE OF LIPASE

Lipases are ubiquitous, since they are produced by virtually every living organism. It is very important to know the three-dimensional structures of lipases in order to make them fit for specific applications. Human pancreatic lipase and the lipase from the fungus *Rhizomucor miehei* were the first ones whose 3D structures elucidated. Various other fungal lipase structures, from *Geotrichum candidum, Fusarium solani, Candida rugosa, Candida antarctica, Humicola lanuginose* and *Rhizopus delemar*, followed them (Jaeger *et al.*, 1999; Jaeger *et al.*, 1994). In contrast, 3D structures of bacterial lipases were determined slowly. The first bacterial lipase structure, from *Pseudomonas glumae*, was clarified in 1993 (Noble *et al.*, 1993). Many bacterial lipases have been studied structurally since 1993.
After the determination of first lipase structures, it was realized that they shared a common folding pattern in spite of not showing sequence similarity (Fan et al., 2008). This folding pattern was identified by Ollis et al., (1992). Ollis and his friends compared five hydrolytic enzymes which were dienelactone hydrolase, haloalkane dehalogenase, wheat serine carboxypeptidase II, acetylcholinesterase and the lipase from *Geotrichum candidum*. They concluded that they share a same folding pattern called α/β hydrolase fold. Because they all catalyze a hydrolysis reaction (Arpigny and Jaeger 1999; Nardini and Dijkstra 1999). So, the alpha/beta-hydrolase fold family consists of structurally related enzymes with diverse catalytic functions.

Despite differences in size, sequence homology, substrates and other properties, most of them adopt a similar core topology, known as the α/β hydrolase fold (Ollis et al., 1992; Gandhi, 1997; Bornscheuer et al., 2002). The interior topology of α/β hydrolase fold proteins is composed largely of parallel β-sheets (at least five in lipases), separated by stretches of α-helix, and forming, overall, a superhelically twisted-pleated sheet. Helicalpeptide sections packed on both faces of this sheet form much of the outer surface of the protein (Cygler et al., 1997; Villeneuve et al., 2000). Lipases belong to different protein families with very low sequence similarity (Arpigny and Jaeger, 1999). However, most lipases have a conserved serine active site region, the Gly/Asp-x-Ser-x-Gly-motif, and a relatively weakly conserved region corresponding to the lipase oxyanion hole, which located 58–100 amino acid residues upstream of serine active site (Jaeger et al., 1999).

The α/β hydrolase fold includes a central, mostly parallel eight- stranded β sheet (only the second β strand is antiparallel ) surrounded on both sides by α helices (Figure
1). The β sheet displays a left-handed superhelical twist and the first and the last allowing it to become accessible for the substrate (Angkawidjaja and Kanaya, 2006). However, this phenomenon does not include all lipases. Remarkable exceptions are the lipases from *P. glumae* and *C. antarctica B*. In spite of having a lid, these lipases do not show interfacial activation (Schmid and Verger, 1998). Some *Pseudomonas* lipases are shown to have a calcium binding site (Figure 2). This calcium binding site is located near the active site, but it is not related to catalytic activity. It is thought to play a role in stabilization of the general structure of the enzyme.

### 2.4 APPLICATIONS OF LIPASE

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications. Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, production of cosmetics, and pharmaceuticals (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.*, 2001) and polyurethane (Takamoto *et al.*, 2001). Major applications of lipases are summarized in Table 1 (Vulfson, 1994; Sharma *et al.*, 2001).
Figure 1 The α/β hydrolase fold (Source: Jaeger et al., 1999)

Figure 2 The 3D structure of the lipase from Pseudomonas aeruginosa (Source: Jaeger and Reetz 1998)
Lipases are used in two distinct fashions. They are used as biological catalysts to manufacture other products (such as food ingredients) and by their application as such (in making fine chemicals). Nowadays commercial use of lipase is a billion dollar business that comprises a wide variety of different applications. About 1000 tons of lipase is sold every year in the field of detergent (Azim et al., 2001; Sharma et al., 2011). Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume. (Jaeger et al., 1999).

The enzymes which are currently used in industry are of microbial origin and are produced in conventional aerobic submerged fermentations, which allow greater control of the conditions of growth than solid-state fermentations (Cheetham, 1995). Lipases have received increased attention recently, evidenced by the increasing amount of information about lipases in the current literature. The renewed interest in this enzyme class is due primarily to investigations of their role in pathogenesis and their increasing use in biotechnological applications.

The usefulness of bacterial lipase in commerce and research stems from its physiological and physical properties.

- A large amount of purified lipase could become available, i.e. ease of mass production.
- Bacterial lipases are generally more stable than animal or plant lipases.
- Lipases are active under ambient conditions and the energy expenditure required to conduct reactions at elevated temperatures and pressures is eliminated that reduces the destruction of labile reactants and products.
• Thermophilic microorganisms and enzymes stable at high temperatures and adverse chemical environments are of advantage in industrial uses.
• Due to specificity of enzymes, unwanted side products that normally appear in the waste stream are reduced or eliminated.
• The use of enzymes can decrease the side reactions and post-reaction separation problems.
• Lipase catalysed processes offer cost-effectiveness too, in comparison with traditional downstream processing.
• Lipases remain active in organic solvents in their industrial applicability.
• When immobilized lipases are used under typical ‘industrial’ conditions, reactor temperatures as high 70°C are possible for prolonged periods.

2.4.1 Lipases in food processing, flavour development and improving quality

In the present day, fat and oil modification is one of the prime areas in food processing industry that demands novel economic and green technologies. Tailored vegetable oils with nutritionally important structured triacylglycerols and altered physicochemical properties have a big potential in the future market. Microbial lipases which are regiospecific and fatty acid specific are of immense importance and could be exploited for retailoring of vegetable oils. Cheap oils could also be upgraded to synthesize nutritionally important structured triacylglycerols like cocoa butter substitutes, low calories triacylglycerols and oleic acid enriched oils. Lipase mediated modifications are likely to occupy a prominent place in oil industry for tailoring structured lipids since enzymatic modifications are specific and can be carried out at moderate reaction conditions (Gupt et al., 2003).
Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheeses, the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream. The free fatty acids generated by the action of lipases on milk fat endow many dairy products, particularly soft cheeses, with their specific flavour characteristics. Thus the addition of lipases that primarily release short chain (mainly C4 and C6) fatty acids lead to the development of a sharp, tangy flavour, while the release of medium chain (C12, C14) fatty acids tend to impart a soapy taste to the product. In addition, the free fatty acids take part in simple chemical reactions, as well as being converted by the microbial population of the cheese. This initiates the synthesis of flavour ingredients such as acetoacetate, beta-keto acids, methyl ketones, flavour esters and lactones.

Lipases have also been used for addition to food to modify flavour by synthesis of esters of short chain fatty acids and alcohols, which are known flavour and fragrance compounds (Macedo et al., 2003). Psychrotrophic Gram-negative bacteria, such as *Pseudomonas* species, pose a significant spoilage problem in refrigerated meat and dairy products due to secretion of hydrolytic enzymes, especially lipases and proteases. This study characterized the enzymes produced by strains of *P. fluorescens* isolated from pasteurized milk (Rajmohan et al., 2002).

In confectionary, 1,3-regioselectivity of lipases was exploited in the process development of a fat production containing high concentration of 1,3-disteraroyl-2-monoloein. This fat could be used as a substitute for sheastearine in the formulation of cocoa butter equivalents. Fats designed to inhibit bloom formation in chocolate
products have also been produced by these types of enzyme esterification reactions. *C. rugosa* lipases have many applications in the food and flavour industry, in the production of ice cream and single cell protein, biocatalytic resolution of life saving pharmaceuticals, carbohydrate esters and amino acid derivatives not obtainable by conventional chemical synthesis (Benjamin and Pandey, 1998; Aravindan *et al.*, 2007).

Uhling (1998) has explained the preparation of lipase modified butter fat which found wide application in various food processes. Chocolates with coco butter substitutes, bread, structured lipids like human milk fat replacers, low calorie health oils, nutraceuticals, EMC, etc. are few examples for lipase mediated food products. Since it is a new technology, more research on usage of lipase to develop new commercial food products must be promoted. Oil from soybean is hydrolyzed by lipase in making Koji, a traditional Asian food (Ang *et al.*, 1999). This Tempeh forms a base material for many delicious, easily digestible and nutritious food preparations, providing a good number of human populations with a valuable and affordable source of protein. In addition to Tempeh, Kenkey and Mave (African food) coupled with fermented vegetables and *salads* should be noted in this context (Pandey *et al.*, 1999).
Table 1 Major application of microbial Lipases
(Vulfson, 1994; Sharma et al., 2001)

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<tr>
<th>Industry</th>
<th>Action</th>
<th>Product of application</th>
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<tr>
<td>Detergents</td>
<td>Hydrolysis of fats</td>
<td>Removal of oil strain from fabrics</td>
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<tr>
<td>Chemicals</td>
<td>Enantioselectivity, synthesis</td>
<td>Chiral building blocks, chemicals</td>
</tr>
<tr>
<td>Dairy foods</td>
<td>Hydrolysis of milk fat, cheese ripening,</td>
<td>Development of flavouring agents in milk, cheese and butter</td>
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<td></td>
<td>modification of butter fat.</td>
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<td>Bakery foods</td>
<td>Flavour improvement</td>
<td>Shelf-life propagation.</td>
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<td>Beverages</td>
<td>Improved aroma</td>
<td>Alcoholic beverages, e.g. sake, wine</td>
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<td>Food dressings</td>
<td>Quality improvement</td>
<td>Mayonnaise, dressings and whippings</td>
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<td>Health foods</td>
<td>Transesterification</td>
<td>Health foods</td>
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<td>Meat and fish</td>
<td>Flavour development</td>
<td>Meat and fish product, fat removal</td>
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<tr>
<td>Fats and oils</td>
<td>Transesterification, hydrolysis</td>
<td>Cocoa butter, margarine, fatty acids, glycerol, mono and diglycerides</td>
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<td>Pharmaceuticals</td>
<td>Transesterification, hydrolysis</td>
<td>Specialty lipids digestive aids</td>
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<td>Cosmetics</td>
<td>Synthesis</td>
<td>Emulsifiers, moisturizers</td>
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<td>Paper</td>
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<td>Paper with improved quality</td>
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<td>Leather</td>
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<td>Cleaning</td>
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<td>Removal of fats</td>
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2.4.2 Detergent industry

After the great commercial success of proteases as detergent additives, the enzyme industry undertook major efforts to introduce lipases as a second group of detergent enzymes. It was hoped that lipases could compete with chemical surfactants in the detergent formulation, and thus respond to changing detergent formula in view of lower wash temperatures and ecologically benign components. Standard wash liquids contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 50°C, which is a rather hostile environment for enzymes. As a result, major screening programs for lipases stable under such conditions were initiated. Early systematic studies with the then available lipases showed that the marginal effect of lipases could be substituted, at lower price, by the addition of suitable chemical surfactants to the formulation (Fujita et al., 1992; Schmid and Verger, 1998).

Because of their ability to hydrolyze fats, lipases find a major use as additives in industrial laundry and household detergents. Detergent lipases are especially selected to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyze fats of various compositions; (2) ability to withstand relatively harsh washing conditions (pH 10-11, 30-60 °C); (3) ability to withstand damaging surfactants and enzymes [e.g., linear alkyl benzene sulfonates (LAS) and proteases], which are important ingredients of many detergent formulations. Lipases with the desired properties are obtained through a combination of continuous screening (Yeoh et al., 1986; Wang et al., 1995; Cardenas et al., 2001) and protein engineering (Kazlauskas and Bornscheuer, 1998).
In 1994, Novo Nordisk introduced the first commercial recombinant lipase ‘Lipolase,’ which originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced ‘Lumafast’ from *Pseudomonas mendocina* and ‘Lipomax’ from *P. alcaligenes* by Genencor International (Jaeger and Reetz, 1998). Jaeger and Reetz (1998) showed that about 1000 tonne of lipases are added annually to approximately 13 billion tonne of detergents produced. Gerritse *et al.*, (1998) reported an alkaline lipase, produced by *P. alcaligenes* M-1, which was well suited to removing fatty stains under conditions of a modern machine wash. The patent literature contains examples of many microbial lipases that are said to be suitable for use in detergents (Bycroft and Byng, 1992).

Godfrey and West (1996) reported that about 1000 tonne of lipases are sold every year in the area of detergents. The use of cold active lipase in the formulation of detergents would be of great advantage for cold washing that would reduce the energy consumption and wear, tear of textile fibers and protect the color of fabrics (Feller and Gerday, 2003). Enzymes can reduce the environmental load of detergent products since they save energy by enabling a lower wash temperature to be used; allow the content of other often less desirable chemicals in detergents. Addition of cold active lipase in detergent become biodegradable, leaving no harmful residues, have no negative impact on sewage treatment processes and do not present a risk to aquatic life. Commercial preparations used for the desizing of denim and other cotton fabrics contain both alpha amylase and lipase enzymes. Lipases are stable in detergents containing protease and activated bleach systems. Lipase is an enzyme, which decomposes fatty stains into more hydrophilic substances that are easier to remove than similar non-hydrolyzed stains (Fuji *et al.*, 1986; Babu *et al.*, 2007).
2.4.3 Medical and pharmaceutical application

Lipases are currently being used by many pharmaceutical companies worldwide for the preparation of optically active intermediates on a kg scale. A number of relatively small biotechnological companies, such as Enzymatix in the U.K, specialise in biotransformations and offer a whole variety of intermediates prepared via lipase mediated resolution. A Regioselective modification of polyfunctional organic compounds is yet another area of expanding lipase application. In many cases, lipases have been shown to acylate or deacylate selectively one or several hydroxyl groups of similar reactivity in carbohydrates, polyhydroxylated alkaloids and steroids. Apart from the synthesis of sugar based surfactants, lipases were successfully applied in the regioselective modification of castanospermine, a promising drug for the treatment of AIDS (Stinson, 1995).

Enantioselective interesterification and transesterification have great significance in pharmaceutical for selective acylation and deacylation (Stinson, 1995). Lipases are important in application in pharmaceuticals in transesterification and hydrolysis reaction. They play a prime role in production of specialty lipids and digestive aids (Vulfson, 1994). The alteration of temperature during the esterification reaction drastically changes the enantiomeric values and also the stereopreference (Yasufuku and Ueji, 1996). Lipases play an important role in modification of monoglycerides for use as emulsifiers in pharmaceutical applications (Sharma et al., 2001).

Psychrophiles producing cold active lipases may be a good source for polyunsaturated fatty acids for the pharmaceutical industry. It is because of their
excellent capability for specific regioselective reactions in a variety of organic solvents with broad substrate recognition makes lipases as an important biocatalyst in biomedical applications (Margesin et al., 2002). A preparation of optically active amines that was intermediate in the preparation of pharmaceuticals and pesticides, which involved in reacting stereospecific N-acylamines with lipases, preferably from Candida antarctica or Pseudomonas sp. (Smidt et al., 1996). In an attempt to determine the substrate specificity for lipases, alkyl esters of 2-arylpionic acids, a class of non-steroidal anti-inflammatory drugs, were hydrolyzed with Caenorhabditis rugosa lipase in which all transformations were highly enantio selective (Botta et al., 1997).

Lipases and/or esterases isolated from the wax moth (Galleria mellonella) were found to have a bactericidal action on Mycobacterium tuberculosis (MBT) H37Rv. This preliminary study may be regarded as part of global unselected screening of biological and other materials for detecting new promising sources of drugs (Annenkov et al., 2004). Lipase from Candida rugosa has been used to synthesize lovastatin, a drug that lower serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride, a widely used coronary vasodilator, was carried out with S. marcescens lipase (Matsumae et al., 1993).

2.4.4 Cosmetics and perfumery

Although the cost of lipase catalyzed esterification remains too high for the manufacturing of bulk products, the synthesis of several specialty esters has found its way in the market place. Unichem International has launched the production of isopropyl myristate, isopropyl palmitate and 2-ethylhexylpalmitate for use as an
emollient in personal care products such as skin and sun-tan creams, bath oils etc. Wax esters (esters of fatty acids and fatty alcohols) have similar applications in personal care products and are also being manufactured enzymatically (Croda Universal Ltd). Metzger and Bornscheuer, (2006) reported that the lipases have potential application in cosmetic and perfumeries because it shows activities in surfactants and in aroma production. Monoacyl glycerol and diacylglycerols are produced by esterification of glycerols and are used as surfactant in cosmetics and perfume industries.

2.4.5 Production of biodegradable polymers

Lipases have become one of the most important groups of enzymes for its applications in organic syntheses. Lipases can be used as biocatalyst in the production of useful biodegradable compounds. 1-Butyl oleate was produced by direct esterification of butanol and oleic acid to decrease the viscosity of biodiesel in winter use. Trimethylolpropane esters were also similarly synthesized as lubricants. Lipases can catalyze ester syntheses and transesterification reactions in organic solvent systems has opened up the possibility of enzyme catalyzed production of biodegradable polyesters. Aromatic polyesters can be synthesized by lipase biocatalysis (Linko et al., 1998).

2.4.6 Use of lipase in textile industry

Lipases are used in the textile industry to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems. Commercial preparations used for the desizing of denim and other cotton fabrics,
contains both alpha amylase and lipase enzymes (http://science.ntu.ac.uk/research/EnzyTex/EnzRep1.html, 2000).

In the textile industry, polyester has certain key advantages including high strength, soft hand, stretch resistance, stain resistance, machine washability, wrinkle resistance and abrasion resistance. Synthetic fibers have been modified enzymatically for the use in the production of yarns, fabrics, textiles, rugs and other consumer items. It relates to modification of the characteristics of a polyester fiber so that such polyesters are more susceptible to post-modification treatments. The use of polyesterase (closely related to lipase) to improve the ability of a polyester fabric to uptake chemical compounds, such as cationic compounds, fabric finishing compositions, dyes, antistatic compounds, anti-staining compounds, antimicrobial compounds, antiperspirant compounds and/or deodorant compounds (http://www.wipo.int, 2000).

2.4.7 Oleochemical industry

Alcoholysis, acidolysis, hydrolysis and glycerolysis are the common reactions associated with oleochemical industry. These reactions are energy intensive with high temperature requirement of 240 to 260°C and high pressure. However, utilization of lipases can minimize the energy consumption and minimizes thermal degradation during alcoholysis, acidolysis, hydrolysis, and glycerolysis (Vulfson, 1994; Bornscheuer, 2000, Sharma et al., 2001). For instance, commercial use of Candida cylindracea lipase in production of soaps was reported. The enzymatic method resulted in a superior product at lesser cost than the conventional chemical process (Saxena et al., 1999). The current trend in the oleochemical industry involves the use of
immobilized lipases to initiate various reactions (hydrolysis, alcoholysis, and glycerolysis) using mixed substrates. Thus, the use of immobilized enzyme ensures high productivity as well as continuous running of the processes. This offers a greatest hope for successful fat splitting/modification without substantial investment in expensive equipment as well as in expenditure of large amounts of thermal energy (Saxena et al., 1999).

Although lipases are designed by nature for the hydrolytic cleavage of the ester bonds of triacylglycerol, lipases can catalyze the reverse reaction (ester synthesis) in a low water environment. Hydrolysis and esterification can occur simultaneously in a process known as interesterification. Depending on the substrates, lipases can catalyze acidolysis (where an acyl moiety is displaced between an acyl glycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an acyl glycerol and an alcohol), and transesterification (where two acyl moieties are exchanged between two acylglycerols) (Balcao et al., 1996).

2.4.8 Lipases in resolution of racemic acids and alcohols

The stereospecificity as a unique feature of lipases is widely employed in identification of racemic organic acid mixtures in immiscible biphasic systems via esterification and transesterification reactions (Klibanov, 1990; Sharma et al., 2001). Racemic alcohols can also be resolved into enantiomerically pure forms by lipase-catalyzed transesterification. Arroyo and Sinisterra (1995) reported that esterification reaction in nonaqueous media using lipase-B from Candida antarctica was stereoselective towards the R-isomer of ketoprofen in an achiral solvent such as isobutyl methyl ketone and (S+)-carvone. Tsai and Dordick (1996) studied the
characteristics of both pure and crude lipases isolated from *Candida rugosa* in aqueous and organic solvents. The purified enzyme was found to be less active than the crude enzyme in organic media, whereas presence of small quantity of water stimulated the activity of the purified enzyme by several folds in the esterification of racemic 2-(4-chlorophenoxy) propanoic acid with nbutanol.

Profens (2-aryl propinoic acids), an important group of nonsteroidal anti-inflammatory drugs, are pharmacologically active mainly in the (S)-enantiomer form (Hutt and Caldwell, 1984). For instance, (S)-ibuprofen [(S)-2(4-isobutylphenyl) propionic acid] is 160 times more potent than its antipode in inhibiting prostaglandin synthesis. Consequently, considerable effort is being made to obtain optically pure profens through asymmetric chemical synthesis, catalytic kinetic resolution (Van Dyck *et al.*, 2001; Xin *et al.*, 2001), resolution of racemate via crystallization, and chiral chromatographic separations. Microorganisms and enzymes have proved particularly useful in resolving racemic mixtures. Thus, pure (S)-ibuprofen is obtained by using lipase-catalyzed kinetic resolution via hydrolysis (Lee *et al.*, 1995) or esterification (Ducret *et al.*, 1998; Xie *et al.*, 1998). Similarly, 2-phenoxy-1-propanol was resolved into its enantiomers using *Pseudomonas* sp. lipase by enantioselective transesterification (Miyazawa *et al.*, 1998). Weber *et al.* (1999) reported solvent-free thioesterification of fatty acids with long-chain thiols catalyzed by lipases from *Candida antarctica* and *R. miehei*. Also, solvent-free trans-thioesterification of fatty acid methyl esters with alkane thiols was reported (Weber *et al.*, 1999).
2.4.9 Ester synthesis

Low molecular weight esters such as ethyl, isobutyl, amyl and isoamyl acetates have been widely used for flavor development in the food industry. Flavour substances such as S-methyl butanethioate and S-methyl 3-methyl butanethioate are important components of the dairy aromas, especially cheese aroma and of fruit aromas like strawberry and banana (Shieh and Chang, 2001; Rajendran et al., 2009). Lipases have been successfully used as catalyst for synthesis of all these esters based on their unique specificity, high reaction rate even at low molar fractions and activity in organic solvents (Salihu and Zahangir, 2012).

The esters produced from short-chain fatty acids have applications as flavoring agents in food industry (Vulfson, 1994). Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels (Vulfson, 1994). From et al., (1997) studied the esterification of lactic acid and alcohols using a lipase of C. antarctica in hexane. Esterification of five positional isomers of acetylenic fatty acids (different chain lengths) with n-butanol was studied by Lie et al., (1998), using eight different lipases. Arroyo et al., (1999) noted that an optimum pre-equilibrium water activity value was necessary for obtaining a high rate of esterification of (R, S)-ibuprofen. Janssen et al., (1999) reported on the esterification of sulcatol and fatty acids in toluene, catalyzed by Candida rugosa lipase (CRL). Krishnakant and Madamwar (2001) reported using lipase immobilized on silica and microemulsion-based organogels, for ester synthesis. Transesterification of hexanol and tributyrin by immobilized lipase (Lipozyme IM-77) from Rhizomucor miehei led to the formation of excellent flavour and fragrance.
Esterification of citronellol and geraniol with short-chain fatty acids are widely applicable in beverages production (Chang et al., 2003).

### 2.4.10 Pulp and paper industry

Pitch or the hydrophobic components of wood (mainly triglycerides and waxes), causes severe problems in pulp and paper manufacture. The enzymatic pitch control method using lipase was put into practice in a large scale paper making process as a routine operation in the early 1990s and was the first case in the world in which an enzyme was successfully applied in the actual paper making process (Bajpai, 1999). Lipases are used to remove the pitch from the pulp produced for paper making. Nippon Paper Industries, Japan, have developed a pitch control method that uses the *Candida rugosa* fungal lipase to hydrolyze up to 90% of the wood triglycerides (Jaeger and Reetz, 1998).

### 2.4.11 Lipases as Biosensor

The quantitative determination of triacylglycerol is of great importance in clinical diagnosis and in food industry. The lipid sensing device as a biosensor are rather cheaper and less time consuming as compared to the chemical methods for the determination of triacylglycerols. Biosensor can be of three types (a) Chemical (b) Biochemical or (c) electronic. Biochemical biosensor utilizes the enzymes or other proteins (antibodies), cells or cell extract immobilized on a suitable matrix linked to a transducer. An analytical biosensor was developed for the determination of lipids for the clinical diagnosis (Masahiko et al., 1995). Here, in quantitative determination of lipases are used to generate glycerol from triacylglycerol in the analytical sample and to
quantify the released glycerol by enzymatic or chemical methods. This principle enabled the physician to diagnose the patients with cardiovascular complaint. *Candida rugusa* lipase biosensor has been developed as a DNA probe (Benjamin and Pandey, 2001).

A biosensor based on the enzyme-catalysed dissolution of biodegradable polymer films has been developed. The polymer enzyme system; poly (trimethylene) succinate, was investigated for use in the sensor, which is degraded by a lipase. Potential fields of application of such a sensor system are the detection of enzyme concentrations and the construction of disposable enzyme based immunosensors, which employ the polymer degrading enzyme as an enzyme label (Sumner *et al.*, 2001).

Radiolabelled polynucleotide probes have been employed extensively for the detection of complementary nucleic acids by specific hybridization. Within the last few years, various methods have been developed using enzyme-labeled probes to avoid unstable and hazardous isotopes. By screening various hydrolytic enzymes to fit the special demands, fungal lipases turned out to be the most practical (Kynclova *et al.*, 1995). Lipases may be immobilized onto pH/oxygen electrodes in combination with glucose oxidase, and these function as lipid biosensors (Karube and Sode, 1998) and may be used in triglycerides (Iwai, 1990) and blood cholesterol determinations (Imamura *et al.*, 1989).

### 2.4.12 Biodiesel production

The process of production of biodiesel as an alternative fuel using natural oils and fats is environment friendly since these substrates are free of nitrogen and sulphur
compounds. This process will markedly reduce the greenhouse effect and air pollution produced by the fossil fuels (Li et al., 2009). The biodiesel production can be achieved by chemical or enzymatic methods. The conversion of oils to methyl- or other short-chain alcohol esters can be obtained in a single transesterification reaction using lipases in organic solvents (Jaeger and Eggert, 2002). Thus, several problems associated with chemical production that impede its continued growth, such as glycerol recovery and the need to use refined oils and fats as main substrates can be overcome by enzymatic transesterification reactions (Kramer, 1995).

Haas and Foglia (2005) reported that residual oil from soy, rapeseed, and palm oil refining waste extracted and recovered from hexane when subjected to methanolysis by R. oryzae lipase in the presence of water content and methanol; highest conversion to methyl esters was observed in palm oil with about 55% yield after 96 h reaction. Thus, several strategies are now suggested to harness some of the production problems especially in increasing the enzyme’s stability upon repeated use by immobilization (Iso et al., 2001) and overproduction of lipases in the target organisms by genetic manipulation for efficient methanolysis in a solvent-free reaction system (Matsumoto et al., 2001).

Immobilized P. cepacia lipase was used for the transesterification of soybean oil with methanol and ethanol (Noureddini et al., 2005). Fatty acid ethyl esters have also been prepared from castor oil using n-hexane as solvent and two commercial lipases, Novozym 435 and Lipozyme IM, as catalysts (de Oliveira et al., 2004). Novozyme 435 have also been used to catalyse the transesterification of crude soybean oils for biodiesel production in a solvent-free medium (Du et al., 2004).
Simple alkyl ester derivatives of restaurant grease were prepared using immobilized lipases from *Thermomyces lanuginose* and *C. antarctica*, as biocatalysts (Hsu *et al.*, 2002). Fatty acids esters were produced from two Nigerian lauric oils, palm kernel oil and coconut oil, by transesterification of the oils with different alcohols using PS30 lipase as a catalyst. In the conversion of palm kernel oil to alkyl esters (biodiesel), ethanol gave the highest conversion of 72%. Some of the fuel properties compared favourably with international biodiesel specifications (Abigor *et al.*, 2000). Despite its importance, studies on the mechanisms of production of microbial lipases and the role of lipidic substances used as inducers in lipase production are scarce (Shimada *et al.*, 1992). Lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, thereby presenting a fascinating field for future research (Jaeger *et al.*, 1994).

### 2.4.13 Environmental applications

There are number of uses of the cold active enzymes, presently it is conceivable that they could be used for environmental bioremediation e.g., as a biodegradable means of treating an oil spill. Bioremediation for waste disposal is a new avenue in lipase biotechnology. Cheng *et al.*, (1997) characterized cold-adapted organophosphorus acid anhydrrolases for application in the efficient detoxification of pesticide and nerve agents. According to Buchon *et al.*, (2000), cold adapted lipases have great potential in the field of wastewater treatment, bioremediation in fat contaminated cold environment, and active compounds synthesis in cold condition. Further, more efforts are needed in identifying and cloning of novel lipase genes for environmental applications.
Suzuki et al., (2001) identified a psychrotrophic strain of the genus *Acinetobacter* strain No. 6, producing an extracellular lipolytic enzyme that efficiently hydrolyzed triglycerides, such as soybean oil during bacterial growth even at 4°C. The strain degraded 60% of added soybean oil (initial concentration, 1% w/v) after cultivation in LB medium at 4°C for 7 days. The psychrophilic microorganisms as well as their enzymes have been proposed as alternative to physicochemical methods for bioremediation of solids and waste waters polluted by hydrocarbons, oils and lipids (Margesin et al., 2002). Ramteke et al., (2005) stated that in temperate regions, large seasonal variations in temperature reduce the efficiency of microorganisms in degrading pollutants such as oil and lipids. The lipase active at low and moderate temperature may also be ideal for bioremediation process.

Oil spills in refinery, shore sand and processing factories could be handled by the use of lipases from different origins (Nakamuara et al., 1994). It has also been used for the degradation of wastewater contaminants such as olive oil from oil mills. Another important application has been reported for the degradation of polyester waste, removal of biofilm deposits from cooling water systems and also to purify the waste gasses from factories (Anonymous, 1995). Margesin et al., (1999) have found that monitoring of soil microbial lipase activity is a valuable indicator of diesel oil biodegradation in freshly contaminated, unfertilized and fertilized soils. Fungal species can be used to degrade oil spills in the coastal environment, which may enhance ecorestoration as well as in the enzymatic oil processing in industries (Gopinath et al., 1998).
2.5 SOURCES OF LIPASES

Lipases occur widely in nature and can be produced by many microorganisms and higher eukaryotes. In animals, lipases obtained from pig and human pancreas are best known and more investigated than all other lipases. In these organisms, they are engaged in several lipid metabolism steps, including fat digestion, adsorption, reconstitution, and in lipoproteins metabolism. In plants, lipases are present in higher plants seeds, as castor bean and canola (Brassica napus). They are also found in several plants energy reserve tissues (Sharma et al., 2001; Jaeger and Eggert, 2002; Villeneuve, 2003; Cavalcanti et al., 2007). However, for the production of industrial enzymes, microorganisms are the preferred source, once they have shortest generation time, high yield of conversion of substrate into product, great versatility to environmental conditions and, simplicity in genetic manipulation and in cultivation conditions. Due to habitats’ multiplicity, microorganisms usually produce various lipases types, with distinct specificity regarding to substrate utilization and also to optimum pH and temperature range. Lipases can be produced by bacteria, filamentous fungi, and yeasts, allowing these microorganisms to use lipids from animal or vegetable origin as carbon and energy sources for their growth. Though many microorganisms have been reported in literature as lipase producers, the genera Candida, Rhizopus, and Pseudomonas are considered the main industrial sources of lipases (Ribeiro et al., 2011).

2.5.1 Animal sources

Higher animal's lipases are well characterized (Ben Bacha et al., 2005; Fendri et al., 2006) in contrast, much less is known about lipases from lower ones. Since the detection of the presence of lipase activity in the gastric juice of some crustaceans
(Homarus americanus) and mollusks (Aplysia californica) (Brockerhoff, 1974), a number of investigations on invertebrate lipases have been reported. Recently, lipases were isolated from digestive glands of lower animals, such as the scorpion (Zouari et al., 2006) and the crab (Cherif et al., 2007). The purified enzymes showed no similarity with any known lipases. Amara et al., (2010) in their study, they purified snail digestive lipase (SnDL) from digestive glands (hepatopancreas) of Eobania vermiculata. Pure SnDL has a molecular mass of 60 kDa; it does not present the interfacial activation phenomenon. It was found to be more active on short-chain triacylglycerols than on long-chain triacylglycerols. Lipase isolated from silkworm larval alimentary canal shows strong antiviral activity against BmNPV, providing evidence that digestive juice may play an important role during peroral infection with BmNPV (Ponnuvel et al., 2003).

2.5.2 Plant sources

In plants mostly lipases are present in the form of food reserve tissues of growing seedlings or especially in those which contains large amount of triacylglycerols. Lipase activity in plant seeds increases during germination because the triacylglycerols are converted to soluble sugars by the action of lipase which is then transported to the growing tissues to supply structural carbon and energy to provide support for the growth of young plants. The sources and properties of plant lipases are given in Table 2. Plant lipases are characterized for pH, temperature and molecular weight. It is interesting to note that, pH range is in between 4.0 to 8.0, temperature range is in between 25 to 60 °C, whereas, molecular weight varies from 40 to 143 kDa. This data indicates that relatively plant lipases are slightly different from bacterial and
fungal lipases (Patil et al., 2011). Pierozan et al., (2011) studied the immobilization and partial characterization of lipases from wheat seeds (Triticum aestivum). The optimum pH and temperature were found to be 5.5 and 32–37 °C, respectively. The stability of the concentrated enzymatic extract to high temperatures (25, 35, 45, and 55 °C) showed that the incubation of the extract at 55°C led to its complete inactivation.

2.5.3 Microbial sources

Since the mid 1980s, there has been a growing interest in lipases, especially in those of microbial origin, due to the vast potential regarding to their usage in different industries. The interest in microbial lipase production has increased in the last decades (Rajesh et al., 2010). Due to the versatility of the molecular structure and catalytic properties, these enzymes have potential biotechnological application in different industrial sectors (Cavalcanti et al., 2005). Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Wiseman, 1995). Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases. Only about 2% of world microorganisms have been tested as enzyme sources. Microbial lipases are produced mostly by submerged culture (Ito et al., 2001). Bacterial strains are generally more used as they offer higher activities compared to yeasts (Frost and Moss, 1987) and tend to have neutral or alkaline pH optima and are often thermostable.

2.5.3.1 Fungal Lipase

Both filamentous fungi and yeasts are potent lipase producers. Most of the fungal enzymes are extracellular in nature. Filamentous fungi are amenable for
cultivation by solid substrate fermentation (SSF) using cheap substrates and therefore have the advantage of considerably reducing cost of lipase production. Even when cultured in submerged fermentation (SmF) mode, fungal lipases are easily amenable for extraction thereby significantly reducing the recovery cost. Thus, low cost production and extraction makes fungal lipases preferable than bacterial lipases (Saisubramanian, 2012).

Lipase-producing fungi are widespread in nature and can be isolated from diverse habitats from oil-contaminated soils to hot springs including compost heaps, coal tips, and industrial wastes (Gunasekaran and Das, 2005). Isolation of 860 lipase-producing filamentous fungal strains from soil samples around the world using enrichment culture techniques have been reported (Cardenas et al., 2001). The nutritional composition of growth media including carbon and nitrogen sources and its physiochemical properties influence the amount of lipase produced by the fungi. Many authors have been reviewed the taxonomic distribution of fungal lipases in a detailed manner (Ghosh et al., 1996; Singh and Mukhopadhyay, 2012). Applications of major classes of fungal lipase have been compiled in an exhaustive manner earlier (Pandey et al., 1999; Gandhi, 1997). A recent review has provided a comprehensive overview of sources, isolation, purification, and applications of fungal lipases (Singh and Mukhopadhyay, 2012). Fungal lipase with thermostable properties has been reported earlier from Rhizopus miehei, H. lanuginosa, Mucor pusillus, Aspergillus terreus, and R. homothallicus. Although thermostable lipases are common, reports on alkaline thermostable lipase are scarce; edible Basidiomycete Antrodia cinnamomea was reported to produce thermostable alkaline lipase upon addition of glucose to the medium (Lin and Ko, 2005).
Yadav et al., (1998) screened *Aspergillus* sp and *Penicillium* sp for extracellular lipase production on agar plates and in liquid media containing olive oil as substrate. Based on the results, ten best lipase-producing *Aspergillus* sp. are *A. alliaceus*, *A. candidus*, *A. carneus*, *A. fischeri*, *A. niger*, *A. ochraceus*, *A. parasiticus*, *A. sundarbanii*, *A. terreus* and *A. versicolor*, and nine best *Penicillium* sp. include *P. aurantiogriseum*, *P. brevicompactum*, *P. camemberti*, *P. chrysogenum*, *P. corynbiferum*, *P. crustosum*, *P. egyptiacum*, *P. expansum* and *P. spiculisporum*. Enrichment culture techniques were used to isolate fifty nine lipase producing fungal strains from Brazilian savanna soil. A strain of *Colletotrichum gloesporioides* was found to be the best in producing alkaline lipases (Colen et al., 2006).

Some of the most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. Lipase production by filamentous fungi varies according to the strain, the composition of the growth medium, cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources (Cihangir and Sarikaya, 2004).

Among yeast, major genera of lipase producers include *Candida*, *Rhodotorula*, *Yarrowia*, *Geotrichum*, and *Trichosporon*. *C. rugosa* lipase (CRL) by virtue of being considered safe is frequently employed in food applications and in a variety of industrially relevant biotransformations (Dominguez de Maria et al., 2006). According to Vakhlu and Kour (2006), the main terrestrial species of yeasts that were found to produce lipases are: *Candida rugosa*, *C. tropicalis*, *C. antarctica*, *C. cylindracea*, *C. parapsilosis*, *C. deformans*, *C. curvata*, *C. valida*, *Yarrowia lipolytica*, *Rhodotorula*...
glutinis, R. pilimormae, Pichia bispora, P. mexicana, P. sivicola, P. xylosa, P. burtonii, Saccharomyces crataegenesis, Torulaspora globosa, and Trichosporon asteroids. The genes that encode lipase in Candida sp., Geotrichum sp., Trichosporon sp., and Y. lipolytica have been cloned and over-expressed (Wang et al. 2007).

Among the yeasts, Candida sp. is the most potential lipase producer reported in the literature. Lipase produced by Candida rugosa is fast becoming one of the most used industrial enzymes due to its high activity, both in hydrolysis as well as synthesis (Vakhlu and Kour, 2006). A Japanese company has used C. rugosa lipase for the production of fatty acids from castor bean as far back as 1985 (Macrae and Hammond, 1985), and there are several reports using C. rugosa for lipase production (Rajendran et al., 2008; Puthli et al., 2006; Benjamin and Pandey, 2001). Other yeasts reported to be potent lipase producers include Trichosporon asahii (Kumar and Gupta, 2008), Candida cylindracea (Salihu et al., 2012; Brozzoli et al., 2009; D’Annibale et al., 2006; Kim and Hou, 2006); Aureobasidium pullulans (Liu et al., 2008), Saccharomyces cerevisiae (Ciafardini et al., 2006); Yarrowia lipolytica (Dominguez et al., 2003).

Other promising lipase-producing yeast include G. candidum, which shows unique preference towards long-chain cis (delta 9) unsaturated fatty acid groups in its substrate (Holmquist, 1998). Another yeast strain isolated from olive oil Williopsis californica was observed to be a good lipase producer (Ciafardini et al., 2006). Kluyveromyces marxianus was shown to produce thermostable acid-tolerant and organic solvent stable lipase (Deive et al., 2003). Of nine yeasts isolated from phylloplane of Hibiscus rosasinensis, three of them, Pseudozyma hubeiensis HB85A, Debaryomyces occidentalis HB83, and Cryptococcus sp. HB80, were observed to be
potent lipase producers. Among them, *P. hubeiensis* HB85 exhibited 3.2-fold increase in lipase production when cultivated by batch mode (Bussamara *et al*., 2010).

Kumar and Gupta (2008) isolated 15 yeasts from petroleum and oil sludge areas in Delphi (India). The isolates were purified and checked for their lipolytic potential. Among these yeast strains, one strain was selected for further studies, based on the largest halo of lipolysis. On the basis of sequence homology, this strain was found to belong to *Trichosporon asahii* genus and share 99% homology with the already existing database.

### 2.5.3.2 Bacterial Lipases

Bacterial lipases are mostly glycoproteins, but some extracellular lipases are lipoproteins. Production of extracellular lipases from bacteria is often dependent upon nitrogen and carbon sources, inorganic salts, presence of lipids, temperature, and availability of oxygen. Most bacterial lipases reported are constitutive, and they are inhibited by some serine hydrolase inhibitors. Bacteria belonging to various genera are potent lipase producers; these include *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium, Pseudomonas, and Staphylococcus* (Jaeger *et al*., 1994; Ertugrul *et al*., 2007; Haba *et al*., 2000).

Despite the enormous number of lipase producing microrganisms, only a few bacteria species were exploited for the production of cold adapted lipases (Joseph *et al*., 2006). Some of the strains reported to produce lipases at temperatures as low as 5°C include *Acinetobacter* sp., *Achromobacter lipolyticum, Aeromonas hydrophila, Bacillus sphaericus, Photobacterium lipolyticum, Morexella* sp., *Pseudomonas fluorescens,* *P.
Pseudomonas okhotskensis, Serratia marcescens and Staphylococcus epidermidis (Joseph et al., 2007).

Aprigny and Jaeger, (1999) have classified the bacterial lipases based on sequence homology and conserved sequence motifs along with similar biological properties into five subfamilies. Within family I, Pseudomonas lipases belong to subfamily I.1; subfamily I.2 includes Burkholderia lipases. Bacillus and Staphylococcus lipases of subfamily I.2 display Ala-X-Ser-X-Gly motif (replacement of glycine with alanine in the conserved pentapeptide sequence). Pseudomonas fluorescens and Serratia marcescens belong to subfamily I.3, which includes Gram-negative bacterial lipases; Bacillus pumilus and B. subtilis represent subfamily I.4; and B. stearothermophilus and B. thermocatenulatus belong to subfamily I.5.

Even though a variety of lipases are produced from both Gram-positive and Gram-negative bacteria, greater part of bacterial lipases comes from Gram-negative bacteria and the most important Gram-negative genus is Pseudomonas which contains at least seven lipase producing species, that are P. aeruginosa, P. alcaligenes, P. fragi, P. glumae, P. cepacia, P. fluorescens and P. putida (Jaeger et al., 1994; Kojima et al., 2003). Pseudomonas lipases are widely used in various industrial applications such as detergent additives and in racemic resolution (Jaeger and Reetz, 1998). Pseudomonas lipases are encoded by bicistronic operon, which codes for a lipase-specific foldase Lip F and are secreted by Type II or Type I secretory system. Pseudomonas and Burkholderia lipases require lipase-specific foldases (Lifs) for their proper folding and enzymatic activity. Lifs are steric chaperones which lower the energetic barrier for the proper folding of cognate lipases (Rosenau et al., 2004). Successful production of
recombinant Pseudomonas lipases includes concomitant expression of cognate lipase-specific foldases (Reetz and Jaeger, 1998).

Pseudomonas lipases are classified into three groups based on their amino acid homologies and some biological properties (Zhang et al., 2008). Group I contains lipases from P.aeruginosa, P.alcaligenes and P.fragi. Lipases in this group are composed of approximately 285 amino acids with a molecular weight of 30 000 dalton. Also, they include two cysteine residues to form a disulfide bond and need another protein called lipase-specific foldase for correct folding and secretion. Moreover, they include a specific sequence at their N-terminal. Group II lipases contain 320 amino acids corresponding to a molecular weight of 33, 000 Da, one disulfide bond and an N-terminal signal sequence like group I lipases. Additionally, there is 60% amino acid homology between group I and group II lipases and they also require lipase specific foldases in order to be active and secreted into extracellular environment. Lipases from P.glumae and P.cepacia are prototypes for group II. Group III Pseudomonas lipases are larger containing about 475 amino acids and a molecular weight of 50 000 Da. P.fluorescens lipases are prototypes of this group (Arpigny and Jaeger, 1999). Group III lipases are separated from group I and group II lipases in several ways a) they do not contain cysteine residues, b) they do not require any lipase specific foldases and c) they do not contain an N-terminal signal sequence. According to these differences, it is said that group III lipases use a different secretion pathway. Group I and II lipases use type II secretion pathway (also called secreton-mediated secretion) while group III lipases are secreted via type I secretion system (also named ABC exporters) (Rosenau and Jaeger, 2000).
As mentioned above, although group III lipases do not have a typical N-terminal sequence, they contain a C-terminal targeting signal sequence responsible for the secretion of lipase by an ABC exporter (Amada et al., 2000, Duong et al., 1994). The type I secretion pathway includes three different proteins a) an inner membrane ATPase confers the substarete specificity to the system b) membrane fusion protein (MFP) functions like a bridge between the inner and outer membrane because it is connected with both the inner and the outer membrane c) the last protein is an outer membrane protein (Rosenau and Jaeger, 2000; Jaeger et al., 1999). Lipases are secreted directly into the extracellular area by this system in which there is no need for an extra step like the formation of enzymatically active periplasmic intermediates appeared in type II secretion pathway.

On the other hand, group I and group II lipases have an N-terminal signal sequence which is used for efficient secretion through the inner membrane by a Sec dependent mechanism. This mechanism contains a multisubunit protein complex called Sec translocase that recognizes the N-terminal sequences of lipases. While lipase is secreted through the inner membrane, signal sequence is removed and lipase interact with its specific foldase as well (Rosenau et al., 2004). As a result, lipases fold into an enzymatically active conformation in the periplasm. Moreover, this process is assisted by the Dsb (disulfide bond formation)-proteins which catalyzes the formation of disulfide bonds. Alternatively, misfolded lipases may be degraded by periplasmic proteases. Finally, lipases are transported through the outer membrane by the help of a complex machinery called secreton which contains different proteins forming type II secretion pathway or secreton mediated- secretion (Figure 3).
Among bacterial lipases being exploited, those from *Bacillus* exhibit interesting properties that make them potential candidates for biotechnological applications. *Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus coagulans, Bacillus stearothermophilus*, and *Bacillus alcalophilus* are the most common bacterial lipases. In addition, *Pseudomonas* sp., *Pseudomonas aeruginosa, Burkholderia multivorans, Burkholderia cepacia*, and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers (Treichel *et al.*, 2010).

*Bacillus* and Geobacillus lipases belong to family I.4 and I.5. Over 70 different *Bacillus* lipases have been reported till date. A comprehensive review on *Bacillus* lipases has been published recently. Among *Bacillus* lipases, *B. subtilis* lipase lacks lid domain and stabilizing disulphide bridges implying that these lipases might possess flexible conformation, which could account for its unique biochemical properties. Due
to stability in the presence of hydrogen peroxide, sodium hypochlorite, surfactants, and extremes of temperature and pH, *Bacillus* lipases are ideally suited for use in laundry detergent formulations. They also display high enantioselectivity and diverse selectivity and are widely employed in racemic resolution of enantiomeric mixtures (Van Pouderoyen *et al.*, 2001; Guncheva and Zhiryakova, 2011).

*Staphylococcal* lipases were initially explored for their physiological significance and to understand their role in pathogenicity and colonization. Lipase-producing strains include *Staphylococcus aureus*, *Staphylococcus warneri*, *Staphylococcus simulans*, *Staphylococcus hyicus*, and *Staphylococcus xylosus*. Among these, *S. xylosus* (SXL1, SXL2, and SXL3) and *S. aureus* (SAL3) enzymes display thermostability. Many *staphylococcal* lipases display unique sn-2 specificity. Staphylococcal lipases are widely used in ester synthesis, antioxidants, and in production of biofuel and biopolymers. An extensive review on various applications of staphylococcus lipases has been recently compiled. A novel lipase from *Staphylococcus* sp. isolated from soil displayed optimal activity at 60 °C and at a pH of 12.0. In addition, the enzyme displayed high stability towards nonionic and anionic surfactants and relative stability towards oxidizing agents, which makes it ideally suited for use as an additive in laundry detergent formulations (Cherif *et al.*, 2011; Horchani *et al.*, 2012).

*Acinetobacter* lipases belong to the same group as *Pseudomonas* lipases. They have been isolated from variety of sources including both pristine and polluted soils. The characteristic features of these lipases are alkaline pH optima and low temperature tolerance. Among different *Acinetobacter* strains, *Acinetobacter calcoaceticus,*
Acinetobacter radioresistens, and Acinetobacter sp. RAG1 have been extensively characterized for their biochemical properties (Snellman and Colwell, 2004; Han et al., 2003). Polysaccharide was shown to increase A. calcoaceticus lipase by two- to fivefold (Martinez and Nudel, 2002). Recently, genome shuffling was shown to increase alkaline cold active lipase production by Acinetobacter johnsonii (Wang et al., 2012). Acinetobacter sp. lipase was successfully employed in ethyl caprylate synthesis and transesterification reactions (Ahmed et al., 2010; Snellman and Colwell, 2008). A cold active alkaline lipase from Acinetobacter sp. was shown to possess optimal activity at pH 8.5 and was found be effective in removing oil stain from soiled fabric by 21–24 %, when used as an additive in laundry detergent formulation under cold wash conditions (15–20°C) (Saisubramanian et al., 2008).

Most bacterial species studied for lipase production are non-pathogenic, mainly because these lipases were aimed to be used for biotechnological applications. It is a fact that extracellular lipases are important microbial virulence factors in addition to their industrial usage. In connection with this aspect, human pathogenic bacteria have been examined recently. One of them is Staphylococcus epidermidis, which is an opportunistic pathogen. During infection, it secretes two lipases that are considered to play a role in the colonization on the skin by breaking sebum-derived triacylglycerols. As well as Staphylococcus, other bacterial species seem to produce lipases for pathogenicity. Propionibacterium acnes, acting as the cause of acne vulgaris, produces an extracellular lipase, which functions in the microbial colonization of human skin (Stehr et al., 2003).
2.6 ISOLATION AND SCREENING OF LIPASE PRODUCING MICROORGANISMS

The industrial demand for new sources of lipases with different catalytic characteristics stimulates the isolation and selection of new strains. Lipase-producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Sharma et al., 2001).

Many microorganisms such as bacteria, yeast and fungi are known to secret lipases. Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil. The oily environment (oil mill effluent) may provide a good environment for isolation of lipase producing microorganisms (Sztajer et al., 1998; Mobarak-Qamsari et al., 2011). Thus, the exploration of biodiversity for searching new microorganisms that are able to produce enzymes is of great interest since most of the lipases used as catalysts in organic chemistry are of microbial origin. Furthermore, the finding of promising newly isolated microorganisms can open promising scientific and commercial perspectives (Ko et al., 2005).

Colen et al., (2006) isolated 59 lipase-producing fungal strains from Brazilian savanna soil using enrichment culture techniques. An agar plate medium containing bile salts and olive oil emulsion was employed for isolating and growing fungi in primary screening assay. Twenty one strains were selected by the ratio of the lipolytic halo radius and the colony radius. Eleven strains were considered and, among them, the strain identified as *Colletotrichum gloesporioides* was the most productive. In another
work, Cihangir and Sarikaya (2004) isolated a strain of *Aspergillus* sp. from soil samples from the different regions of Turkey and obtained an expressive activity of 17 UmL\(^{-1}\).

Mohan *et al.*, (2008) were used enrichment culture technique to isolate extracellular lipase producing strains from the soil sample of coconut oil industry with lipolytic activity in tributyrin media plates. In total, 28 isolates were collected from the soil sample and among them; five isolates (B1 to B5) showed high lipolytic activity. The lipolytic microbes were further screened and characterized by their features and reactions and then identified as Gram positive, rod shaped motile organisms. Finally the morphological and biochemical test indicated that the suspected organisms were *Bacillus* sp.

Rigo *et al.*, (2010) they were isolated 203 microorganisms (96 filamentous fungi, 3 bacteria, and 104 yeasts) from several contaminated sources as olive oil, cheese, tomato extract, soybean oil, milk cream, meat, soybean meal, and culture medium. Among the 203 isolated strains, yeasts generally presented higher potential for lipases production than bacteria and fungi, considering the synthesis activity of n-propyl oleate. The isolated strains 69F and 161Y showed ability to efficiently catalyze the reaction for production of n-propyl oleate. Other strains can also be considered of potential interest, as 74F, 111Y, and 186Y.

Ertugrul *et al.*, (2007) isolated 17 bacterial strains that could grow on media based on OMW and selected the most promising strain for lipase production. After screening in tributyrin agar medium, a strain of *Bacillus* sp. was identified as the best lipase producer. After the medium optimization, the intracellular activity found was
Kiran et al., (2008) isolated 57 heterotrophic bacteria from the marine sponge *Dendrodoris nigra*, of which 37% produced a clear halo around the colonies on tributyrin agar plates for lipase production. Particularly, the strain *Pseudomonas MSI057* exhibited large clean zones around the colonies. Then, this strain was selected for further studies, and after optimization, a maximum lipase activity was found as 750 UmL$^{-1}$.

Carvalho et al., (2008) isolated a bacterium strain from petroleum-contaminated soil and codified as Biopetro-4. After investigation of several inducers on lipase activity, the maximum value obtained was 1,675 UmL$^{-1}$ after 120 h of fermentation. Abada (2008) produced lipase from a strain of *B. stearothermophilus AB-1* isolated from air and obtained a maximum lipase activity of 1585 U mL$^{-1}$ in 48 h of fermentation.

Takac and Marul (2008) isolated microbial cultures from soil enriched by periodic sub-culturing of samples in nutrient broth containing 1% (v/v) tributyrin. The isolation process was performed by serial dilution samples on tributyrin agar (TBA) plates. *Bacillus* sp. was selected towards producing the largest opaque halo. Active colonies were re-streaked on TBA agar for purification. Shariff et al., (2007) isolated a thermophilic bacterium, *Bacillus* sp. strain L2 from a hot spring in Perak, Malaysia. An extracellular thermostable lipase activity was detected through plate and broth assays at 70 °C after 28 h of fermentation.

Lipase-producing microorganisms include bacteria, fungi, yeasts, and actinomyces. A simple and reliable method for detecting lipase activity in microorganisms has been described by Sierra (1957). This method uses the surfactant
Tween 80 in a solid medium to identify a lipolytic activity. The formation of opaque zones around the colonies is an indication of lipase production by the organisms. Modifications of this assay use various Tween surfactants in combination with Nile blue or neet’s foot oil and Cu$^{2+}$ salts. Also, screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas et al., 2001) and clear zones around the colonies indicate production of lipase. Screening systems making use of chromogenic substrates have also been described (Yeoh et al., 1986). Wang et al., (1995) used plates of a modified Rhodamine B agar to screen lipase activity in a large number of microorganisms. Other versions of this method have been reported (Kouker and Jaeger, 1987; Hou, 1994).

Microbial lipases are more stable as compared to plant and animal lipases and they can be obtained cheaply. Table 2 shows some of the commercially important lipase-producing bacteria have been isolated and characterized from various sources.

Microorganisms are often tested for lipase production on solid media. All methods containing agar can be grouped into two categories; (1) Methods based on substrate changes in appearance as a result of lipolysis and (2) methods including the usage of an indicator dye to detect lipolysis (Thomson et al., 1999). In all methods, it is important that there must be a contact between substrate and enzyme. Thus the agar content in screening media can be reduced for increased diffusibility of extracellular lipase (Hou and Johnston, 1992).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Isolated from</th>
<th>Reference</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>Bacillus</em> sp. MPTK 912</td>
<td>Oil mill effluent</td>
<td>Mukesh Kumar <em>et al.</em>, (2012)</td>
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<tr>
<td><em>Alcalignes viscosus</em> (DOGE-1)</td>
<td>Dairy industry waste waters</td>
<td>Sekhar, (2012)</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em> (Ps5)</td>
<td>Wastewater</td>
<td>Zouaout and Bouziane, (2012)</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em> KGSHW-1</td>
<td>Slaughter house wastes</td>
<td>Bayoumi <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Fish Gut</td>
<td>Selva Mohan and Palavesam, (2012)</td>
</tr>
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<td><em>Burkholderia anthina</em></td>
<td>Soil</td>
<td>Jin <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em>, <em>Serratia sp.</em>, <em>Pseudomonas sp.</em> and <em>Staphylococcus sp.</em></td>
<td>Industrial effluents</td>
<td>Prasad and Manjunath, (2012)</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> MTCC-10498</td>
<td>Hot-spring of Tattapani</td>
<td>Sharma <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> KM110</td>
<td>Wastewater of an oil processing plant</td>
<td>Mobarak-Qamsari <em>et al.</em>, (2011)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em> 4</td>
<td>Marian samples (Soil and water)</td>
<td>Kavitha and Shanthi (2013)</td>
</tr>
<tr>
<td><em>Bacillus flexus, Bacillus pseudofirmus, Bacillus pumilus, Bacillus halodurans, Bacillus cereus</em> and <em>Bacillus sp.</em> <em>Aeromonas sp.</em> EBB-1</td>
<td>Lake water (salinity and alkalinity)</td>
<td>Tambekar and Dhundale, (2012)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em> MPTK 912</td>
<td>Marine sludge</td>
<td>Jittima <em>et al.</em>, (2011)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> KM110</td>
<td>Oil mill effluent</td>
<td>Mukesh Kumar <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em> KGSHW-1</td>
<td>Wastewater of oil processing plant</td>
<td>Mobarak-Qamsari, <em>et al.</em>, (2011)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Slaughter house wastewater</td>
<td>Bayoumi <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em>, <em>Serratia sp.</em>, <em>Pseudomonas sp.</em> and <em>Staphylococcus sp.</em></td>
<td>industrial effluents</td>
<td>Prasad and Manjunath, (2012)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em> PD-12</td>
<td>Soil-oil industry</td>
<td>Praveen and Sharmishta (2011)</td>
</tr>
<tr>
<td><em>Pseudomonas xinjiangensis</em> CFS14</td>
<td>oil contaminated soil</td>
<td>Lomthaison et al (2012)</td>
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<tr>
<td><strong>Fungi</strong></td>
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<tr>
<td><em>Trivhoderma virers</em></td>
<td>Oil polluted soil</td>
<td>Christianah <em>et al.</em>, (2012)</td>
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</tbody>
</table>
In the first method to visualize lipase producing microbial colonies on solid media is depending on the used lipase substrate. A growth medium is prepared with tributyrin or triolein and inoculated the culture. Lipase activity is defined by a clear zone formation around colonies after incubation. This method has several advantages like the elimination of the requirement for specific dyes that can reduce the cost of analysis and prevent the inhibition of microbial growth because microorganisms may be sensitive to dyes (Thomson et al., 1999).

The agar media can be useful for only screening of lipase production, but not to measure lipase activity. By direct observation method, the formation of clear or turbid zones around colonies, or the production of crystals on the agar surface displays the presence of lipolytic activity. Triolein is used as the substrate in lipase detection methods; however, it is difficult to visualize zones of hydrolysis. When Tweens are used as lipase substrates, clear zones are easy to observe (Shelley et al., 1987).

In the second using different specific dyes like victoria blue B, spirit blue, nile blue sulfate, and night blue to detect the lipase producing microorganisms. These dyes can be used by either directly adding into the growth medium or applying to the plate after incubation. In dye-based methods, indicator dyes must show a clear and distinguishable color changes as a result of pH changes that occur due to the release of free fatty acids from triacylglycerols during lipolysis. Various combinations of substrates like tributyrin, tweens and dyes such as Victoria blue B and night blue can be used in these methods (Thomson et al., 1999). However, these substrates are not suitable to detect true lipases because they are hydrolyzed by esterases too. The formation of clear zones around colonies against an opaque background on tributyrin
agar lacking Victoria blue B indicates lipolytic microbes, but in the presence of Victoria blue B lipolytic colonies are surrounded by dark zones against an opaque, light blue background (Jones and Richards, 1952).

Microbial lipase activity can also be identified by using fluorogenic dye Rhodamine B. The method containing Rhodamine B as an indicator of the presence of lipase and olive oil as lipid substrate was firstly found by Kouker and Jaeger (1987). Agar plates containing olive oil and rhodamine B are opaque and pink colored. Lipase producing bacteria forms orange fluorescent holes around their colonies under UV light, but lipase negative bacteria do not show orange fluorescence upon UV irradiation (Kouker and Jaeger, 1987). The fluorescence is related to the formation of a rhodamine B-long chain fatty acid conjugate (Jaeger et al., 1994). Methods used to find lipolytic microbes with triacylglycerols and pH indicator dyes such as Victoria blue B are sensitive to any pH changes and can inhibit the growth of some bacteria. The rhodamine B plate method is not affected by pH changes and does not inhibit the growth of test microorganism or change its physiological properties (Kouker and Jaeger, 1987; Thomson et al., 1999).

2.7 PRODUCTION OF MICROBIAL LIPASE

Microbial lipases are produced mostly by submerged culture (Ito et al., 2001), but solid state fermentation methods (Chisti, 1999a) can be used also. Immobilized cell culture has been used in a few cases (Hemachander et al., 2001). The effects of the cultivating conditions in shake flask experiments are commonly investigated by
subjecting the microorganism to different environmental conditions (Yang et al., 2003). The optimal culture conditions and nutritional requirement are most important for lipase production by submerged culture. Because, microbial lipases are mostly extracellular and their production is greatly influenced by medium composition as well as physicochemical factors such as temperature, pH, dissolved oxygen, agitation, inoculums size, different carbon sources, different nitrogen sources, metal ions and inducers (Elibol and Ozer, 2001). These enzymes are generally produced in the presence of lipid substrates such as oils or any inducers in form of triacylglycerols, fatty acids, hydrolysable esters, Tweens, bile salts, and glycerol (Gupta et al., 2004; Sharma et al., 2001). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils (Sharma et al., 2001).

The catalytic properties of lipases, such as selectivity and estereoespecificity, can be controlled through manipulation of reaction conditions. The influence of the reaction medium composition on lipases properties is associated to a complex mechanism of enzymes action, which involves conformational changes in their structures (Vargas et al., 2008).

Xu et al., (2008) reported that the production cost is one relevant limitation to industrial application of microbial lipases. It is determined by the production yield, experimental conditions of the process and enzyme stability. Therefore, it is interesting to develop strategies to enhance the process productivity by employing newly isolated high enzyme producer microorganisms as well as to perform the process optimization through manipulation of the main variables that affect the process. Besides, the
application of lipases in large-scale industrial processes requires careful investigation of
potential techniques suitable for the reuse and increase the enzyme stability, such as
concentration/purification and immobilization strategies.

Hahn-Hagerdal et al., (2005) reported that the media components have a strong
impact on economics of industrial fermentation processes and can account for up to
30% of the total production cost. Hence, the design of an economic and efficient
growth/production medium compatible with commercial needs must be considered in
the early steps of process development. Cavalcanti et al., (2005) has studied a fixed-bed
bioreactor to improve productivity and scaling-up of lipase production using P. simplicissimum in SSF. The influence of temperature and air flow rate on lipase
production was assessed by statistical experimental design, and an empirical model was
proposed to validate the experimental data. Higher lipase activities have achieved at
lower temperature and higher air flow rates. A maximum lipase activity of 26.4 U g\(^{-1}\)
was reported at 27 °C at an air flow rate of 0.8 L min\(^{-1}\).

2.8 EFFECT OF TEMPERATURE ON LIPASE PRODUCTION

Most lipase producing organisms are mesophilic in nature (growing in moderate
temperature typically between 25 and 40°C). However, some psychrophilic and
thermophilic organisms have been reported in the literature (Salihu and Zahangir,
2012). Joseph et al., (2007) reported about some psychrophilic strains which are
producing lipases include Acinetobacter sp., Achromobacter lipolyticum, Aeromonas
hydrophila, Bacillus sphaericus, Photobacterium lipolyticum, Morexella sp.,
Pseudomonas fluorescens, Pseudomonas fragi, Psychrobacter okhotskensis, Serratia
marcescens and Staphylococcus epidermidis.
Cherif et al., (2011) mentioned that, the increase in temperature seemed to have a negative effect on biomass production and also lipolytic activity. Decrease in lipase production can be associated to either decrease in cell growth or inactive nature of enzyme itself. Shafei and Rezkallah, (1998), have reported similar results. Kim et al., (2000), reported a \textit{B.stearothermophilus} that showed optimum growth at 55 °C, but showed almost no activity at the same temperature. On the other hand Sifour et al., (2010) reported a \textit{B. stearothermophilus} that produced high yields of lipase at 55 and 60 °C.

\section*{2.9 EFFECT OF PH ON LIPASE PRODUCTION}

Lipases are active over broad pH and temperature range and they have molecular weight ranging from 94 to 840 kDa. From available literature it can be interoperated that generally lipases have neutral pH optima but the pH and temperature optima of lipases depends on the habitat of its sources. Lipases posses stability over a wide range of pH from 4 to 11 (Patil et al., 2011).

The effect of pH on the growth and enzyme production by \textit{GeoBacillus stearothermophilus} KGSHW-1 was assessed in a wide range of pH. Lipolytic productivities were found at maximum at initial pH 10, since the lipase yields reached up to 1477.9 (UmL\(^{-1}\)). Below and above this particular value, the enzyme productivity decreased gradually. From this result, it was concluded that the microorganism needs neutral or alkaline pH values. For pH values outside this range, cell growth seems to be completely inhibited a fact which reveals the importance of studying this factor in cultures of \textit{G. stearothermophilus} strain and the controlling the pH variations during its cultivation (Kim et al., 2000). Ramani et al., (2010) reported maximum \textit{Pseudomonas}
gessardii lipase activity of 156UmL$^{-1}$ at acidic pH of 3.5 using slaughterhouse wastes at 0.31 g substrate concentration. Table 3 gives an overview on recent literature regarding the effect of temperature and pH on lipases production.

<table>
<thead>
<tr>
<th>Name of the bacteria</th>
<th>Temperature ($^\circ$C)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter sp. CR9</td>
<td>40</td>
<td>8.0</td>
<td>Kasana et al., (2008)</td>
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<tr>
<td><strong>Bacillus licheniformis</strong></td>
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<tr>
<td><em>B. licheniformis</em> MTCC-10498</td>
<td>25</td>
<td>7.0</td>
<td>Sumanjelin et al., (2013)</td>
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<td><strong>Bacillus sp.</strong></td>
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<tr>
<td>B. licheniformis MTCC-10498</td>
<td>55</td>
<td>7.5</td>
<td>Sharma et al., (2012)</td>
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<td><strong>Bacillus sp.</strong></td>
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<tr>
<td><strong>Bacillus sp. MPTK 912</strong></td>
<td>30</td>
<td>8.0</td>
<td>Mukesh Kumar et al., (2012)</td>
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<tr>
<td><strong>Bacillus sp. PD-12</strong></td>
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<td></td>
<td>Praveen and Sharmishtha (2011)</td>
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<tr>
<td><strong>B. stearothermophilus</strong></td>
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<tr>
<td><strong>Burkholderia multivorans</strong></td>
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<td><strong>Burkholdria cepacia</strong></td>
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<td><strong>Fervidobacterium changbaicum</strong> Lip1</td>
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<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
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<tr>
<td><strong>P. aeruginosa</strong> MTCC 10055</td>
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<tr>
<td><strong>P. aeruginosa</strong> NRRL-B727</td>
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<tr>
<td><strong>P. fragi</strong> NRRL-B727</td>
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<tr>
<td><strong>Pseudomonas sp.</strong></td>
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<td><strong>P. xinjiangensis</strong> CFS14</td>
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<tr>
<td><strong>Staphylococcus epidermidis</strong></td>
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<td><strong>Thermobifida fusca</strong></td>
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<tr>
<td><strong>Salinvibrio sp. strain SA-2</strong></td>
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2.10 **EFFECT OF AGITATION SPEED ON LIPASE PRODUCTION**

Alonso *et al.*, (2005) studied the lipase production by *Y. lipolytica* in a stirred tank reactor at different agitation speeds and an air flow rate has been investigated. The most pronounced effect of oxygen on lipase production is determined by stirring speed. A maximum lipase activity has been detected in the stationary phase at 200 rpm and an air flow rate of 0.8–1.7 vvm, when the lipid source has been fully consumed. Higher stirring speeds resulted in mechanical or oxidative stress, while lower speeds seemed to limit oxygen levels. An increase in the availability of oxygen at higher air flow rates has increased lipid uptake and anticipation of enzyme release in culture medium. The maximum lipase production has reported at 200 rpm and 1dm$^3$min$^{-1}$.

2.11 **EFFECT OF INOCULUM SIZE ON LIPASE PRODUCTION**

The nature of inoculum as well as its size may affect the microbial process and could play crucial role in enzyme production (Elibol *et al.*, 1995). In a study of lipase production by *Bacillus thermoleovorans* ID-1, Lee *et al.* (1999) obtained optimum results when 1% (v/v) inoculum was used to inoculate the fermentation medium. At a suitable inoculum size, the nutrient and oxygen levels support sufficient growth of bacteria and therefore, enhance the lipase production. Higher inoculum size may cause insufficiency of total dissolved oxygen and nutrient supply in the culture media resulting in poor product yield (Baharum *et al.*, 2003). If the inoculum size is too small, insufficient biomass lead to reduced level of secreted lipase.
2.12 EFFECT OF CARBON SOURCES ON LIPASE PRODUCTION

Carbon sources serve as important substrates for energy production in microorganisms. Lipidic carbon sources serve as inducers and are generally essential for obtaining a high lipase yield. Ghosh et al., (1996) reported that the requirement of sugar as a carbon source in addition with lipids varies with the microorganism. But generally, media supplemented with glucose along with triglycerides stimulate the lipase production in *Rhizopus nigricans*. But Enzyme activity was not detected when glucose was the sole carbon source, confirming that the presence of an inducer is necessary for *Penicillium aurantiogriseum* to produce lipases (Lima et al., 2003).

Sugihara et al., (1991) reported lipase production from *Bacillus* sp. in the presence of 1% olive oil in the culture medium. Little enzyme activity was observed in the absence of olive oil even after prolonged cultivation. Fructose and palm oil were reported to be the best carbohydrate and lipid sources, respectively, for the production of an extracellular lipase by *Rhodotorula glutinis*. When the two carbon sources were compared, palm oil at a concentration of 2% was found to yield 12-fold more lipase than the fructose medium (Papaparaskevas et al., 1992).

Various authors have been reported the influence of different carbohydrates sources on lipases production. Among the carbohydrates used as carbon source, it was found that sucrose resulted in maximal lipase production (Fadiloglu et al., 1999). The production of lipase from *Candida rugosa* was highest with enzyme activity 5.58UmL\(^{-1}\) in a media containing yeast extract, protease-peptone and olive oil whereas minimum lipase activity (2.81UmL\(^{-1}\)) was observed with tryptone and lactose medium (Fadiloglu
et al., 2002). Espinosa et al., (1990) reported that the lipase production by *R. delemar* was 1.6 times higher with dextrin as compared to glucose.

Bhushan et al., (2011) used various growth media (M1-M5) containing different amount and sources of carbon, nitrogen and other nutrients were used for the optimum cell biomass and lipase production from *Arthrobacter sp.* (RRLJ-1/95). In batch fermentor, the maximum cell biomass 10gL$^{-1}$ and lipase activity 1800 Ug$^{-1}$ was obtained using media M5 which contained 20g L$^{-1}$ sucrose than other media. Lin et al., (2006) reported maximum lipase production by *Antrodia cinnamomea* with sucrose among the various carbohydrates including glucose, galactose, fructose and xylose used as carbon source.

Bisht et al., (2012) studied the effect of different carbon sources such as fructose, glucose, lactose, mannitol, soluble starch, sorbitol and sucrose on alkaline lipase production by *Pseudomonas aeruginosa* MTCC 10,055. Maximum lipase production was obtained with starch (960.8UmL$^{-1}$) followed by glucose (550.9 UmL$^{-1}$) and lactose (476.1UmL$^{-1}$), whereas, mannitol (448.1UmL$^{-1}$), sucrose (380.4 UmL$^{-1}$) and fructose (253.9UmL$^{-1}$) had negative effect on lipase production. Similar Pogaku et al. (2010) have also been reported the lipase production by *Staphylococcus* sp. Lp12 and Rathi et al. (2001) also reported increase in lipase production from *Burkholderia cepacia* in the presence of oil and glucose as sugar additive.

Various authors reported that the presence of glucose in the cultivation medium depressed the production of lipase compared to olive oil. Glucose supplementation to the basal production medium inhibits lipase production, perhaps by catabolic repression. This occurred with reports for other lipase-producing organisms for which a
high glucose concentration caused reduced lipase production (Chen et al., 1992; Dharmsthit et al., 1998; Lottrakul and Dharmsthiti, 1997; Mobarak-Qamsari, et al., 2011). Christianah et al., (2012) studied the nutrient utilization for growth and lipase production by some lipolytic fungi isolated from oil polluted environmental samples and found that the sucrose and xylose was best utilized for growth and lipase production by Trichoderma virens and Hypocre a patella.

2.13 EFFECT OF NITROGEN SOURCES ON LIPASE PRODUCTION

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used, such as peptone and yeast extract, which have been used for lipase production by various thermophilic Bacillus sp. and various Pseudomonads (Sugihara et al., 1991; Sharma et al., 2002). A study by Mobarak-Qamsari, et al., (2011) found that the peptone was the most suitable nitrogen source for lipase production by newly isolated bacterium Pseudomnas aeruginosa KM110.

Inorganic nitrogen sources such as ammonium chloride and ammonium dihydrogen phosphate have been also reported to be effective in some microbes for lipase production (Dong et al., 1999; Rathi et al., 2001; Mobarak-Qamsari, et al., 2011). Bayoumi et al., (2012) studied the effect of different organic and inorganic nitrogen sources on lipase production by the thermoalkaliphilic B. stearothermophilus KGSHW-1 and found maximum productivity of lipase(15024.67 UmL⁻¹) at potassium nitrate present culture among eleven different nitrogen sources were applied.

Bisht et al., (2012) used various nitrogen sources to enhance the alkaline lipase production by Pseudomonas aeruginosa MTCC 10055 and found a significant increase
in growth and lipase production with yeast extract. Likewise, maximum lipase production with 0.1% (w/v) and 0.5% (w/v) yeast extract have also been reported earlier by *Bacillus* strain A30-1 (Wang *et al.*, 1995) and *P. citrinum* (Pimentel *et al.*, 1994), respectively. According to Gupta *et al.*, (2007) yeast extract apart from acting as a nitrogen source also supplies vitamins and trace metals, thereby affecting the growth of organism in turn, increase in lipase production.

Among the inorganic nitrogen sources, the ammonium sulphate was most suitable for lipase production by *Pseudomonas aeruginosa* MTCC 10055, whereas ammonium di-hydrogen orthophosphate inhibited the lipase production and others had insignificant effect (Bisht *et al.*, 2012). Lima *et al.* (2003) also reported that ammonium salt was necessary for growth of *P. aurantiogriseum*. Rohit *et al.*, (2001) reported that lipase yield and stability could be improved by supplementing the preferred organic nitrogen source with ammonium. Guptal *et al.*, (2004) and Christakopoulos (1992) also reported that the ammonium sulfate and ammonium chloride are the best nitrogen sources for lipase production. In contrast, Pogaku *et al.* (2010) found that potassium nitrate was better than ammonium sulphate for increased lipase production by *Staphylococcus* sp. LP12. Inorganic nitrogen sources such as sodium nitrate have also been reported to be effective in some microbes (Sharma *et al.*, 2011).

Christianah *et al.*, (2012) studied the nutrient utilization for growth and lipase production by some lipolytic fungi isolated from oil polluted environmental samples and found that the urea was the best utilizable nitrogen source for lipase production (2.35U mL⁻¹) followed in order by ammonium chloride (2.05U mL⁻¹) and ammonium sulfate (2.00U mL⁻¹). Sharma *et al.* (2001) have also been reported that the urea
stimulates lipase production in both bacterial and fungal isolates. Babu and Rao (2007) reported maximum lipase production in the presence of urea in case of *Y. lipoLytica* NCIM 3589.

### 2.14 EFFECT OF TRACE ELEMENTS ON LIPASE PRODUCTION

Metallic ions are believed to influence enzyme production and according to Tan *et al.*, (2003), magnesium (Mg\(^{2+}\)), sodium (Na\(^{+}\)) and potassium (K\(^{+}\)) are beneficial for the biosynthesis of lipases. Balaji and Ebenezer (2008) reported that the metal ions, magnesium sulphate yielded 1240 Ug\(^{-1}\) Dry Matter (DM) followed by zinc sulphate 1220 Ug\(^{-1}\) DM, calcium chloride 925 Ug\(^{-1}\) DM, Potassium chloride 909 with pongamia oil cake as the substrate by endophytic fungi *Colletotrichum gloeosporioides* isolated from oil seeds. Lipase production by a thermophilic *Bacillus* species was increased several fold when magnesium, iron, and calcium ions were added to the production medium. Lipase production by *Bacillus* species A 30-1 (ATCC 1841) required a complex medium that contained Ca\(^{2+}\), Mg\(^{2+}\), Na\(^{+}\), Co\(^{2+}\), Fe\(^{2+}\), K\(^{+}\), Mn\(^{2+}\), Mo\(^{2+}\) and Zn\(^{2+}\) (Rooney and Weatherley, 2001). Lipase production by a thermophilic *Bacillus* sp. was increased several fold when magnesium, iron, and calcium ions were added to the production medium (Janssen *et al.*, 1994). Similarly, Pokorny *et al.*, (1994) reported that lipase production by *A. niger* was enhanced in the presence of Mg\(^{2+}\). Production of an extracellular lipase by *Acinetobacter Calcoaceticus* BD 413 was enhanced when the medium was supplemented with Mg\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\), and Co\(^{2+}\) (Kok *et al.*, 1995). The enzyme hydrolyzed long acyl chain p-nitrophenol (pNP) esters, such as pNPP and its optimal
activity occurred between pH 7.8 and 8.8 (Kok et al., 1995). The *A. calcoaceticus* lipase was quite similar to *Pseudomonas* lipases.

Lipase production by *P. pseudoalcaligenes* F-111 was enhanced when a phosphate containing medium was provided with 

Mg$^{2+}$ (Lin et al., 1995). This alkaline lipase was most active and stable in the pH range 6-10 and its optimal reaction temperature was 40°C. Lipase production by *Bacillus* sp. A 30-1 (ATCC 53841) required a complex medium that contained Ca$^{2+}$, Mg$^{2+}$, Na$^+$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, K$^+$, Mn$^{2+}$, Mo$^{2+}$, and Zn$^{2+}$ (Wang et al., 1995). The source bacterium, isolated from a mineral-rich hot spring (Yellowstone National Park), grew optimally at 60°C (pH 9) (Wang et al., 1995). Maximal lipase production by *P. pseudoalcaligenes* KKA-5 occurred at Mg$^{2+}$ concentration of 0.8 M (Sharon et al., 1998). Exclusion of the magnesium ions from the medium caused approximately 50% reduction in lipase production (Sharon et al., 1998), but supplementing the medium with calcium ions did not affect lipase production. In one case, presence of Ca$^{2+}$ was reported to enhance lipase production by the thermophilic *Bacillus* sp. RS-12 (Sidhu et al., 1998a, b).

As metal ions are known to influence lipase synthesis, activity and secretion mechanism (Lee et al., 1999), various metal salts in the medium were evaluated for their effect on alkaline lipase production. Maximum enzyme production was observed in medium containing MgSO$_4$ followed by FeSO$_4$ (Bisht et al., 2012). On the other hand, influence of Ca$^{2+}$ in addition to Mg$^{2+}$ was found to enhance lipase production by *P. aeruginosa* (Kulkarni and Garde, 2002).

Gupta et al. (2004) who reported that calcium and magnesium ion had the best stimulation effect on lipase production by *Burkhodleria* sp. Similarly they reported that
Fe$^{2+}$ plays a critical role in lipase production of *Pseudomonas* sp. Utilization of metal ions for lipase production, Zn$^{2+}$ was observed to stimulate lipase production by *Hypocrea patella* (Christianah *et al.*, 2012). A stimulatory effect of divalent ions on lipase production has been reported (Sangeetha *et al.*, 2011).

Iron (III) represses extracellular enzyme production by many psychrotrophs. Lipase and protease production by *P. fluorescens* B52 is strongly repressed by 20 μM Fe$^{3+}$, while growth was fastest at 2 μM (McKellar *et al.*, 1987). Up to 400 μM Fe$^{3+}$ had only a slight effect on the lipase enzyme itself, and no effect on protease. B52 was also found to produce a siderophore, pyoverdine, which facilitates iron uptake in low-iron growth conditions. Addition of this compound to a B52 culture stimulated lipase production.

### 2.15 IMMOBILIZATION OF BACTERIAL CELLS FOR LIPASE PRODUCTION

Immobilization of cells would provide a number of advantages; higher cell densities at lower broth viscosities would result in faster reaction rates of mass transfer. On this basis, the efficiency of a biocatalyst reflects the complexity of the catalytic system, composed not only of the microbial cells, but also of the immobilizing matrix and the reaction medium. Biocatalyst immobilization provides a tool to reduce the deleterious effect of the organic phase, by providing cells with a protective environment. This approach additionally facilitates biocatalyst separation from the production media. Immobilization is very important in the industrial application of enzymes, as immobilized biocatalysts offer unique advantages in terms of better process control, enhanced stability, cell-free products, predictable decay rates, and
improved economics. It was found that whole cells in a gel matrix lived for a month under certain conditions. Immobilized living whole cells have a potential application to a new type of fermentation. Production of biologically active substances can be performed with a fermentation system using immobilized whole cells (Joseph et al., 2006).

Yang et al., (2005) studied the repeated-batch lipase production by immobilized mycelium of Rhizopus arrhizus in submerged fermentation. The time to replace the volume, the volume of the replaced medium, and the optimal composition of the medium were optimized. Immobilized cells showed high stability for repeated use. Nine repeated batches were carried out in flasks for 140 h and six repeated batches in a 5-L bioreactor. The lipase productivity increased from 3.1UmL⁻¹h⁻¹ in batch fermentation to 18 UmL⁻¹h⁻¹ in repeated-batch fermentation.

Ellaiah et al., (2004) used the whole immobilized biomass of Aspergillus niger to produce lipase and obtained similar activities for both free and immobilized biomass cultivations (4 U mL⁻¹). Elitol and Ozer (2000) immobilized the whole cell of R. arrhizus and the rate of lipase production was constant through several repeated batch experiments.

Wolski et al., (2008) reported the use of response surface methodology to optimize the lipase production by submerged fermentation using immobilized biomass of a newly isolated Penicillium sp. At the optimized experimental conditions, the authors reached a lipase activity around 21UmL⁻¹, higher than the activity obtained by the same microorganism before immobilization. Da Silva et al., (2009) were examined and compared five inexpensive materials viz Accurel EP-100, Amberlite MB-1, Celite,
Montmorillonite K10 and Silica gel as supports for the immobilization of a native *Aspergillus niger* lipase. Amberlite MB-1 was found to be the best supporter, with a conversion of 38.2%, enantiomeric excess of 50.7% and enantiomeric ratio \((E\text{ value})\) of 19 in 72 h of reaction. After a thorough optimization of several parameters, the \(E\) value of the immobilized *A. niger* lipase was increased \((E = 23)\) in a shorter reaction period \((48 \text{ h})\) at 35 °C. Moreover, the immobilized *A. niger* lipase maintained an esterification activity of at least 80% after 8 months of storage at 4 °C and could be reused at least six times.

Li *et al.*, (2005) used repeated fed-batch strategy to produce lipase from *Acinetobacter radioresistens*. A constant cell concentration was shown to be a pre-requisite to extend the number of repeated cycles, and adequate cell growth rate was critical for obtaining high lipase yield. Benjamin and Pandey (1997) carried out experiments in batch and repeated-batch (fed-batch type) for lipase production using immobilized *C. rugosa* cells in packed-bed bioreactor. A maximum enzyme activity \((17.9 \text{ U mL}^{-1})\) was obtained when the fermentation was carried out in repeated-batch mode using a feed medium containing arabic gum and caprylic acid, keeping the flow rate of the feed at 0.4 mL min\(^{-1}\) and allowing each cycle to run for 12 h.

### 2.16 PURIFICATION OF LIPASE

Purification of the enzyme is essential for various industries like fine chemical, pharmaceutical, detergent and cosmetics and also to understand the 3D structure and the structure function relationships of proteins (Aires-Barros *et al.*, 1994; Saxena *et al.*, 2003). Most commercial applications of lipase require homogeneous lipase preparations with a certain degree of purity to enable efficiency. Further, purified lipase
preparations are needed for biocatalytic production of pharmaceuticals, cosmetics, leather, detergents, foods, perfumery, medical diagnostics, and other organic synthetic materials (Singh and Mukhopadhyay, 2011).

The main constraints in traditional purification strategies include low yields and long time periods. Alternative new technologies such as membrane processes, immune purification, and aqueous two-phase systems are gradually coming to the purification of lipases. For industrial purposes, the enzyme purification strategies employed should be inexpensive, rapid, high-yielding and amenable to large scale operations. There are various purification strategies have been used for lipases and highlighted clearly the importance of designing optimal purification schemes for various microbial lipases (Palekar et al., 2000; Saxena et al., 2003; Singh and Mukhopadhyay, 2011).

In many cases, many lipases have been extensively purified to homogeneity and crystallized. Purification methods used depends on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration, and ion exchange chromatography. Precipitation was used as a fairly crude separation step, often during the early stages of a purification method, followed by chromatographic separation. Affinity chromatography was used in some cases to reduce the number of individual purification steps needed (Woolley and Peterson, 1994).

Activity of lipase depends on the concentration of ammonium sulfate solution used (Pabai et al., 1995). Precipitation method often gives average yield of 87% with limited purification and such enzyme preparations are used in detergent formulations. However, for certain applications, such as synthetic reactions in pharmaceutical, food, and leather industry, further purifications are needed. Since lipases are known to be
hydrophobic in nature, with large hydrophobic surfaces around the active site, the purification of lipases can best be achieved by opting for affinity chromatography such as hydrophobic interaction chromatography. Affinity methods can be applied at an early stage but as the hydrophobic matrices are expensive; alternatively ion exchange and gel filtration are usually preferred after the precipitation step (Shu et al., 2006). Although gel filtration has a lower capacity for loaded protein, it can be used at an early stage in the purification or as one of the last steps for fine polishing of the protocol.

There are many reports on lipases purification by ion exchange chromatographic technique (Sharma et al., 2001). Lee and Rhee (1993) used ion exchange and gel filtration to purify a lipase from *P. putida* 3SK. Kim et al. (1996) purified a highly alkaline extracellular lipase of *Proteus vulgaris* by ion exchange chromatography. The purified lipase had a maximum hydrolytic activity at pH 10.0 and its molecular mass was 31 kDa by SDS-PAGE. A *Pichia burtonii* lipase was purified to homogeneity by a combination of DEAE-Sephadex A-50 ion exchange chromatography, Sephadex G-100 gel filtration, and isoelectric focusing. The purified enzyme was monomeric and had a molecular mass of 51 kDa by SDS-PAGE (Sugihara et al., 1995).

Chartrain et al., (1993) purified a lipase from *Pseudomonas aeruginosa* MB5001 using a three-step procedure. Concentration by ultrafiltration was followed by ion exchange chromatography and gel filtration. The purified lipase had a molecular mass of 29 kDa by SDS-PAGE. Similarly Sharon et al., (1998) purified an extracellular lipase from *P. aeruginosa* KKA-5 by ammonium sulfate precipitation and successive chromatographic separations on hydroxyl appetite. After a 518-fold purification, the
enzyme was homogenous electrophoretically and its molecular mass was estimated to be 30 kDa.

A three-step procedure involving ammonium sulfate precipitation, DEAE Sephacel ion exchange chromatography, and Sephacryl S-200 gel filtration chromatography was used to purify a lipase from a thermophilic *B. thermoleovorans* ID-1 to homogeneity. The protein was purified 223-fold. The molecular mass of the lipase was 34 kDa (SDS-PAGE) (Lee *et al.*, 1999). A lipase produced by *Staphylococcus epidermidis* RP 62A was purified to homogeneity by a combination of precipitation techniques, metal affinity chromatography, and gel filtration (Simons *et al.*, 1998). The purified enzyme had a pH optimum of 6.0 and required calcium as a cofactor for catalytic activity (Simons *et al.*, 1998).

As well as the desired components, the fermentation process generates by-products that frequently hinder the use of the fermented broth in industrial procedures. For the success of commercial production of enzymes and proteins, efficient downstream processing techniques are needed. When these processes are applied to biological materials, purification steps that are delicate enough to preserve the biological activity are required. However, these protocols increase the cost of the process and decrease the yield of the reaction. The conventional procedure includes ammonium sulphate precipitation, chromatography, dialysis and filtration (Lima *et al.*, 2002; Liu *et al.*, 2008; Koblitz and Pastore, 2006; Saxena *et al.*, 2003).

An effective and economically viable method for the separation and purification of lipase is the partitioning in an aqueous two-phase system (ATPS). The ATPS can be formed by combining either two incompatible polymers or a polymer and salt in water,
above a certain critical concentration. These systems are attractive due to low costs, rapid phase disengagement and the availability of commercial separators, which allow faster continuous protein separation (Silva and Meirelles, 2001).

De Souza et al., (2010) they were found that the aqueous two-phase system is a good process to use for lipase purification because a single step promoted a purification factor similar to that found in the pre-purification steps and the best enzyme recovery (83.40%). When increase the PEG molecular weight from 1,500 to 8,000gmol$^{-1}$ they find the best values of purification factor, recoveries and efficiency.

Sztajer et al., (1992) has purified a lipase enzyme produced by P. simplicissimum by ammonium sulfate precipitation, phenyl-SepharoseCL-4B, Ultrogel AcA-54, and hydroxyapatite. The molecular weight of the enzyme was estimated at 56,000gmol$^{-1}$ by SDS-PAGE with recovery of 20%. A lipase produced by a Penicillium expansum DSM 1994 was purified to 219-fold by cross-flow filtration, ammonium sulfate precipitation, and phenyl-Sepharose with a specific activity of 558Umg$^{-1}$.

Ohnishi et al., (1994b) has reported an A. oryzae strain that produced at least two kinds of extracellular lipolytic enzymes, L1 and L2. The lipolytic enzyme L1 has purified from culture filtrate to homogeneity by ammonium sulfate and acetone fractionation, ion exchange chromatography, and gel filtration. Lipase L1 has a monomeric protein (24,000gmol$^{-1}$ molecular weight) and preferentially cleaved all the ester bonds of triolein.

Shimada et al., (1993) have purified a lipase produced by Fusarium heterosporum by SP-Sephadex chromatography, gel filtration, and isoelectric focusing
with recovery yield of 38%. The lipase has a monomeric protein with a molecular weight of 31,000 g mol\(^{-1}\) estimated by SDS-PAGE. A lipase produced by *A. terreus* was purified to electrophoretic purity by means of ammonium sulfate and acetone precipitation, gel filtration (G-100) and ion exchange chromatography (Q-Sepharose). The molecular mass by SDS-PAGE was reported as 41,000 g mol\(^{-1}\) with the specific activity of 250 Um g\(^{-1}\) of protein (Yadav *et al.*, 1998). A lipase produced by *A. carneus* was purified by a simple two-step procedure involving ammonium sulfate precipitation and hydrophobic interaction chromatography on octyl-Sepharose. The purified lipase has a molecular weight of 27,000 g mol\(^{-1}\). A lipase monomer of apparent molecular weight of 29000 g mol\(^{-1}\) was purified to homogeneity from extracellular culture of *A. nidulans* WG312 by phenyl-Sepharose chromatography and affinity binding on linolenic acid agarose (Mayordoma *et al.*, 2000).

Hiol *et al.*, (2000) purified an extracellular lipase produced by *Rhizopus oryzae* by ammonium sulfate precipitation, sulfopropyl sepharose chromatography, Sephadex G-75 gel filtration, and a second sulfopropyl sepharose chromatography step. The enzyme was purified 1200-fold and had a molecular mass of 32 kDa by SDS-PAGE and gel filtration. The enzyme had an isoelectric point of pH 7.6. A thermostable lipase produced by a thermophilic *Bacillus* sp. J33 was purified to 175-fold by ammonium sulfate and phenyl Sepharose column chromatography (Nawani and Kaur, 2000). The overall recovery was 15.6%. The enzyme was shown to be a monomeric protein of 45 kDa molecular mass. The enzyme hydrolyzed triolein at all the positions.
2.17 CHARACTERIZATION OF LIPASE

Chahinian et al., (2000) reported that the enzyme lipase II which was produced by *Penicillium cyclopium* was characterized and observed the activity of Lipase II was maximal at pH 7.0 and 40°C. The enzyme was stable between pH values of 4.5 and 7.0. Activity was rapidly lost at temperatures greater than 50°C. Similarly Lee et al., (1999) characterized the lipase produced by thermophilic *B. thermoleovorans* ID-1. The enzyme showed optimal activity at 70 – 75°C and pH 7.5. The enzyme retained 50% of its original activity after 1-h incubation at 60°C and 30-min incubation at 70°C. Kaminishi et al., (1999) found that the purified enzymes from *Aspergillus repens* had a pH optimum of 5.3 and temperature optimum of 27°C. The *Eurotrium hebariorum* NU-2 lipase had a pH optimum of 5.2 and a temperature optimum of 37°C.

Mobarak-Qamsari et al., (2011) studied the effect of pH on the activity of lipase with four different buffers covering the range of pH 3.0 to 12.0. The enzyme was most active at pH 6.0 and 9.0, increasing activity from pH 7-9 can be characterized as an alkalophilic enzyme but high lipase activity at pH 6 can be made the lipase applicable at acidic pH conditions. The remarkable stability of *Psudomonas aeruginosa* KM110 lipase in this range has proved it to be a potential alkaline lipase. In general bacterial lipases are stable in a wide range of pH from 4 to 11. A comprehensive review of all bacterial lipase done by Gupta et al., (2004), states that maximum activity of lipases at pH values higher than 7 has been observed in many cases.

Bacterial lipases have a neutral or alkaline optimum pH with the exception of lipase from *P. fluorescens* SIK W1 that has an acidic optimum pH 4.8. Interestingly, other *Pseudomonas* lipases designated as alkaline, e.g., *P. fluorescens* HU380 (Kojima
and Shimizu, 2003), P. mendocina PK-12CS (Jinwal et al., 2003) and P. fluorescens 2D (Makhzoum et al., 1996) have lower pH optima of 8.5, 8.0, 8.5 and 9.0, respectively. Lipases from P. pseudomalei 12 (Kanwar and Goswami, 2002) and P. aeruginosa YS-7 (Shabtai and Daya-Mishne, 1992) both isolated Pseudomonas growing in different water restricted environments are stable within the pH ranges of 7-10.5 and 6.5-7.5, respectively.

Lipases are able to work in both organic solvents and in aqueous solutions. Their stability in organic solvents is desirable especially in reactions involving synthesis. Various water-miscible and water-immiscible solvents were tested for their effects on lipase activity and stability. A marked stimulation of the enzyme was observed upon addition of hexane up to 80% to the assay mixture. In contrast, ethanol, methanol and isopropanol inhibited the enzyme at concentrations of 50% and above but no effects were observed at 20% in all the solvents (Shailu et al., 2012). Thus, water miscible solvents (ethanol, acetone and isopropanol) appeared to be concentration dependent, where low concentration of these solvents showed some stimulatory effects on the lipase activity. Similar trend in stability was observed in P. chrysogenum 9' lipase, where the enzyme was more than 90% stable in the presence of hexane, 1,4-dioxane and almost 70% in the presence of cyclohexane, butanol, pentanol and xylene inhibited the enzyme activity completely at higher concentrations (Bancerz et al., 2005).
Sztajer et al., (1992) showed that *P. simplicissimum* lipase has low stability in water-miscible organic solvents with good stability in water immiscible solvents such as hexane, heptane, benzene and iso-octane. Bussamara et al., (2010) reported that the lipase from yeast *P. hubeiensis* was enhanced up to 123.4% by EDTA, while Ca$^{2+}$ showed inhibitory effects on the enzyme activity. In case of *C. verticillata* lipase, the enzyme activity was strongly inhibited by AgNO$_3$, NiCl$_2$, HgCl$_2$, CdCl$_2$ and EDTA; while the presence of Ca$^{2+}$, Mn$^{2+}$ and Ba$^{2+}$ ions enhanced the activity of the enzyme (Gopinath et al., 2002).