1.1 Enzymes

Enzymes, the biological catalysts are remarkable molecular devices that determine the patterns of chemical transformations. These catalysts also mediate the transformation of one form of energy into another. The most striking characteristics of enzymes are their catalytic power and specificity (Berg and Tymoczko, 2007). They are produced by living organisms to catalyze the biochemical reactions required for life. Thus, enzymes mainly formed within living cells, can continue to function *in vitro* (in the test-tube). Their ability to perform highly specific chemical transformations making them increasingly useful in industrial processes. Enzyme-catalyzed processes are gradually replacing chemical processes in many areas of industries.

Enzymes offer substantial and significant advantages over chemical catalysts. It can be derived from renewable resources and are biodegradable. The enzymes can operate at ambient temperature and pH, and tend to offer exquisite selectivity in both reactant and product stereochemistry unlike their chemical counterparts. Growing environmental concern due to population explosion and global industrialization advocate the need to find more benign methods of processing. This leads to the escalating interest in enzymes (Cherry and Fidantsef, 2003)**.** Of all the enzymes, the group hydrolases are most often employed for industrial biotransformations. It is estimated that approximately 80 % of all industrial enzymes belongs to the group hydrolases (Krishna, 2002)**.** Within the hydrolases, lipases are most explicitly used for food applications as well as in the area of fine chemicals manufacture (Schulze and Wubbolts, 1999)**.**

1.2 Lipolytic enzymes

Lipases are glycerol ester hydrolases (E.C. 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface (Reis *et al*., 2009). The increasing demand of lipases has attributed to their efficiency in catalyzing hydrolysis along with various reverse reactions, such as esterification, transesterification and aminolysis in organic solvents (Mala and Takeuchi, 2008). Moreover, the endowed substrate specificity, regioselectivity and enantioselectivity of microbial lipases contribute to their catalytic activity under mild temperatures with reduced side products at low waste treatment costs (Aloulou *et al*.,

2006). The activity retainment in organic solvents extend the range of applications of lipases as commercial biocatalysts in industry (Hasan *et a*l., 2006; Kumari *et al*., 2009a & 2009b; Jegannathan *et al.*, 2008; Mahapatra *et al*., 2009a & 2009b).

Lipases catalyze particularly the hydrolysis of the ester bond of tri-, di- and monoacylglycerols (glycerides). Generally, lipases have a preference for tri- and diglycerides rather than monoglycerides (Svendsen, 2000). Lipases are often 1,3-regioselective, acting on the positions *sn*-1 and *sn*-3 (end positions) rather than on *sn*-2 (mid position). Lipases also catalyse the hydrolysis or synthesis of a rather broad range of substrates containing ester linkages, such as aliphatic, alicyclic, bicyclic and aromatic esters (Schmid and Verger, 1998) along with naturally occurring triglycerides, the often preferred substrates. Lipases are also effective biocatalysts for the acylation and deacylation of a wide range of synthetic substrates, while still showing high regioselectivity and chiral recognition. The catalytic actions of lipases are affected by the water content of the reaction mixture. In aqueous media, lipases catalyze the hydrolysis of triacylglycerols into diglycerides, monogylcerides, glycerol and fatty acids (Fig. 1.1). In non-aqueous environment, lipases catalyze ester synthesis (Jaeger *et al.,* 1994).

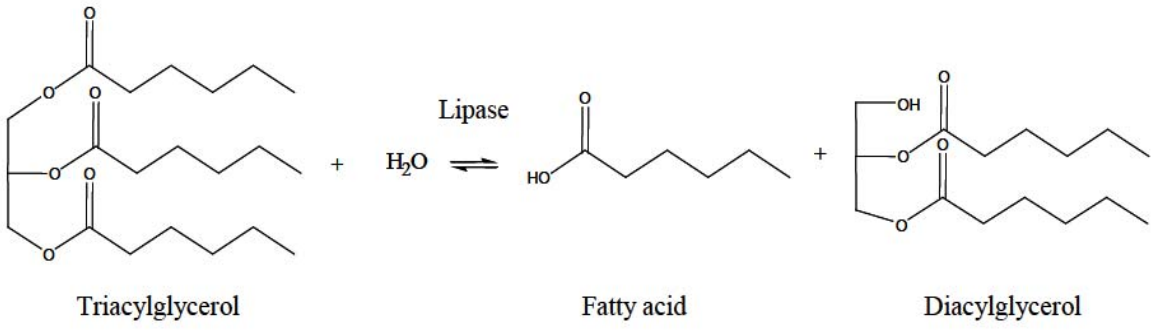

Figure 1.1: General enzymatic reaction of a lipase

Lipase mediated reactions may include ester synthesis from glycerol and fatty acids, transesterification involving the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis) and interesterification in which an acyl group is transferred to a fatty acid (acidolysis) or an ester of fatty acid (Fig. 1.2). The last two described reactions are most important for industrial applications (Jaeger and Eggert, 2002).

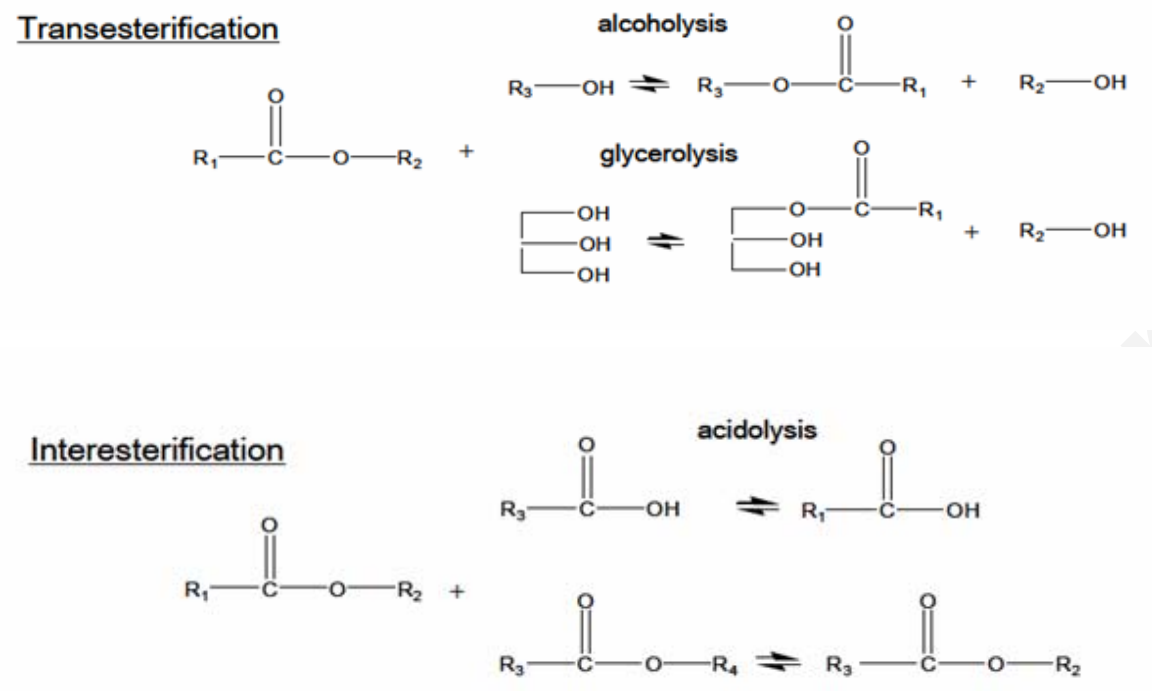

Figure 1.2: Different types of synthesis reactions catalyzed by lipases. Transesterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis) whereas in interesterification an acyl group is transferred to a fatty acid (acidolysis) or a fatty acid ester

1.3 Lipase specificity

Lipases usually carry out all the reactions with a high chemo-, regio- or enantio-selectivity. The specificities can be different between the reactions of hydrolysis and the reactions of synthesis or acyl exchange (Gunstone, 1999). Thus, lipases can be:

- **Substrate specific**: Lipases act on different compounds such as triacyl glycerols (TAGs), diacyl glycerols (DAGs), monoacyl glycerols (MAGs), or other esters by displaying a different ratio of lipolysis (Pandey *et al*., 1999a).
- **Nonspecific or regiospecific**: Regiospecific lipases are usually 1,3-specific lipases acting only on primary ester bonds (i.e. ester bonds at atoms C1 and C3 of glycerol) thus hydrolyzing TAGs into FAs, 1,2 (2,3)-DAGs and 2-MAGs. However, there are some 2-specific lipases, which hydrolyze only secondary bonds (i.e. ester bonds at atom C2 of glycerol) producing FAs and 1,3-DAGs (Gunstone, 1999). While the nonspecific lipases

act at random on TAGs, resulting in their complete breakdown to FAs and glycerol, regiospecific lipases have a positional preference for the ester bonds they hydrolyze.

- **Fatty acid-specific**: Lipases exhibit a pronounced preference for one fatty acid (FA) or for a specific range of FAs (Gupta *et al*., 2004). They are capable of distinguishing structural features of acyl chains such as length, number, position, or configuration of double bonds or the presence of branched groups, as well as the nature of the acyl sources such as free acid, alkyl ester, glycerol ester, etc (Gunstone, 1999).
- **Enantio / stereoselective**: Lipases are capable of discriminating the enantiomers of a racemic mixture. The stereospecificity of a lipase depends largely on the structure of the substrate, on the interactions at the active site, and on the reaction conditions. Stereospecificity is one of the most important properties of lipases for their application in biotechnology (Muralidhar *et al*., 2002; Gupta *et al*., 2004).

1.4 Sources of lipase

The sources of lipases can be divided into plant, animal, and microbial depending upon the environmental conditions. The lipases obtained from plants and animals are intracellular in nature whereas, the microbial sources may be intracellular or extracellular. It can further seen that the acid stable lipases are from plant sources such as castor bean, dehulled oats and rapeseed etc. Lipases from animal sources such as pancreas of pig and human are mainly used in pancreatic insufficiency such as cystic fibrosis and in enzyme replacement therapies (Abdelkafi *et al.,* 2009; Tursi *et al.,* 1994; Rosnitschek and Theimer, 1980). Although pancreatic lipases have been traditionally used for various purposes, it is now well established that microbial lipases are preferred for commercial applications due to their multifold properties, easy extraction procedures and unlimited supply (Macrae and Hammond, 1985).

The demand for microbial lipases is ever increasing owing to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH, organic solvents, chemo-, regio- and enantioselectivity, which facilitates the catalyzing of various industrial processes (Saxena *et al*., 1999). Lipase-producing microorganisms have been found in diverse habitats such as oil processing industries, dairies, soil contaminated with oil, decaying food (Sztajer *et al*., 1988), compost heaps, coal tips, and hot springs (Wang

et al., 1995). Microbial Lipases occur widely in various organisms such as filamentous fungi, yeast and bacteria and actinomycetes (Jaeger and Eggert, 2002). Among bacteria, Gram-positive species such as *Bacillus, Staphylococcus, Lactobacillus, Streptococcus, Micrococcus* ((Nawani and Kaur 2000; Lee *et al*., 1999; Manco *et al*., 1998; Simons *et al*., 1998; Hou, 1994) and *Gram*-negative species such as *Pseudomonas, Chromobacterium* (Ito *et al*., 2001; Yang *et al*., 2000; Diogo *et al*., 1999) have been exploited for the production of lipases. The utilization of yeasts such as *Candida, Yarrowia, Pichia, Saccharomyces* species (Takahashi *et al*., 1998; Weber *et al*., 1999; Pignede *et al*., 2000; Tahoun *et al*., 1985) and *Streptomyces sp.* of actinomycetes (Sommer *et al*., 1997) for production of lipases are also acknowledged in various research studies.

Filamentous fungi have been widely used in industry for several years because many strains have been classified as GRAS (generally regarded as safe). In addition, they can rapidly grow on relatively inexpensive substrates and secrete a remarkable amount of heterogeneous proteins.

Table 1.1: List of fungal species used for lipase production

Genus	Species (References)
Rhizopus	*R. delemar* (Espinosa *et al*., 1990; Klein *et al*., 1997) *R. oryzae* (Coenen *et al*., 1997; Beer *et al*., 1998; Hiol *et al*., 2000) *R. nigricans* and *R. chinensis* (Ghosh *et al*., 1996) *R. niveus* (Kohno *et al*., 1999) *R. arrhizus* (Elibol and Ozer, 2002) *R. oligosporus* (Ul-Haq *et al.,* 2002)
Aspergillus	*A. oryzae* (Ohnishi *et al*. 1994) *A. flavus* (Long *et al*., 1998) *A .niger* (Chen *et al.,* 1995) *A. carneus* (Helisto and Korpela, 1998) *A. nidulans* (Mayordomo *et al.,* 2000)
Pencillum	*P. camambertii* (Ghosh *et al.,* 1996) *Penicillium sp.* (Helisto and Korpela, 1998) *P. wortmanii* (Costa and Peralta, 1999) *P. cyclopium* (Chahinian *et al.,* 2000)
Mucor	*M. circinelloides* (Balcao *et al.,* 1998)
Geotrichum	*Geotrichum sp.* (Macedo *et al.,* 1997)
Rhizomucor	*Rhizo. miehei (*Weber *et al.,* 1999)

The genus of *Rhizopus*, *Aspergillus*, *Peniciliium*, *Mucor*, *Geotrichum* and *Rhizomucor*, serves as the major fungal sources for lipase production, which have tremendous industrial applications are presented in Table 1.1.

1.5 Fungal lipase production through solid–state fermentation (SSF)

Most microbial lipases are produced extracellularly in large quantities and are quite stable under non-natural conditions such as high temperature and in nonaqueous organic solvents (Gutarra *et al*., 2005**)**. Stability, inexpensive manufacturing, as well as broad synthetic potential of microbial lipases make them ideal biocatalysts for various industrial applications (Illanes, 2008).

Filamentous fungi are widely distributed metabolically versatile organisms. Many fungi are regarded as nature's primary degraders because they secrete a wide variety of hydrolytic enzymes that degrade waste organic materials (Archer *et al.,* 2008). Fungi serve as the source of many commercial enzymes (Archer and Peberdy, 1997 and Peberdy *et al.,* 2001) acted as hosts for production of heterologous enzymes which accounts for multifold industrial applications (Godfrey and West, 1996; Lowe, 1992).

The utilization of agricultural residues as the carbon/energy source or the solid support/substrate offers the advantage of combining the utilization of low-valued problematic residues for the production of costly enzymes and chemicals. For their ability to secrete a large amount of hydrolytic enzymes, filamentous fungi are capable of utilizing the cellulosic materials, starchy products, and pentose sugars present in agricultural residues and plant biomass (Holker *et al.,* 2004). Therefore, direct fermentation by filamentous fungi is a promising process, leading to many applications.

Enzyme production is a growing field of biotechnology. Annual world sales figures are close to a billion dollars (Layman, 1990). Traditionally, enzymes are produced using the submerged fermentation (SmF), in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. In recent years, a significant interest has been evolved in using solid-state fermentation (SSF) technique to produce a wide variety of

enzymes, mainly from mold origin (Pandey *et al.,* 1999b). Moreover, research on SSF has led to a wide range of laboratory scale applications and comparative studies between SmF and SSF claim higher yields for products made by SSF (Viniegra-González, 1998 and Viniegra-González *et al.,* 2003).

1.5.1 General aspects and advantages of SSF

SSF is generally defined as the growth of microorganisms (often fungi) on solid substrates in systems with a continuous gas phase and no free-flowing water by employing either a natural support or an inert support as a solid material (Couto and Sanorman, 2005; Pandey *et al*., 2000). The non-inert materials such as wheat bran, baggase and oil cakes used in SSF serve as an attachment as well as supplier of some nutrients to the microorganism (Durand *et al*., 1993 and Ralph, 1976). Rodriguez *et al*. (2006) utilized sugarcane bagasse with liquid medium as the substrate for lipase production by *Rhizopus homothallicus* through solid state fermentation. Rice and wheat bran supplemented with rice bran oil were utilized as the substrates for lipase production by SSF from *Candida* sp. (Bhusan *et al.,* 1994). Enhanced lipase activity was reported in case of lipase production by *R. pusillus* and *R. rhizopodiformis* with the mixture of olive oil cake and sugarcane baggase as the substrate for the solid state fermentation (Cordova *et al*., 1998). Several researchers utilized wheat bran as the substrate for lipase production by *Rhizopus oligosporus* (Beuchat, 1982 and Ul-Haq *et al.,* 2002), *P. candidum* (Ortiz-Vazquez *et al.,* 1993) and *A. niger* NCIM 1207 (Mahadik *et al*., 2002) through solid state fermentation.

In addition to above features, costs are much lower due to the efficient utilization and value-addition of wastes in case of SSF (Holker and Lenz, 2005). Compared to SmF, SSF have certain advantages and drawbacks (Viniegra-González *et al.,* 2003 and Robinson *et al*., 2001).

The advantages include:

- High volumetric productivity.
- Low capital investment and energy requirements.
- Ease of product recovery.

- Less waste-water output.
- Fermentation conditions well adapted to the requirements of fungal cultures.
- Possibility of using new substrates (e.g. solid byproducts, plants) without the need for pretreatments.

The drawbacks associated with the SSF include:

- Difficulties in fermentation control and automation.
- Limited mass and heat transfer.
- Necessity for adequate aeration to remove excess heat;
- Sterilization and contamination problems;
- Problems with downstream processing, for some applications and difficulties with scale-up of the process.

The advantages of higher titer values in case of SSF compared to SmF was cited in several instances, while comparing the same strain and fermentation broth in case of different fungal enzymes. Viniegra-Gonz´alez *et al*., (2003) reported the higher titres values of invertase, pectinase and tannase in SSF than in SmF. Further, the comparative economic analysis of SSF and SmF processes for the production of lipases by *Penicillium restrictum* as reported by Castilho *et al*., (2000) indicated the better economic efficiency of the SSF. Higher lipase titer values were also reported in case of fungal lipase production by *P. candidum*, *P. camembertii* and *M. miehei* through SSF over SmF (Rivera-Munoz *et al*., 1991).

The production of lipases through SSF is influenced by various fermentation conditions such as pH, temperature, liquid to solid ratio and incubation time, presence of lipids. Lipases are mostly induced in presence of fats and oils (Lotti *et al*., 1998) in the culture medium. Sztajer *et al*., (1993) reported the maximal lipase activity by *Penicillium expansum* upon addition of 0.1% olive oil in the culture medium. The extracellular lipase production by *Candida rugosa* was induced by addition of fatty acids to the culture medium (Lotti *et al*., 1998) resulted in different isoforms. In another study, rapeseed and corn oils were reported as the inducers for lipase production (Essamri *et al*., 1998). The optimal temperatures of 30 °C and 37 °C were reported for lipase production by *Geotrichum sp.* (Chen *et al*., 1998) and *Aspergillus carneus* (Burkert *et al*., 2004)

respectively through SSF. The alkaline and acidic pH was preferred for lipase production by *Bacillus sp* (Sugihara *et al*., 1991) and *Rhizopus chinensis* (Tenga and Xu, 2008) respectively. An optimum incubation times of 96 h and 72 h were reported for the lipase production by *P. fluorescens* BW 96CC (Dong *et al*., 1999) and *Aspergillus carneus* (Kaushik *et al*., 2006).

1.6 Application of statistical and evolutionary approaches for modeling and optimization approaches

Modeling and optimization are two of the most vital steps in a fermentation process for maximizing the efficacy of the process. Most of the studies carried out till date were using one variable at a time approach that is invariably time consuming, requiring more number of experimental runs and often fails to give information regarding the interactions between the variables. These limitations can be overcome by developing a non-linear multivariate process model for optimization. Thus, the experiment may be modeled for obtaining the second order polynomial equation, so as to simulate the experimental data by using statistical, evolutionary and swarm intelligence approaches. Response surface methodology (RSM) is a statistical technique which defines the effect of the independent variables (alone or in combination), on the processes and generates a mathematical model in addition to analyzing the effects of the independent variables of the process (Myers and Montgomery, 2005). Evolutionary and swarm intelligent optimization approaches such as Genetic Algorithms (GA), Differential Evolution (DE) and Particle Swarm Optimization (PSO), have attracted a great deal of attention in recent times. With their better global search abilities these techniques successfully overcome the local optima obstacle and thus replace the traditional gradient optimization approaches (Tang and Wu, 2009). These approaches can find global optima more quickly through cooperation and competition among the population of potential solutions of the search space even for complex optimization problems such as fermentation processes (Back, 1996). Genetic Algorithm, originally developed by Holland, is a computerized search and optimization technique. It works on the "survival of the fittest" concept of natural selection namely Darwinian evolution (Holland, 1992). Differential Evolution is a stochastic, vector population based optimization technique with a conceptual base of simulating the

evolution of a population of individuals using a predefined set of operators namely selection and search (Storn and Price, 1997). It works on a particular way of constructing so-called mutant vectors by using differences between randomly selected elements from the current population. Particle Swarm Optimization proposed by Kennedy and Eberhart in 1995, is a swarm intelligence optimization based on the simulation of the social behavior of birds within a flock, whose basic thinking was to find the optimal value through cooperation and sharing information among individuals of swarm (Kennedy and Eberhart, 1995).

The relatively prohibitive cost of native enzyme has been the chief obstacle hindering more rapid expansion of industrial lipase technology. Optimization of fermentation and extraction conditions of lipase production through solid state fermentation (SSF) represents one of the promising options for production of inexpensive lipases in bulk as far as energy requirements, volumetric productivity and product recovery is concerned (Sun *e t al.,* 2009). An increased thermal stability was reported by Mateos Diaz et *al*., (2006), in case of extracellular lipase produced through solid state fermentation than the submerged fermentation. Lipase production by *Rhizopus homothallicus* cultured under SSF was improved by optimizing the nutrient sources (Rodriguez *et al*., 2006). Kempka *et al*., (2008) utilized RSM to predict the optimal conditions of lipase production by *Penicillium verrucosum* through SSF using soybean bran as the substrate. Enhanced enzyme recovery was reported in case of transglutaminase (Volken de Souza *et al*., 2008) and xylanase (Heck *et al*., 2005) through optimization of extraction variables from the SSF biomass of *Bacillus circulans* BL32 and *Bacillus circulans* respectively. Optimization of α-amylase and protease production through GA and PSO approaches was reported by Skolpap *et al*., (2008).

An efficient lipase extraction from fermented biomass enhances concentration and recovery of lipase at reduced capital cost. The influence of temperature, pH, type of solvent, solid to liquid ratio, soaking time and interrelation among them on lipase recovery need to be taken into account during extraction process (Castilho *et al*., 1999). Illustrative effect of different extraction variables on enzyme recovery from the

fermented biomass has been reported in case of glutathione (Xiong *et al*., 2009), exo-polygalacturonase (Gupta *et al*., 2008), xylanases (Heck *et al*., 2005), transglutaminase (Volken de Souza *et al*., 2008), protease (Monteiro *et al*., 2005) and amylase (Palit and Banerjee, 2001). Vardanega *et al*., (2009) assessed the effect of solvent, pH, agitation, temperature and solid to liquid ratio on lipase yield produced through SSF of soybean meal by a newly isolated strain of *Penicillium* sp. In another study, aqueous two-phase system of polyethylene glycol (PEG) and phosphate was utilized for the extraction of α-galactosidase by *Aspergillus oryzae* (Naganagouda and Mulimani, 2008). The extraction of chitosanase from the fermentation biomass of *Bacillus cereus* NTU-FC-4 using reversed micelles was reported by Chen *et al.,* (2006).

1.7 Structure of lipases

From the X-ray crystallographic and NMR studies, almost all lipases have the similar structural fold namely α/β hydrolase fold although they display low sequence similarity (Jaeger *et al*., 1999). The α/β hydrolase fold is common for lipases and many other hydrolases (Ollis *et al*., 1992). In general, this α/β hydrolase fold consists of a central, mostly parallel β-sheet of eight strands with the second strand being antiparallel. The parallel strands β3 to β8 are connected by α-helices, which are located on either site of the central β-sheet (Fig.1.3). The curvature of the β-sheet as well as the positions of the α-helices varies considerably among the different lipases (Schrag and Cygler, 1997).

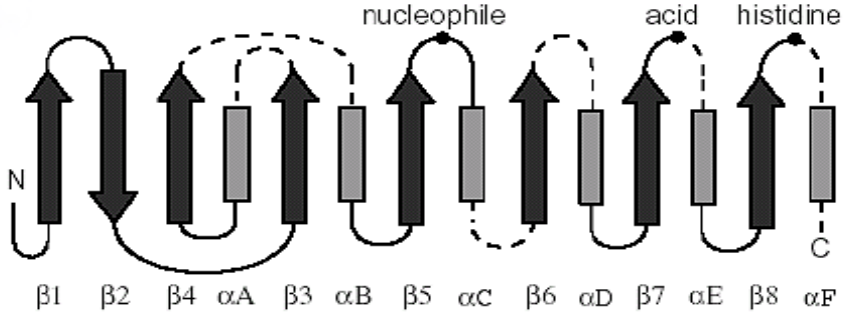

Figure 1.3: General folding pattern of α/β hydrolases (Van Pouderoyen *et al*., 2001). α-Helices are indicated by bars and β-sheets by arrows. The topological position of the active site residues are shown by a solid circle

Active site of lipases is located in the central β-sheet and consists of three catalytic amino acid residues:

- a nucleophilic serine
- a histidine
- a catalytic acid residue that is either an aspartate or glutamate.

The nucleophilic serine residue is located in a highly conserved Gly-X-Ser-X-Gly pentapeptide. This consensus sequence is part of the β-ε-Ser-α motif, which can be found in all serine hydrolases playing an important role for the catalytic activity by providing the steric requirements (Ollis *et al*., 1992). β3 to β8, the parallel strands are connected by α helices, which pack on either side of the central β sheet. The β sheet has a left-handed superhelical twist such that the surface of the sheet covers about half a cylinder and the first and last strands cross each other at an angle of ~90º. Curvature of the β sheet may differ significantly among the various enzymes, and also, the spatial positions of topologically equivalent α helices may vary considerably. Variations of the peptide chain at the C-terminal ends of strands in the C-terminal half of the β sheet form the binding subdomains of the α/β hydrolase fold proteins. They differ substantially in length and architecture, in agreement with the large substrate diversity of these enzymes (Jaeger *et al*., 1999). The lipase structures known so far obey the α/β hydrolase fold although they can display additional variations in the number or in the disposition of the α helix and β strands (Schrag and Cygler, 1997).

Furthermore, most lipases have a particular structure, designated as "lid", located at the protein surface, close to the active site. The lid consist of a single α helix, two helices, or a loop region, which is mainly hydrophobic on the side directed towards the active site, and hydrophilic on its external face. The lid displays a variable position depending on the physicochemical environment of the enzyme (Grochulski *et al*., 1994; Cygler and Schrag, 1997). Thereby, the lid covers the active site of the enzyme in the absence of an interface. On the contrary, the presence of an interface produces a structural rearrangement that displaces the lid, which is stabilized by hydrophilic interactions with the enzyme surface, allowing a free accession of the substrate and the solvent to the active site of the enzyme. However, exceptions such guinea pig pancreatic lipases do not have lid (Verger, 1997; Jaeger *et al*., 1999; Cygler and Schrag., 1999).

1.8 Catalytic mechanism of lipase

Evidence of the mechanism of ester hydrolysis or synthesis has come from crystallographic analyses of inhibitor–lipase complexes. It is constituted by the four steps represented in Fig. 1.4. During the first step, the substrate binds to the nucleophilic serine yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residues, the helix C, and the oxyanion hole. Next, the alcohol is released and an acyl–enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol or ester in trans- or interesterification) forms again a tetrahedral intermediate, which after resolution yields the final product (an acid or an ester) and free enzyme (Bornscheuer, 2002).

The detailed steps of lipase mechanism of action in hydrolysis reaction are summarized below:

Step 1: The hydrolysis starts with the lipid binding and the activation of nucleophilic serine residue by the neighbouring active histidine, to which a proton from the serine hydroxyl group is transferred. Proton transfer is facilitated by the presence of the catalytic acid, which precisely orients the imidazole ring of the histidine and partly neutralizes the charge. Activation is followed by an attack by the oxygen atom (O^-) of the serine hydroxyl group on the activated carbonyl carbon of the susceptible lipid ester bond (Jaeger *et al*., 1999).

Step 2: A transient tetrahedral intermediate is formed, which is characterized by a negative charge on the carbonyl oxygen atom of the scissile ester bond and four atoms bonded to the carbonyl carbon atom arranged as a tetrahedron. The intermediate is stabilized by the macrodipole of helix C, and by hydrogen bonds between the negatively charged carbonyl oxygen atom (oxyanion) and at least two main-chain NH groups (oxyanion hole).

Step 3: The additional proton of histidine is subsequently donated to the ester oxygen of the susceptible bond, which is thus cleaved. At this stage, the acid component of the substrate is esterified to the nucleophilic serine (the covalent intermediate), whereas the alcohol component diffuses away (Jaeger *et al*., 1999).

Step 4: The histidine residue donates its additional proton to the oxygen atom of the active serine residue, which breaks the ester bond between serine and the acyl component, and releases the acyl product. After diffusion of the acyl product, the enzyme is ready for another round of catalysis (Jaeger *et al*., 1999).

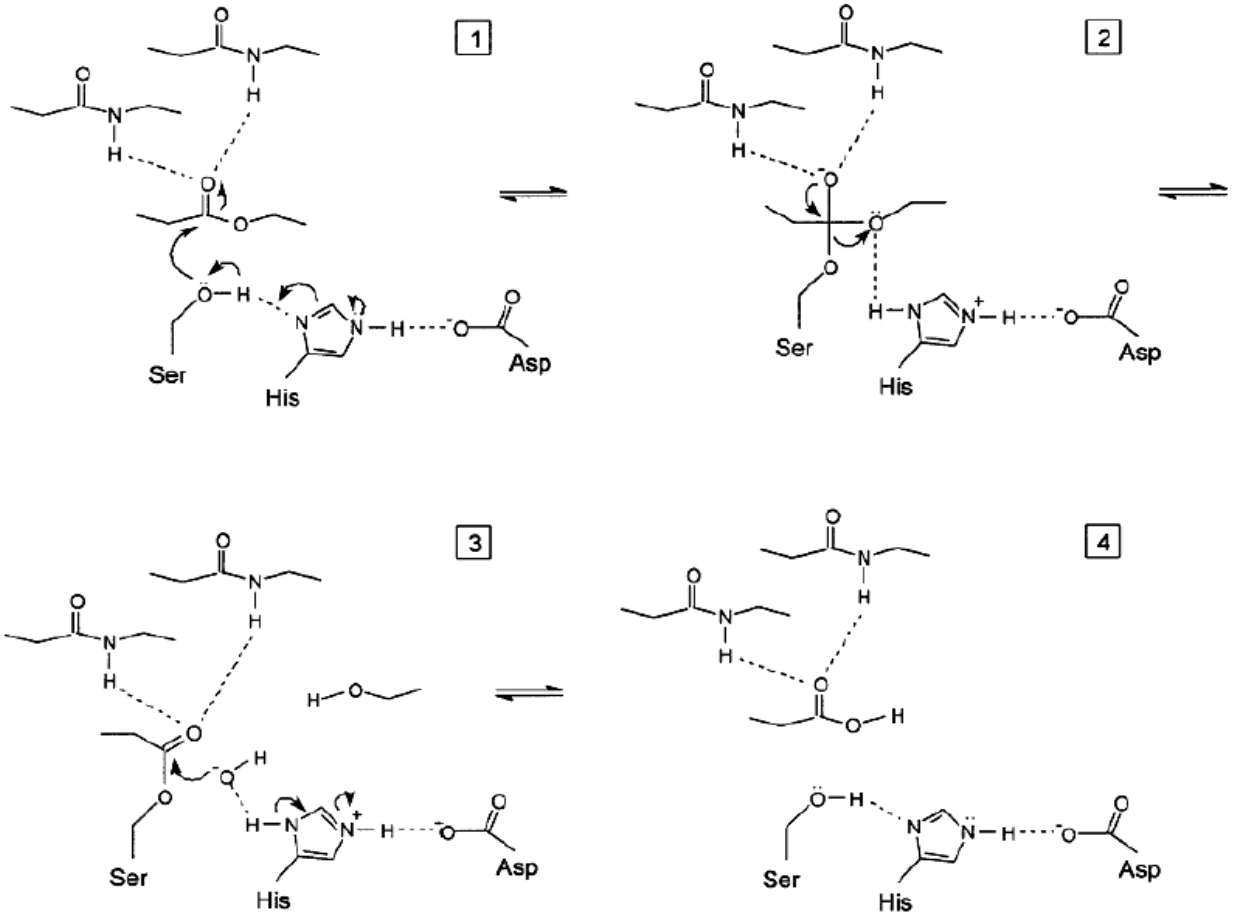

Figure 1.4: Mechanism of action of lipase in hydrolysis reaction

1.9 1nterfacial activation of lipases

A notable characteristic of lipase has been documented as when the substrate concentration exceeds the critical micelle concentration the enzyme increases its catalytic activity (Sarda and Desnuelle, 1958) (Fig. 1.5) because of the phenomenon of interfacial activation. This increase in enzymatic activity is triggered by certain structural rearrangements of the lipase active site region, as witnessed from the crystal structures of

lipases complexed with small transition state analogs (Jaeger *et al*., 1999), and it is facilitated by the particular amino acid composition of the enzymes themselves (Fojan *et al*., 2000). However, this phenomenon depends highly on the quality of the interface of the lipids used as substrates, as well as on the reaction conditions i.e., ionic strength, presence of detergents or emulsifying agents, stirring, etc (Verger, 1997).

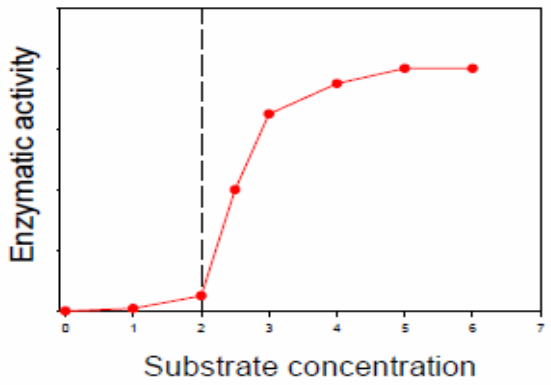

Figure 1.5: Interfacial activation of lipase: Lipases display low activity until their substrate reaches a concentration high enough to form an emulsion or micelles (dashed line), then their activity increases drastically

Lipases display an enhanced content of short, non-polar residues (usually valine, leucine and isoleucine) which cluster at the protein hemisphere where the active site is located. These residues could facilitate the lipase attachment to the hydrophobic substrate aggregate, which then undergo the structural rearrangements responsible for the opening of the lid (Fojan *et al*., 2000). Once lid is opened, the active site becomes accessible to the substrate, which explains the resulting increased activity (interfacial activation) of the enzyme. For this reason, lipases that do not have a lid do not show interfacial activation (Verger, 1997; Jaeger *et al*., 1999).

Various crystallographic structures of fungal (Brzozowski *et al*., 1991; Grochulski *et al*., 1993; Schrag and Cygler, 1993; Grochulski *et al*., 1994; Derewenda *et al*., 1994a) lipases with and without ligands have shown that the interfacial activation can be partially attributed to a conformational change in the lipases. In fungal lipases, the interfacial activation is caused by the opening of an alpha-helical, amphipathic lid by a rotation around two hinge regions (Derewenda, 1994b). In the open active form a significant

hydrophobic surface is revealed. In the case of pancreatic lipases a more complex model including effects mediated by a collapse has been proposed (Hermoso *et al*., 1997 and Pignol *et al*., 2000). Recent studies on the activation of fungal and pancreatic lipases have indicated that the activation mechanism is controlled by a combination of hydrophobic and electrostatic interactions (Berg *et al*., 1998 and Cajal *et al*., 2000) and is possibly influenced by the curvature of lipid interface (Hermoso *et al*., 1997 and Cajal *et al*., 2000). The hydrophobic surface that is exposed in the open form of the lipase interacts with the lipid interface. Electrostatic interactions between the anionic groups of the lipid interface and the positively charged arginines in the hinge regions of the lid have been found to stabilize the open form (Berg *et al*., 1998 and Cajal *et al*., 2000). The importance of the electrostatic interactions in the activation of lipase is supported by experiments which show that lipase activation does not occur in the zwitterionic surface (Berg *et al*., 1998 and Cajal *et al*., 2000). Although the importance of the hydrophobic and electrostatic interactions in the activation mechanism is now known, the exact mechanism of the interfacial activation remains unclear.

The closed conformation of lipases (with the active site covered by the lid), predominates in aqueous solution, although persistence of a low level of activity in these solutions shows that open conformations are also present, at least transiently. However, the open conformation of lipases produced by lid opening predominates when the protein contacts the hydrophobic components of lipid-water interfaces. The open conformation makes the catalytic residues accessible to substrate and rearranges the oxyanion hole in a more suitable conformation. It also exposes the large hydrophobic surface surrounding the catalytic residues. Because close contacts exist between the residues of the active-site region, the amino acids located two positions before and two positions behind the nucleophile are usually glycine, or occasionally other small residues such as alanine or valine (Jaeger *et al*., 1999). Moreover, the increased content of small non-polar residues in the active site region of lipases in comparison with that of carboxyl esterases and other enzymes enhances even more the interaction between active site of TLs and the substrate, thus increasing the enzyme activity when the lid is moved away (Fojan *et al*., 2000). The fact, that evolution of lipases chose small non-polar residues to populate the region

around the active site is attributed to several reasons. Small non-polar residues have a smaller entropic penalty for their solvation, are flexible and can more easily intercalate into an interface than bulky amino acids, which tend to stack with membrane-like structures. This intercalation of small residues into hydrophobic interfaces disturbs them and facilitates the acquisition of triacylglycerol molecules into the active site. Furthermore, these residues are very unspecific, and no predefined attachment point on the hydrophobic surface has to be present (Fojan *et al*., 2000).

1.10 Immobilization of lipases

Technical applications of lipases in the industries are feasible only if the lipases are stabilized against extreme temperature, pH and presence of salts, alkali and surfactants. Utilization of non–aqueous conditions for lipase mediated reactions facilitate the increased solubility of apolar substrates, shift of thermodynamic equilibrium to favor synthesis over hydrolysis, suppression of many water dependent side reactions, decreased microbial contamination in addition to high selectivity, high specificity and catalytic rates under mild conditions. Exploiting such advantages is often limited by the low stability and/or activity of lipase under non-aqueous conditions.

Immobilization to solid carriers is perhaps the most used strategy to improve operational stability of lipases, better operation control, easier product recovery without catalyst contamination and flexibility of reactor design. Further, decreased inhibition by reaction products, selectivity towards non-natural substrates and better functional properties compared to the corresponding soluble enzymes, all make immobilization one of the most preferred methods of enzyme improvement towards stabilization (Fagain, 1995). Any immobilized enzyme, by definition, must comprise two essential functions, namely the non-catalytic functions (NCF) and catalytic functions (CF). NCF are designed to aid in separation (e.g. separation of catalysts from the application environment, reuse of the catalysts and control of the process) whereas, CF are designed to convert the substrates within the desired time and space (Fig. 1.6) (Cao *et al*., 2003). NCF are strongly connected with noncatalytic part of the immobilized enzymes namely the geometric properties such as shape, size, thickness and length of the selected carrier. The catalytic

properties of enzyme i.e. activity, selectivity, and stability, pH and temperature profiles are connected with the CF. The general criteria for selection of NCF and CF for robust immobilized enzymes are presented in Table 1.2.

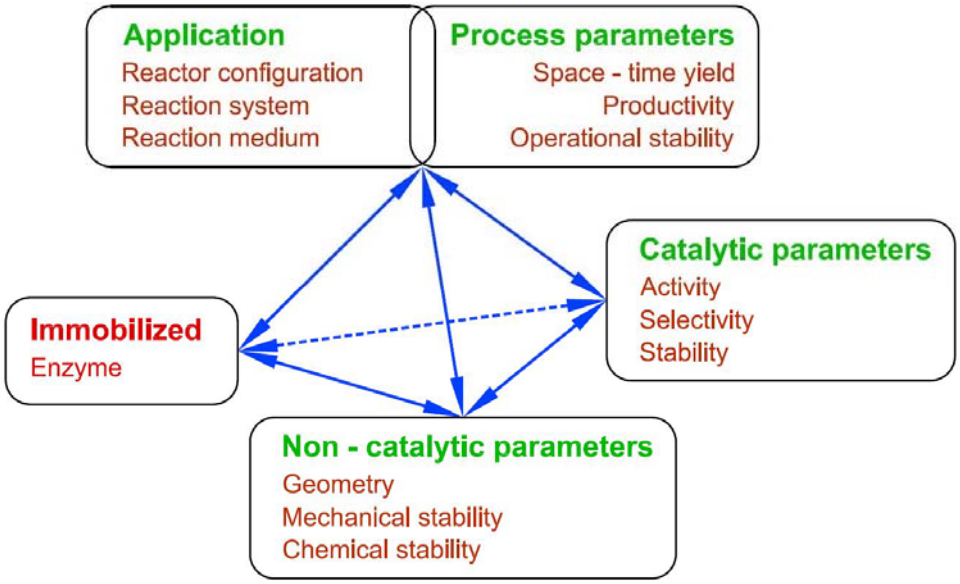

Figure 1.6: Relationship between NCF and CF of an immobilized enzyme and its applications

The four principal methods of immobilization are (Fig. 1.7):

(a) Adsorption

(b) Covalent binding

(c) Cross-linking

(d) Entrapment.

1.10.1 Adsorption

Physical adsorption of an enzyme onto an insoluble, prefabricated carrier, without covalent binding, is the oldest commercial application for the immobilization of an enzyme. The process is a very simple, easy to perform, economical procedure for the immobilization of enzymes, with wide applicability and capabilities of high enzyme loading, whilst not grossly altering the biocatalysts activity under the correct conditions. In practice, the enzyme is simply mixed with a suitable adsorbent under appropriate conditions of pH and ionic strength for a sufficient incubation period. After which, the

loosely bound and unbound enzyme is washed off to produce the immobilized enzyme in a directly usable form. The particular choice of adsorbent, a variety of which are available, depends principally upon minimizing leakage of the enzyme during use. Although the physical links between the enzyme molecules and support are often very strong, they may be reduced by many factors including the introduction of the substrate. Additional care must be taken so that the binding forces are not weakened during use by inappropriate changes in pH or ionic strength.

Table 1.2: Criteria for robust immobilized enzymes (Cao, 2005)

Parameter	Requirement	Benefits
Non-catalytic function	Suitable particle size and shape	Aid separation, easy control of the reaction
	Suitable mechanical properties	Flexibility of the reactor design
	Low water regain capability	Easy removal of the water
	High stability in a variety of organic reactions	No change of pore radius and fewer diffusion constraints
Catalytic function	High volume activity (U g^{-1})	High productivity and space time yield
	High selectivity	Fewer side reactions, easier downstream processing and separation of products and less pollution
	Broad substrate specificity	Tolerance of structural variation of the substrates
	Stability in organic solvents	Shift of reaction equilibrium with the use of organic solvents
	Thermostability	Short reaction time by increasing temperature
	Operation stability	Cost-effective and lower cost contribution for the product
	Conformational stability	Modulation of enzyme properties
Immobilized enzyme	Recyclability	Low cost-contribution of catalyst
	Broad applicability	Tolerance of process variation
	Reproducibility	Guarantee product quality

Suitable adsorbents include ion exchange matrices, porous carbon, clays, hydrous metal oxides, glasses and polymeric aromatic resins. Stability of the adsorbed enzyme derivative will depend on the strength of the non-covalent bond formed between the support and the amino acid residues on the surface of the protein. Therefore, adsorption is not permanent like covalent bonding. The driving force causing this bonding is usually a combination of hydrophobic effects and the formation of several salt links per enzyme

molecule. The binding forces may be ionic, hydrogen bonds or van der Waal's interactions.

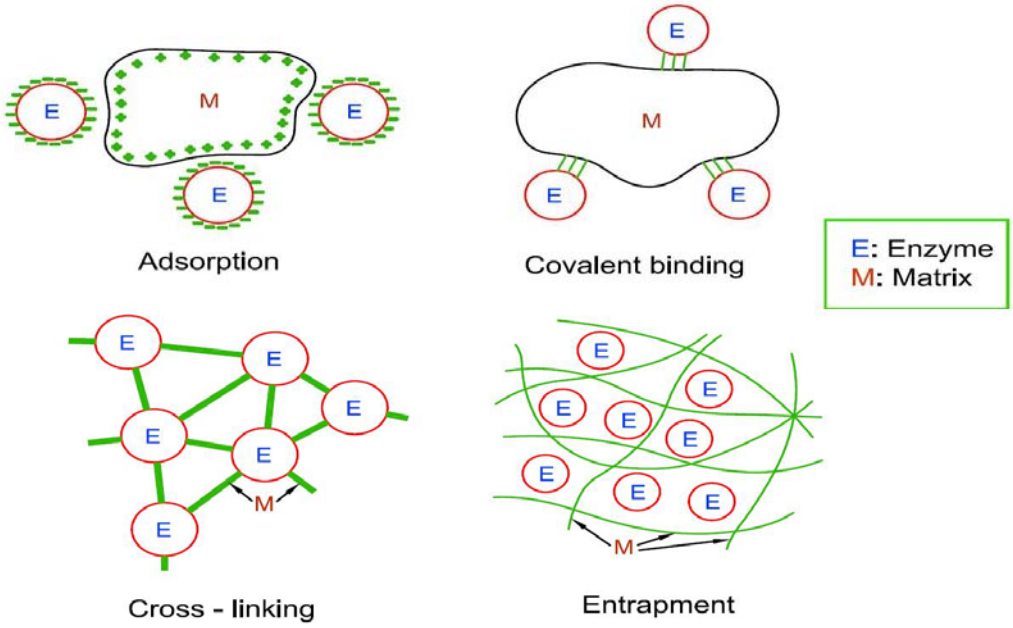

Figure 1.7: Enzyme immobilization techniques

The number and nature of bonds formed are controlled by the iso-electric point of the protein and the pH at which immobilization occurs, as both factors determine the respective surface charges. In addition to simplicity, wide applicability for both whole cells and isolated enzymes and low cost, adsorption has the advantages of mild operating conditions, the availability of a ready prepared polymer material suitable for use in column reactors and the potential for regeneration of the immobilised enzyme or support as the binding is reversible. Additionally, there are minimum limitations for access of the substrate to the bound enzymes whilst causing no harm to the protein itself as chemical attachment might do. There is however, one principal disadvantage associated with adsorption as a method of immobilization. That is, the reversible nature of the interactions between support and enzyme which are much weaker than in the case of the covalent binding. This can result in the adsorbed enzymes easily being desorbed from the support surface if there are changes in temperature, pH, ionic strength or even in the presence of substrate, may be at a critical time. Also, the amount of enzyme bound per unit of adsorbent is often low so the enzyme is often partially or totally inactivated. A further disadvantage to mention is non-specific further adsorption of other proteins or

other substances that may take place and subsequently alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, then the rate will probably decrease depending on the surface mobility of the enzyme and substrate. Although this method is usually used for scientific studies or for so called 'disposable' enzymes, adsorbed enzymes have been very useful in several areas of biotechnology and are still considerably more stable than an enzyme in solution (Bickerstaff, 1997).

1.10.2 Covalent binding

Covalent binding is the covalent chemical attachment of an enzyme to a water insoluble supporting material through the activated groups of the support and some external functional groups of the protein. Attachment of an enzyme to a carrier through covalent binding is probably the most extensively studied and used method. Immobilization of an enzyme *via* covalent binding generally involves two steps; activation of the support and coupling of the enzyme to the activated support. In order to make the functional groups on the chosen support strongly electrophilic, which is necessary for the second step to take place, the functional group of the support must be activated with a specific reagent. As the activated supports have unstable and reactive functional groups, immobilization of the enzyme should follow immediately after activation, unless steps are taken to preserve the activated support.

The second step is the coupling reaction where the external functional nucleophilic groups of the enzyme which are not essential for catalytic activity are covalently attached to the activated support material *via* a chemical reaction. Usually, this step is carried out using a bi-functional agent as a bridge between the enzyme and support. The range of chemical coupling procedures used is enormous. However, the selection of methodology is dependant on the nature of the support and enzyme and it is important to study the active sites of the enzyme prior to choosing a coupling method as the immobilization method must not involve any group at that point and bonding must take place through other groups in the enzyme, such as hydroxyl, phenolic, thiol, carboxyl or amino groups.

Coupling reactions involved in covalent immobilization fall into the following categories (Kennedy and Cabral, 1983)

- Diazotization
- Peptide bond formations
- Alkylation or arylation
- Schiff base formations
- Ugi reactions
- Amidation reactions
- Thiol-disulphide interchange reactions
- Mercury-enzyme interactions and
- γ- irradiation induced coupling.

Covalent binding is very strong with minimal leakage of enzyme from the support. Theoretically, this method of immobilization should not alter the enzymes conformational flexibility thereby yielding stable, insolubilised enzyme derivatives that maintain activity for several cycles without enzyme desorption. Disadvantages however, are that the covalent coupling of an enzyme can produce a considerable loss of activity due to the influence of the coupling conditions and to conformational changes in the enzyme structure. Also, irreversible binding of the enzyme to the carrier during covalent coupling does not allow for the recovery of the carrier from the carrier-enzyme complex and extensive, difficult and tedious preparations are often involved with high associated costs. Covalent binding is only applicable to a handful of enzymes with only small amounts of enzyme being immobilized by this technique. In addition, immobilization through covalent binding does not offer microbial protection and it is not a good technique for the immobilization of whole cells.

1.10.3 Cross-linking

Intermolecular cross-linking of an enzyme is essentially an extension of covalent bonding techniques. To achieve immobilization, the enzyme forms links to an insoluble, solid support matrix or itself, using homo- and hetero-bifunctional cross linking agents such as glutaraldehyde. In practice, the enzyme is crystallised before being chemically cross-

linked using a bifunctional reagent such as dialdehydes like glutaraldehyde or diamines activated with carbodiimide to 'lock' the crystalline state, obtaining an insoluble active protein matrix in the form of homogenous enzyme crystals (Sheldon *et al*., 2006). Immobilization of enzymes *via* cross-linking is a particularly attractive, relatively cheap immobilization alternative. However, the actual development of an adequate crystallisation protocol is expensive and time consuming and often yields very little bulk of immobilized enzyme with a very high intrinsic activity. The cross-linking of enzyme aggregates developed by Tischer and Wedekind (1999) is basically the covalent cross-linking of a precipitated enzyme without the need for crystallisation, based on the amply demonstrated notion that enzymes and proteins in general can be precipitated by agents such as inorganic salts or organic solvents without undergoing denaturation. The resulting cross linking enzyme aggregates tend to have high enzymatic activity but lower stability than the cross linking enzyme crystals.

1.10.4 Entrapment

The immobilization of a biocatalyst *via* entrapment within the gels, membranes or fibres to generate hydro-gels or water-soluble polymers, is in principle, an easy to perform process. The enzyme or whole cell becomes confined within a matrix by dissolving the biocatalyst in a solution of the chemicals required for the synthesis of the enzyme phase and then treating this so that a distinct phase is formed. The process may be purely physical caging or involve covalent binding, resulting in a system where in theory the enzyme cannot escape, whilst substrates and products have the ability to diffuse in and out. The costs associated with entrapment are usually quite low, the method is applicable to a wide variety of enzymes, is simple and requires mild conditions whilst providing microbial protection. The method is desirable as a high enzyme loading is usually achieved with high activity.

The entrapment of enzymes is a convenient method for use in processes involving low molecular weight substrates and products. In practice, the biocatalyst is dissolved in a solution of the polymer precursors, after which polymerization is initiated. The polymeric mass is then broken up to the desired particle size, whilst the enzyme remains 'trapped' in

the gel, 'net-like' matrix, which in theory, has pores that are large enough to permit entry of low molecular weight substrates but too small to allow the escape of the enzyme. A wide variety of natural polymers like agar, agarose and gelatine have been used for polymer entrapment, as well as a number of synthetic polymers, such as the photo-crosslinkable resins, polyurethane prepolymers and acrylic polymers like polyacrylamide (Mateo *et al.,* 2007). The broad distribution in pore size of synthetic gels of the polyacrylamide type inevitably results in leakage of the entrapped enzyme, even after prolonged washing. This may be overcome by cross-linking the entrapped protein with glutaraldehyde.

Immobilization of lipases has been reported in several instances, which shows the potential application of immobilization techniques in improving the enzyme activity and stability. Mateo *et al.*, (2007) reported the improvement of enzyme activity, stability and selectivity by using different immobilization techniques. They devised different immobilization strategies, which results in the enhancement of different critical enzyme properties like stability, activity, inhibition by reaction products and selectivity towards non-natural substrates. The biochemical properties of free and immobilized *Rhizopus oryzae* lipases on $CaCO_3$ were investigated which suggested the improvement of thermal stability and hydrolytic activity of immobilized enzyme (Ghamgui *et al*., 2007). Torres *et al*., (2006) obtained higher enantioselectivity with immobilized lipase compared to free lipase. Wilson *et al*., (2006) studied immobilization of lipase from *alcaligenes sp.* on hydrophobic supports via strong adsorption. The immobilized preparation exhibited a 135 % of catalytic activity for the hydrolysis of *p*-nitrophenyl propionate as compared to the soluble enzyme; the thermal stability of the immobilized enzyme was highly improved. Won *et al.,* (2005) optimized the lipase entrapment in calcium alginate beads. They found that increasing in alginate concentration and calcium chloride concentration raised loading efficiency, but decreased immobilization yield. With increasing bead size, immobilization yield decreased due to mass transfer resistance, but loading efficiency was unchanged. Yesiloglu (2005) proposed bentonite as an immobilization matrix for *Candida rugosa* lipase due to its low cost, its lack of toxicity and chemical reactivity and obtained better enzymatic properties. The kinetics and mechanism of reactions catalyzed

by different lipases were studied by Paiva *et al.,* (2000), which provide structural features of lipases to understand immobilization, interfacial activation, catalytic performance, physicochemical and statistical significance of parameters in rate expressions. The lipase from *Rhizopus japonicus* immobilized on celite (Khare and Nakajima, 2000) was used for the enrichment of docosahexaenoic acid in soybean oil.

1.11 Industrial applications of lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) constitute a most important group of hydrolytic enzymes with immense biotechnological applications in the health care/pharmaceutical, biodiesel, oleo-chemical, food, cosmetic and agrochemical industries (Hasan *et al*., 2006; Jaeger and Reetz, 1998). The attractive biotechnological potential of lipase and/or lipase mediated reactions is mainly attributed to its exquisite catalyzing efficiency of aqueous and non-aqueous reactions under mild conditions (Reis *et al*., 2009 and Verma *et al*., 2008). Utilization of wide spectrum of substrates, higher stability at extreme conditions of temperature, pH, substrate-, regio- and enantio selectivities of microbial lipases makes the microbes the predominant sources for lipase production (Mala and Takeuchi, 2008). Fungal lipases, especially *Rhizopus* lipases from GRAS organisms are preferred in oleaginous (Villeneuve, 2007), food (Torres *et al*., 2009 and Mahapatra *et al*., 2009a), biopharmaceutical (Mahapatra *et al*., 2009b) and biofuel industries (Fjerbaek *et al*., 2009 and Kumari *et al*., 2009a) due to their 1,3-(regio)-specificity.

The positive attributes of lipase mediated reactions are summarized below:

(i) **Versatility:** lipases are versatile biocatalysts which can carry out many different reactions of hydrolysis in organic solvents, synthesis and acyl exchange using a wide range of natural and non-natural compounds (Bornscheuer, 2002; Gupta *et al*., 2004).

(ii) **Specificity and selectivity:** some of them show high substrate specificity, or high chemo-, regio- and stereoselectivity (Gunstone, 1999; Jaeger and Eggert, 2002).

(iii) **Absence of subproducts:** most lipases do not perform lateral reactions (Jaeger and Eggert, 2002).

(iv) **Stability:** lipases are active and stable in organic solvents, and in a wide range of pH

and temperature (Schmidt-Dannert, 1999; Gupta *et al*., 2004).

(v) **Knowledge**: their structure and function is well known, and they can be modified to adapt them to novel uses (Schmidt-Dannert, 1999; Jaeger and Eggert, 2002).

(vi) Availability: lipases, mainly those which are secreted, are available in large amounts by fermentation processes of natural or recombinant strains (Schmidt-Dannert, 1999; Gupta *et al*., 2004).

(vii) **No cofactors**: most lipases do not require cofactors (Gupta *et al*., 2004).

(viii) **Low-cost and green:** processes involving the use of lipases have a lower cost and are less polluting, because these enzymes act under mild conditions and with low energy and equipment requirements (Gunstone, 1999; Pandey *et al*., 1999a; Jaeger and Eggert, 2002).

The most common industrial applications of lipases are summarized below:

- **Food industry:** Lipases are used *in situ*, and sometimes together with other enzymes, during the elaboration of bread, cheese, and other foods to improve their shelflife and their rheological properties, or to produce aromas or emulgents. Moreover, they are used *ex situ* to produce flavours, and to modify the structure or composition of acyl glycerols (AGs) by inter- or transesterification (Gunstone, 1999 and Reetz, 2002).
- **Organic chemistry:** The industrial applications of lipases in organic chemistry include production of specific products whose elaboration by classical chemical means is difficult or expensive. For example, they are used in pharmaceutical and agrochemical industries to the modification or synthesis of antibiotics, anti-inflammatory compounds, pesticides, and to the production of enantiopure compounds or the resolution of racemic mixtures (Gunstone, 1999; Pandey *et al*., 1999b; Reetz, 2002).
- **Detergency and cleaning:** Lipases resistant to high temperature, proteolysis, and denaturation by surfactants find applications in the composition of laundry detergents (together with proteases) to improve the removal of lipid stains. They are also used in the synthesis of surfactants for soaps, shampoos and dairy products (Schmidt and Verger, 1998; Pandey *et al*., 1999a).

- **Paper industry:** Lipolytic enzymes are used to remove the "pitch", the lipid fraction of wood that interferes during the elaboration of paper pulp. They also help in the removal of lipid stains during paper recycling and to avoid the formation of sticky materials (Pandey *et al*., 1999a and Gutiérrez *et al*., 2001).
- **Management of waste and toxic compounds:** Lipases can be used in the management of waste produced during fat or food processing, in the treatment of lipid–contaminated waters and sludge. Moreover, they are useful in the treatment of biofilm deposits, oil-contaminated soils, and poisonous gases (Pandey *et al*., 1999a).
- **Other applications:** lipases can synergistically collaborate with cellulases, pectinases, and proteases in the elaboration and bleaching of cotton fibres (Li and Hardin, 1997). Lipases are also used as components of biosensors, in biodiesel production, in leather processing, in hard-surface cleaning, in single-cell protein production, and in the synthesis of polymers, biodegradable plastics, lubricants or cosmetics (Schmidt and Verger, 1998; Schmidt-Dannert, 1999; Pandey *et al*., 1999a; Reetz, 2002).

Lipase mediated transesterication reactions offer numerous possibilities for the biotechnological production of useful commodities such as fatty acid methyl esters and food flavour esters (Hasan *et al*., 2006; Jaeger and Eggert, 2002)

1.11.1 Lipase mediated synthesis of fatty acid methyl esters (FAME's)

The indiscriminate exploration and consumption of fossil fuels has led to a reduction in petroleum reserves. From the point of view of protecting the global environment and the concern for long-term supplies of conventional diesel fuels, it becomes necessary to develop alternative fuels comparable with conventional fuels. Alternative fuels should be, not only sustainable but also eco-friendly.

Recently, the methyl esters from renewable resources such as edible/non-edible/ waste fried oils have become more attractive because of their environmental benefits. Vegetable oils have the potential to be substituted for a fraction of the petroleum distillates and

petroleum based petrochemicals in the near future. Vegetable oil fuels are not competitive fuels because they are more expensive than petroleum fuels. With the recent increase in petroleum prices and the uncertainties concerning petroleum availability, there is a renewed interest in using vegetable oils in diesel engines. There are more than 350 oil bearing crops identified, among which only sunflower, safflower, soybean, cottonseed, rapeseed and peanut oils are considered as potential alternative fuels for diesel engines (Asakuma *et al*., 2009 and Fukuda *et al*., 2001). A number of studies have shown that triglycerides hold promise as alternative diesel engine fuels ((Lv *et al.,* 2010; Singh and Singh, 2010; Barnal and Sharma, 2005; Iso *et al.,* 2001). The advantages and disadvantages of vegetable oils are summarized below (Murugesan *et al.,* 2009):

The advantages of vegetable oils as diesel fuel include

- Portability
- Heat content equivalent to 80% of diesel fuel
- Ready availability and
- Renewability.

The disadvantages of vegetable oils as diesel fuels are

- Higher viscosity
- Lower volatility and
- The reactivity of unsaturated hydrocarbon chains.

Problems appear only after the engine has been operating on vegetable oils for long periods, especially with direct-injection engines. The direct use of vegetable oils and/or oil blends is generally considered to be unsatisfactory and impractical for both direct-injection and indirect type diesel engines. The problems include (Ma and Hanna, 1999; Srivastava and Prasad, 2000):

- Injector coking and trumpet formation on the injectors to such an extent that fuel atomization does not occur properly or is even prevented as a result of plugged orifices
- Carbon deposits
- Oil ring sticking
- Thickening and gelling of the lubricating oil as a result of contamination by the vegetable oils.

Different ways have been considered to reduce the high viscosity of vegetable oils i.e., dilution, micro emulsions, pyrolysis, catalytic cracking and transesterification. Among all these alternatives, transesterification seems to be the best choice, as the physical characteristics of fatty acid esters (biodiesel) are very close to those of diesel fuel and the process is relatively simple. Transesterification is the process of reacting triglycerides such as one of the vegetable oils with alcohol in presence of a catalyst such as enzyme, strong acid or base producing a mixture of fatty acids alkyl esters and glycerol (Lam *et al*., 2010 and Leung *et al.,* 2010). In this process, there is the displacement of alcohol by a monohydric alcohol that yields three alkyl esters from one triglyceride molecule. Fukuda *et al.,* (2001) reported that the molecular weight of typical esters molecules is roughly one third that of straight vegetable oil molecules and the viscosity is nearly twice to that of diesel fuel instead of 10-20 times in case of neat vegetable oil. This process has been widely used to reduce the high viscosity of triglycerides.

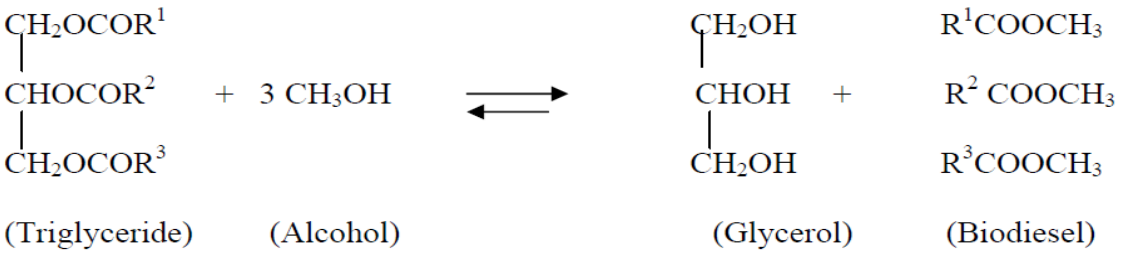

Figure 1.8: Transesterification reaction

The overall process is a sequence of three consecutive and reversible reactions, in which diglycerides and monoglycerides are formed as intermediates (Lam *et al*., 2010). The stoichiometric reaction requires 1 mole of a triglyceride and 3 moles of the alcohol (Fig. 1.8). However, an excess of the alcohol is used to increase the yields of the alkyl esters and to allow its phase separation from the glycerol formed.

Transesterified vegetable oils are known as biodiesel, which are generally using as alternative fuel resources (Ma and Hanna, 1999). It is biodegradable and nontoxic, has low emission profiles and so is environmentally beneficial (Meher *et al*., 2006). Alcoholysis of vegetable oils is an important reaction that produces fatty acids alkyl esters like methyl and ethyl esters, which are excellent substitutes for conventional fossil diesel fuels (Murugesan *et al.,* 2009; Leung *et al.,* 2010). The use of biodiesel results in

substantial reduction of unburnt hydrocarbons, carbon monoxide and particulate matters. It has almost no sulphur, no aromatics and has about 10 % built in oxygen, which helps it to burn fully. Its higher cetane number improves the combustion (Knothe *et al*., 2003). Several methods of transesterification using alkali and acids as catalysts (Marchetti *et al*., 2007) and enzymatic transesterification using lipase in presence and absence of solvents have been reported (Chen *et al*., 2009; Modi *et al.,* 2007; Shah and Gupta, 2007).

Several researchers utilized immobilized lipases as the biocatalysts for the synthesis of fatty acid methyl esters, flavor esters and optically active pharmaceutical ingredients due to their technical (enhanced catalytic properties and easy separation) and economical (reusability) advantages. The immobilized pig pancreatic lipase showed a remarkable stability as well as a great reusability (more than 11 successive reuses) in alcoholysis of sunflower oil (Caballero *et al*., 2009). Dizge *et al*., (2009) utilized covalently immobilized *Thermomyces lanuginosus* lipase on microporous polymeric matrix in the synthesis of fatty acid methyl esters (biodiesel) from various vegetable oils. The immobilized enzyme retains its activity up to 10 repeated batch reactions, each lasting 24 h. The optimization studies of aromatic ester synthesis using *Candida rugosa* lipase (CRL) and porcine pancreatic lipase (PPL) entrapped in calcium alginates beads has been reported by Ozyilmaz and Gezer (2010). The *Candida rugosa* lipase immobilised onto layered double hydroxides (LDHs) has been utilized for the synthesis of methyl adipate via green esterification of adipic acid and methanol in hexane (Rahman *et al*., 2008).

The different possible methods for biodiesel production were explained by Marchetti *et al*. (2007), by giving advantages and disadvantages of different technolgies and kinetic models of those technologies. The kinetic mechanism for the production of biodiesel from palm oil as substrate using *Mucor miehei* lipase was (Al-Zuhair *et al*., 2007) was explained by a Ping Pong mechanism. The whole-cell catalyzed methanolysis of soyabean oil for biodiesel production was optimized by Li *et al*., (2007) using response surface methodology and obtained a maximum of 72 % biodiesel yield under the optimal conditions**.** The lipase-catalyzed biodiesel production from soybean oil in ionic liquids using 23 ionic liquids was reported by Ha *et al*., (2007). Modi *et al.* (2007) studied the

lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetae as acyl receptor. The maximum yield of ethyl esters was obtained was 92.7 % with crude sunflower oils among different vegetable oils. Shah and Gupta (2007) studied the lipase-catalyzed preparation of biodiesel from Jatropha oil in a solvent free system. They obtained higher yield 98 % (w/w) by using *Pseudomonas cepacia* lipase immobilized on celite at 50 °C in the presence of 4–5 % (w/w) water in 8 h. Royon *et al*., (2007) reported the enzymatic production of biodiesel from cottonseed oil with t-butanol as a solvent using immobilized *Candida antarcitica lipase* as a biocatalyst. They obtained methanolysis yield of 97 % after 24 h at 50 °C with a reaction mixture containing 32.5 % t-butanol, 13.5 % methanol, 54 % oil and 0.017 g enzyme. The lipase-catalyzed transesterification of rapeseed oils for biodiesel production was studied by Li *et al*., (2006) using a novel organic solvent as the reaction medium. The highest biodiesel yield of 95% was achieved under the optimum conditions of tert-butanol/oil volume ratio 1:1; methanol / oil molar ratio 4:1; 3 % Lipozyme TL-IM and 1 % Novozym 435 based on the oil weight; temperature 35 °C; 130 rpm, 12 h. Nie *et al.,* (2006) optimized the biodiesel production from plant oil and waste oil using immobilized *Candida antarcitica* lipase as biocatalyst. The transesterification of sunflower seed oil to biodiesel catalyzed by silica aerogel encapsulated lipases using methyl acetate as solvent was reported by Orcaire *et al*., (2006). The biodiesel production from triolein and short chain alcohols using immobilized *Pseudomonas cepacia* lipase was investigated by Salis *et al*., (2005). Xu *et al*., (2005) proposed a simplified model based on Ping Pong mechanism to understand the kinetics of enzymatic interesterification of triglycerides for biodiesel production. A comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors was presented by Du *et al*., (2004) and obtained a 92 % methyl ester yield using methyl acetate as acyl acceptor, with a molar ratio of methyl acetate to oil of 12:1. Using three-step methanolysis Watanabe *et al.,* (2002) obtained a 93.8 % molar conversion of methyl esters from degummed soyabean oil using immobilized *Candida antarctica* lipase as a biocatalyst. Kose *et al.,* (2002) used immobilized *Candida antarctica* lipase in catalyzing alcoholysis of cottonseed oil in a solvent-free medium and obtained a methyl esters (ME) yield of 91.5 % at the optimum conditions of 30 % enzyme based on oil weight; oil/alcohol molar ratio 1:4; temperature:

50 ^{o}C and reaction time: 7 h. Enzymatic biodiesel production was studied using lipases immobilized onto different supports such as hydrophobic microporous styrene–divinylbenzene copolymer (Dizge *et al.,* 2009), hydrotalcite and zeolites (Yagiz *et al.,* 2007), and silica aerogel by encapsulation (Orçaire *et al.,* 2006).

1.11.2 Lipase mediated solvent-free synthesis of flavour esters

Now a days, natural flavours and fragrances play an important role in the quality of food and beverages. Due to food-processing operations such as premature harvesting, extended storage and physical treatment, aromas may be lost and the addition of flavour supplements to foodstuff is often required (King and Berger, 1998). Additionally, consumers are more concerned about food quality and prefer natural food additives to chemically synthesized compounds. As a result, the "natural" label allocated by the European and US Food legislation represents a strong marketing advantage. In USA, a distinction between natural and artificial flavour compounds is made and according to the "Code of Federal Regulations" a natural flavour is "the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate of any product of roasting, heating or enzymolysis, which contains the flavouring of constituents derived from a spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products or fermented products thereof, whose significant function in food is rather flavouring than nutrition". With respect to the bioproduction of flavour compounds, it can be concluded from this definition that products obtained by reactions of enzymes or microorganisms can be considered natural as long as natural raw materials are used and the resulting molecule itself occurs in nature. Products that occur in nature but that are produced in a non-natural process are called "nature-identical" (Cheetham, 1997). Because the isolation of natural flavours from plants is limited and the flavour market is increasing rapidly, alternative sources for natural flavours are required. Thus, the biotechnological generation of aroma compounds, by means of microorganisms, isolated enzymes or plant cell cultures, receives much attention (Häusler and Münich, 1997**)**.

In recent times, biotechnologically synthesized flavour compounds are occupying a promising role in the food industry. This is the result, among other things, of scientific advances in biological processes, making use of microorganisms or enzymes as an alternative to chemical synthesis, combined with recent developments in analytical techniques such as GC-MS, LC-MS, IR or mass spectrometry (Christen and López-Munguía, 1994). Operation under mild conditions to protect unstable products, i.e. 'green' processes, and functionalization of even complex precursor molecules in a highly specific way (chemo-, regio-, stereo-) are two important factors allowing biocatalysis to outplay chemical approaches in certain cases (Schrader *et al.,* 2004)

Pires-Cabra *et al.,* (2007) optimized the ethyl butyrate production by using RSM catalyzed by immobilized *Candia rugosa* lipases. Ester production increased with increasing a_w of the biocatalysts. Higher conversions (>95 %) were observed for low initial butyric acid concentration values. In other instance, ethyl butyrate production was improved using non-ionic surfactant-coated lipase immobilized on silica (Thakar and Madamwar, 2005). A low amount of surfactant proved to be most effective. A maximum conversion yield of 60 % of butyl acetate was obtained in a solvent-free system was obtained under the conditions of 500 IU immobilized lipase, 45 % of initially added water, acetic acid/butanol molar ratio: 1:1; and an incubation temperature of 37 °C using novel strain of *Rhizopus oryzae* (Salah *et al.,* 2007). Compared with a solvent-free system, the synthesis activity was improved in the presence of heptane and hexane with conversion yields of 80 % and 76 %, respectively. Optimized synthesis of citronellyl flavour esters using free and immobilized lipase from *Rhizopus sp*. was performed by Melo *et al.,* (2005) using RSM based on a five-level, two variable central composite rotatable design (CCRD). The optimum conditions were 2.41:1 molar ratio of alcohol: acid and 6.12 % (w/w) lipase amount and achieved 100% (molar conversion) yield in 48 h. Rodriguez-Nogales *et al.,* (2005) utilized statistical approach for optimization of immobilized *Candida antarcitica* lipase catalyzed synthesis of ethyl butyrate. The optimal conditions for the enzymatic reaction were obtained at 0.04 M substrate concentration using 7 % enzyme concentration at 34 °C for 96 h. The lipase entrapped in sol-gel polymer showed enhanced reusability and operational stability than the free

enzyme in the flavour ester synthesis (Chen and Hwang, 2003). Yadav and Trivedi (2003) developed a kinetic model for transesterification of n-octanol with vinyl acetate catalyzed by immobilized-lipase in non-aqueous media.

The maximal molar conversion of 80 % was reported by Guvenc *et al.* (2002) in case of immobilized lipase catalyzed solvent free synthesis of isoamyl acetate, with an acid–alcohol molar ratio of 1:2, 5 % Novozym 435 (g/g substrates), 30 °C and 150 rpm. Krishna *et al.,* (2001) and Chowdary *et al.,* (2000) utilized immobilized *Rhizomucor miehei* for the synthesis of isoamyl acetate and isoamyl valerate respectively. By using a new esterase from *Bacillus licheniformis,* Alvarez-Macarie and Baratti (2000) obtained *higher* yields of short chain flavour esters. The immobilized lipase mediated solvent-free ester synthesis is more frequent during last decade. Yadav and Dhoot, (2009) obtained high yields of cinnamyl laurate by using immobilized enzyme in non-aqueous media. Dandavate and Madamwar (2007) reported the synthesis of ethyl isovalerate by surfactant coated *Candida rugosa* lipase immobilized in microemulsion based organogels.

Fungal lipases with high stability and versatility find a better diversified industrial application in comparison to their counterparts. Also the applicability of lipases to combat the global energy crisis and environmental issues make them an enticing area to be delved into. Advanced research efforts to minimize the high cost of the lipase and enhancement of its utility are the existing areas of interests. Hence, the present research work was an attempt to address the issue.

Objectives

- Modeling and optimization of lipase production by *R. oryzae* NRRL 3562 under SSF
- Modeling of lipase extraction from fermented biomass
- Immobilization of *R. oryzae* NRRL 3562 lipase
- Utilization of *R. oryzae* NRRL 3562 lipase for synthesis of fatty acid methyl esters (FAME's) (Bio-diesel) and flavour esters.