1.1 Enzymes and their properties

Enzymes are biological macromolecules produced by living cells, which acts as catalyst to bring about specific biochemical reactions. Enzymes are generally known as “Biocatalyst”. They accelerate the rate of a reaction by lowering its activation energy. All metabolic processes in the living systems need enzymes to sustain life. Most of the known enzymes are proteins, few are catalytic RNA molecules. Enzymes are highly specific in their action on substrates and reactions they catalyze. During participation in a reaction enzymes are neither consumed nor permanently altered. Specificity of enzymes comes from their unique three-dimensional structures. Enzymes have specific sites called active sites that contain catalytical residues where the substrates bind. The catalytic activity of many enzymes depends on the cofactors. Cofactors can be metals or small organic molecules. Cofactors are also called coenzymes.

The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes. Every enzyme is designated with a EC number based on the chemical reactions they catalyze.

According to the enzyme commission the enzymes are divided into 6 Classes (Webb, 1992):-

1) Oxidoreductase (EC1): Catalyze oxidation and reduction reactions which involve the transfer of electrons from one molecule to another eg:- catalases, glucose oxidases and laccases

2) Transferase (EC2): Catalyzes the transfer of groups of atoms from one molecule to another. eg:- fructosyltransferases and glucosyltransferases

3) Hydrolase (EC3): Catalyze hydrolysis of larger molecules into smaller fragments. The reactions include the cleavage of glycosidic bonds in carbohydrates, peptide bonds in proteins and ester bonds in lipids. e.g. amylases, cellulases, lipases, mannanases, pectinases, phytases, proteases, pullulanases and xylanases

4) Lyase (EC4): Catalyze the formation of double bonds by the removal of groups and the addition of groups to double bonds. e.g. pectate lyases, alpha-acetolactate and decarboxylases

5) Isomerase (EC5): Catalyze the transfer of groups in the same molecule from one position to another. Thus change the structure of a molecule by rearranging its atoms. e.g. glucose isomerases, epimerases, mutases, lyases and topoisomerases.

6) Ligase (EC6): Catalyze joining of molecules together with covalent bonds e.g. argininosuccinate and glutathione synthase
1.2 Biological significance of enzymes

Enzymes are involved in many biosynthetic pathways. Fructose-2,6-bisphosphatase, catalyzes the hydrolytic release of the phosphate from fructose 2,6-bisphosphate in gluconeogenesis. The synthesis of malonyl-CoA first reaction of fatty acid biosynthesis is catalysed by acetyl-CoA carboxylase. In glycolysis, hexokinase catalyses interconversion of glucose and ATP with glucose-6 phosphate and ADP. The pyruvate dehydrogenase complex oxidizes pyruvate to acetyl-CoA and CO$_2$ in Krebs cycle. Protein kinases phosphorylate proteins by catalyzing transfer of the terminal phosphoryl group of ATP to the hydroxyl groups of seryl, threonyl, or tyrosyl residues. In oxidative phosphorylation ATP synthase catalyzes the formation of ATP from ADP and Pi.

Some of the principal enzymes used in clinical diagnosis (Harper et al., 2003):

1) Myocardial infarction: Aspartate aminotransferase, lactate dehydrogenase and creatine kinase
2) Viral hepatitis: Alanine aminotransferase
3) Acute pancreatitis: Amylase, lipase
4) Hepatolenticular degeneration (Wilson’s disease): Ceruloplasmin
5) Varous liver diseases: γ-Glutamyl transpeptidase
6) Metastatic carcinoma of the prostate: Acid phosphatase
7) Various bone disorders and obstructive liver diseases: Alkaline phosphatase

Thousands of enzymes are present in the body that carry functions necessary to cell vitality and to sustain life. Common enzymes that carry different metabolic process in the body are amylases, proteases and lipases.

Amylases are glycoside hydrolases that act on α-1,4-glycosidic bonds. Amylase catalyses the breakdown of starch into sugars. Amylase is divided into three sub classes α, β, -amyrase. α-amyrase is major form of amylase found in humans and other mammals. β-amyrase is synthesized by bacteria, fungi, and plants. Salivary glands release saliva containing salivary amylase, which begins the digestive process by breaking down starch present in food into maltose, maltotriose and limit dextrins. Pancreas release pancreatic amylase which hydrolyse starch into disaccharides and trisaccharides. These disaccharides and trisaccharides are converted by other enzymes to glucose to provide energy to living systems (Gurung et al., 2013).
Protease breaks down protein into amino acids. Human digestive tract produces three main proteases such as pepsin, trypsin and chymotrypsin. An inactive enzyme, pepsinogen, is produced by the cells of stomach which changes into pepsin when it contacts the acid environment in the stomach. Pepsin breaks chemical bonds present in proteins, producing smaller molecules called peptides. Pancrea release trypsin and chymotrypsin, into small intestine through the pancreatic duct. When partially digested food moves from stomach into intestine, trypsin and chymotrypsin breaks protein into simple amino acids that are absorbed in the body. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen in virtually all living organisms which are exposed to oxygen. Cellulose is abundant and found in all plants.

Cellulase breaks down cellulose into monosaccharides such as β-glucose, cellobiose and cellooligosaccharides by hydrolysis of the 1,4-β-D-glycosidic (Sukumaran et al., 2005). Thus cellulases are very important for plants. Lipase is an enzyme that breaks down dietary fats into fatty acids and glycerol. Hydrolysis of food starts in stomach, where gastric lipase, cleaves 15–20% of the fatty acids. Partially hydrolysed food passes to small intestines, where the emulsion mixes with pancreatic juice containing lipases secreted by pancreas which hydrolyse triglycerides into fatty acids and glycerol (Lowe, 2002). Several other types of lipases, such as phospholipases and sphingomyelinas es exist in nature however, these are usually treated separately from conventional lipases (Gurung et al., 2013).

1.3 Industrial properties and application of enzymes

Enzymes offer many advantages in industrial processes such as:-
1) They are of natural origin and are nontoxic.
2) They have high specificity of action, hence can bring about reactions that cannot be easily carried out.
3) They work best under mild conditions of moderate temperature and mostly near neutral pH, thus do not require expensive equipment.
4) They act rapidly at relatively low concentrations, and the rate of reaction can be readily controlled by adjusting temperature, pH, and amount of enzyme and substrates.
5) They are easily inactivated when reaction has to be stopped.

Enzymes show wide range of applications in different industries such as food, detergent, medicine, cosmetics and paper etc. Bacteria produce different classes of enzymes.
Hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases occupy the major part of the industrial enzyme market (Gupta et al., 2004). Amylases have a wide range of application in various industries in food, paper industries, pharmaceutical, drug delivery, detergents, chemical industries, as well as agriculture and environmental engineering. In clinical, medicinal, and analytical chemistry bacterial α-amylases are being used (Pandey et al., 2000). Most common and important application of amylases is in the starch industry. During starch hydrolysis in the starch liquefaction process amylases convert starch into fructose and glucose syrups. Amylases are extensively used in processed food industry for baking, brewing, preparation of digestive aids and fruit juices and production of cakes (Van Der Maarel et al., 2002).

Amylases constitute 90% of all liquid detergents and are the second type of enzymes used in the detergent formulation. They are used in laundry and automatic dishwashing for cleaning residues of starchy foods and smaller oligosaccharides (Mitidieri et al., 2006). In textile industry amylases are utilized for desizing process. Desizing is the process in which removal of starch from the fabric takes place, it prevents breaking of the warp thread during the weaving process (Ahlawat et al., 2009). In the pulp and paper industry, α-amylases are mainly used for modification of starch of coated paper, to produce low-viscosity, high-molecular weight starch (Mitidieri et al., 2006). One of several medical conditions, including perforated peptic ulcer, acute inflammation of the pancreas, macroamylasemia, torsion of an ovarian cyst, macroamylasemia, and mumps may be predicted by a higher than normal concentration of amylases. The level of α-amylase activity is of clinical importance in human body fluids e.g. in pancreatitis, diabetes and cancer research (Singh et al., 2011).

Proteases have important biotechnological applications in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Proteases are widely used in laundry detergents for removing protein based stains from clothing (Banerjee et al., 1999). In biopharmaceutical products such as enzyme cleaners, enzymic debriders and contact-lens, proteases are important constituents (Anwar and Saleemuddin, 2000). In textile industry, the stiff and dull gum layer of sericine can be removed from the raw silk fibre by using proteases to achieve improved luster and softness. Protease treatments can provide new and unique finishes to wool and silk fibres by modifying the surface of wool and silk fibres. Proteases have been used in the hide dehairing process.
Cellulases find applications in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textile, and laundry. In agriculture cellulases are used for disease control, enhanced seed germination and improved root system, enhanced plant growth and flowering. It also helps in improving soil quality and reducing dependence on mineral fertilizers. Cellulases help in conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein and lipids. Cellulases help in the production of energy-rich animal feed, improved nutritional quality of animal feed. Alkaline cellulases have potential application as detergent additives. Cellulase preparations are capable of modifying cellulose fibrils thus improving color brightness and removing dirt from the cotton blend garments. In pulp and paper industry cellulases are used as coadditive in pulp bleaching, in biomechanical pulping and reduced chlorine requirement. Cellulases help in production of biodegradable cardboard, paper towels, and sanitary paper. In textile industry cellulase help in biostoning of jeans and biopolishing of textile fibers. In food industry cellulase helps in controlling bitterness of citrus fruits and in improving flavour and aroma of fruits and vegetables (Kuhad et al., 2011).

1.4 Introduction to lipases

Carboxylesterases (EC 3.1.1.1) are subclass of hydrolases and are the enzymes which hydrolyse small ester containing molecules (Arpigny and Jaeger, 1999). Lipases belong to subclass 1 of hydrolytic enzyme (class 3) and have been assigned sub-sub class 3.1.1 due to their specificity for carboxylic acid ester bonds (Patil et al., 2011). Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes having great physiological significance and industrial potential. They display maximal activity towards water-insoluble long-chain triglycerides. In the turnover of water-insoluble compounds lipases play an important role (Hasan et al., 2006). At an oil-water interface, lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids and also catalyze esterification and transesterification reactions in non aqueous media (Reis et al., 2009; Kharrat et al., 2011; You et al., 2013)(Fig. 1.1 A, B). Lipases can exhibit regiospecificity, specificity in terms of fatty acids, nature of the alcohol, stereospecificity (distinction between sn-1 and sn-3 position on the triglyceride), chemo and enantioselectivity.
Figure 1.1: Hydrolysis, esterification and transesterification reaction of triglyceride by lipase. A triglyceride is hydrolysed into free fatty acids and glycerol by lipase in the presence of water which is shown by forward arrow. Lipases also catalyse esterification of free fatty acids and glycerol into triglyceride with liberation of water shown by backward arrow (A). Transesterification of triglyceride by lipase in the presence of methanol produces fatty acid methyl esters and glycerol (B). $R_1$, $R_2$, $R_3$ are different alkyl groups.

Lipases are produced by various plants, animals and microorganisms and are omnipresent (Gupta et al., 2004). Plant sources include oil seed lipase eg. beans (*Phaseolus vulgaris*), sunflower (*Helianthus annuus* L.), canola (*Brassica napus* L.), barbados nut (*Jatropha curcas* L.) etc. and cereal seed lipase: rice (*Oryza sativa*), wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), barley (*Hordeum vulgare*) (Barros et al., 2010) etc. Animal sources include edible fore stomach of calves (cow), kids (goat) and lambs (sheep) and animal pancreatic tissue. Fungal sources include organisms such as *Aspergillus, Rhizopus, Candida, Mucor, Humicola, Geotrichum, Pencillium* species (Gosh et al., 1996). A new strain of *Penicillium Cyclopium* was isolated from mouldy Grenoble walnuts. It produced two extracellular lipases and one cell-bound themostable lipase linked to mycelium (Druet et al., 1992). Bacterial lipase includes organisms such as *Arthrobacter, Alcaligenes, Achromobacter, Burkholderia, Chromobacterium, Bacillus, and Pseudomonas* sp. (Gupta et al., 2004). Lipase producing *Staphylococcus chromogenes* O1A was isolated from oil spilled soil (Golani et al., 2016).
Bacillus sp. MPTK 912 producing lipase was isolated from oil mill effluent (Kaleeswari oil refineries, Chennai) (Kumar et al., 2012a).

Lipases of microbial origin have great industrial application due to their widely diversified enzymatic properties and substrate specificity. Bacterial lipases have received more attention, since they have great variety of catalytic activity, easy to manipulate genetically, capable of rapid growth on inexpensive media and are not affected by seasonal fluctuations (Hasan et al., 2006). Lipases can be extracellular or intracellular, but bacterial lipases are mostly extracellular. Extracellular lipases have advantages over intracellular lipases, that they can be easily separated from product, have low production cost and high catalytical activity.

1.5 Molecular mechanism of lipases

Microbial lipases have a molecular weight range of 19 kDa (Kawasaki et al., 2002) to 300 kDa. Oligomeric forms have molecular weight above 300 kDa with subunits of around 50 kDa (Salameh and Wiegel, 2007). Lipases belong to serine hydrolases, therefore they do not require any cofactor (Gosh et al., 1996). All exhibit a characteristic folding pattern known as the α/β hydrolyase fold (Ollis et al., 1992). The core of the lipase is composed of a central β sheet consisting of eight different β strands (β1–β8) which are connected by six α helices.

Lipases contain the consensus sequence G–X1–S–X2–G. The active site of the α/β hydrolyase fold enzymes contains three catalytic residues which are nucleophilic residues: serine, cysteine or aspartate, catalytic acid residue X2 = glutamic or aspartic acid and histidine residue -X1 (Svendsen, 1994). The nucleophilic residues has been determined to be a serine residue in lipases. At the C-terminal end of β5 strand the nucleophilic Ser residue is present in a highly conserved pentapeptide GXSXG, which forms a characteristic β-turn–α motif named the ‘nucleophilic elbow’. A nucleophilic attack by the oxygen of catalytic serine on the carbonyl carbon atom of the ester bond leads to substrate hydrolysis (Jaeger and Reetz, 1998).

Molecular modelling is a tool to visualize three dimensional structure of molecules and to stimulate, predict and analyse the properties and behaviour of the molecules. In pharmaceutical research and development, understanding the principles by ligands recognize and interact with macromolecules is of great importance (Blaney, 2012). In structural molecular biology and computer assisted drug designing, molecular docking is a
key tool. The structure of the intermolecular complex formed between ligands and protein is predicted by molecular docking. The Docking explores possible orientations of a molecule within a macromolecular active site by superimposing atoms onto precomputed site points. Molecular docking has wide range of applications in drug discovery, lead optimization, finding potential leads by virtual screening, in mutagenesis studies, chemical mechanism studies and combinatorial library design (Morris and Lim-Wilby, 2008). The docking process can be divided into two parts: a search algorithm and a scoring algorithm. The search algorithm samples the degrees of freedom of the ligand in the active site of the protein to include the true binding modes. The scoring algorithm represents the thermodynamics of interaction sufficient to distinguish the true binding modes from all others explored (Ewing and Kuntz, 1997).

1.6 Purification and application of lipases

Purification of enzymes is required to obtain pure biological catalyst, for determination of their primary amino acid sequence, understanding three-dimensional structure and purified lipase preparations are needed in industries such as pharmaceutical and cosmetics (Saxena et al., 2003). Lipase has been purified by different chromatographic methods such as ion exchange, gel filtration and affinity chromatography. Among which affinity chromatography especially hydrophobic interaction chromatography is used in most of cases. Lipases constitute the most important group of enzymes for biotechnological applications and find applications in the detergent, food, flavour industry, resolution of pharmaceuticals, esters and amino acid derivatives, production of fine chemicals, agrochemicals, biosensor, bioremediation, cosmetics and perfumery (Hasan et al., 2006).

Lipase catalyzed hydrolysis, esterification and transesterification are the important group of reactions for food technology applications in fats and oil industry, dairy industry and bakery industry. Lipases are extensively used in the dairy industry for the hydrolysis of milk fat to modify the fatty acid chain lengths and to enhance the flavours of various cheeses (Aravindan et al., 2007; Ray, 2012). In the food industry, lipases have been used for synthesis of esters of short chain fatty acids and alcohol moieties, which are used as flavour and aroma constituents. During manufacturing of sausage, lipases play an important role in the fermentative steps (Gurung et al., 2013). In the food and pharmaceutical industry, lipases play a major role in the processing of γ-linolenic acid, a polyunsaturated fatty acid (PUFA) and in the production of
monoglycerides for use as emulsifiers (Sharma et al., 2001). Lipases from different microbial sources were used previously for improving rice flavour, modifying soybean milk, and for enhancing the aroma and speeding the fermentation of apple wine (Seitz, 1990). Lipases are a particular useful category of enzymes in organic synthesis, as they accept broad spectrum of substrates and are relatively stable in aqueous media where lipolytic substances can be readily processed. Lipase has been widely applied for the kinetic resolution of racemic acid, alcohol and esters by hydrolysis, esterification, or transesterification reaction in aqueous or organic medium (Tsai and Wei, 1994). In the medical field lipases are considered as important drug targets or marker enzymes. The high levels of lipases can indicate certain infection or disease and can be used as a diagnostic tool. They are used in determining serum triglycerides by the liberation of glycerol which is detected by enzyme-linked colorimetric reactions. Level of lipases in blood can be used for determining acute pancreatitis and pancreatic injury (Lott and Lu, 1991). Lipases can be used as digestive aids. Lipases are activators of tumor necrosis factor thus can be used in the treatment of malignant tumors. Candida rugosa lipase is used for synthesizing lovastatin, a drug that lowers serum cholesterol level. Lipase from Serratia marcescens is used for the synthesis of 3-phenylglycidic acid ester. The asymmetric hydrolysis product of 3-phenylglycidic acid ester, is a key intermediate in the synthesis of diltiazem hydrochloride and is widely used as coronary vasodilator (Matsumae et al., 1993).

The additions of hydrolytic lipases have been exploited in detergents. Enzymes reduce use of chemicals in detergents, mostly biodegradable, leaving no harmful residues and does not possess any kind of risk to aquatic life and reduce the environmental load of detergent products, as they save energy by enabling a lower wash temperature to be used (Gurung et al., 2013). Lipases find applications in the textile industries for removing the size lubricants, which increases fabrics absorbance ability for improved levelness in dyeing. Lipase is used to reduce the frequency of cracks and streaks in the denim abrasion systems (Raja et al., 2012). In pharmaceuticals and cosmetics such as skin care products, retinoids (vitamin A and derivatives) are commercially very important. For the preparation of water-soluble retinol derivatives, immobilized lipases are used. Lipases have been used as an ingredient of topical antiobese creams and in hair waving preparation (Smythe, 1951). A biosensor is an integrated electronic device employing biological elements like enzymes, antibodies receptor proteins, nucleic acids, cells or tissue as the analyte sensor. It couples a biological element to a transducer for detecting signal and displays it on a panel after processing and amplification (Arya et al., 2008). Lipases immobilized on pH/oxygen
electrodes along with glucose oxidase, can serve as lipid biosensors and used for
determination of triglycerides and blood cholesterol (Imamura et al., 1989).
In the recent past worldwide biodiesel, an environment friendly diesel fuel similar to petro-
diesel, has received considerable attention (Parawira, 2010). Transesterification of vegetable
oils is the most popular method of producing biodiesel. Transesterification is the
reaction of a fat or oil (triglyceride) with an alcohol to form fatty acid alkyl esters, methyl
and ethyl esters (which are excellent substitutes for biodiesel) and glycerol (Parawira,
2010). Recently enzymatic transesterification using lipase has attracted much attention for
biodiesel production (Devanesan et al., 2007). One of the non-edible feedstocks that has
received great attention as a source of renewable energy is Jatropha curcas (Makkar and
Becker, 2009; Devappa et al., 2010). In fuel industry, lipase is used for transesterification of
Jatropha oil, to produce biodiesel and also for biodegradation of phorbol esters present in
seed cake left after biodiesel extraction.
Lipases play key role in waste treatment. Lipases are highly specific for their substrates,
specificity allows lipases to remove target pollutants selectively, while utilizing any
required chemical reactants with very high stoichiometric efficiency. Lipases hydrolyze
triacylglycerides in effluents with high lipid content, reducing levels of suspended solids and
lipids. In the enzymological remediation of polluted soils, lipase producing microorganisms
play a key role (Nagar et al., 2013). Lipases are used for cleaning of wastes of factories and
industries, etc. Microbial lipase activity in soil can be an important indicator of diesel oil
biodegradation in freshly contaminated, unfertilized, and fertilized soils (Margesin et al.,
1999).
Industrialized world is facing the major problem of environmental pollution due to oil
effluents today. New technologies that focus on the detoxification and destruction of the
contaminants rather than the conventional approach of disposal have been developed to
remediate these effluents. Bioremediation, the use of microorganisms or microbial process to
detoxify and degrade the oil effluents is among the innovative technologies. Wastewaters
from dairies are rich source of biodegradable organic molecules and nutrients. They also
contain high levels of fats and proteins having low biodegradability coefficient. If waste water
is not treated, it will cause severe pollution of land and water with their high biochemical
oxygen demand (BOD) and chemical oxygen demand (COD). In countries where food habits
result in a large amount of residual fat and oil in wastewater, it has become very difficult to
fulfill the discard requirements. There are few studies which describe the degradation of fats
and oils by alkali/acid/enzymatic hydrolysis. The treatment of effluents from different origins is a new and promising area for lipases. The oily environment provides a good habitat for isolation of lipase producing microorganisms. Lipase producing bacteria have been isolated from different industrial wastes. There is no previous report of lipolytic bacteria isolated from paint industrial waste which mostly contains oil and grease-10%, phenolic compounds-1% and other traces of metals. These ingredients being the inducer and substrate for lipase/esterase enzymes. The most important stage in a biological process is optimization to improve and increase the efficiency of the process without increasing the cost. Bacterial lipases are mostly extracellular and are greatly influenced by physico-chemical factors, such as temperature, pH, nutritional factors such as nitrogen and carbon sources, inorganic salts, agitation, and dissolved oxygen concentration (Gupta et al., 2004). The lipase production requires the study of fermentation condition which relate carbon and nitrogen sources to temperature and pH conditions (Silva et al., 2005). It is essential to biochemically characterize and optimize enzyme before using it for hydrolysis, esterification or any other application as each application requires unique properties with respect to specificity, stability, temperature and pH-dependence. Hence the present study was undertaken to identify and characterize lipase producing bacteria from paint industrial waste. As well to study the application of these lipolytic bacteria in biowaste management and biotransformation.

1.7 Objectives of the study

Keeping in view the advantages and diverse applications of microbial lipases, the present study was proposed to accomplish the following objectives:

- Isolation, screening and characterization of lipase producing bacterial isolates from effluent waste of paint industry.
- Characterization and partial purification of lipase from bacterial isolates
- Application of lipase in industrial waste water treatment and biotransformation of *Jatropha curcas* ester.
- *In silico* studies and molecular evolution of bacterial lipases