This thesis has been divided into three independent chapters. Section 1 of each chapter is a mini-review on the topic of that chapter. Methodologies and experimental details are provided in section 2 of each chapter. A brief discussion on the results obtained and conclusions drawn is given below.

Chapter 1: Characterization of Thioesterase of *Alcaligenes faecalis*: The Physiological Role

Thioesterases are a group of enzymes that hydrolyze acyl-CoA esters of various chain lengths to the free fatty acids and CoASH. Although this enzyme activity has been shown to be widely distributed among organisms and cell types, the physiological function of these enzymes remains largely unknown. The only established function for acyl-CoA thioesterases is in termination of fatty acid synthesis in eukaryotes. In addition to these enzymes with established function, a number of thioesterase activities of less defined function have been characterized from mammalian cells and yeast. Among bacteria, two thioesterases, thioesterase I and II that cleave acyl-CoA molecules *in vitro* have been characterized from *E. coli*. Thioesterase I, encoded by *tesA* gene is a periplasmic protein of 20.5 kDa and has active site similar to serine proteases, consistent with its inhibition with diisopropylflurophosphate (DFP). Thioesterase II, encoded by *tesB* gene is a tetrameric protein having identical subunits of 32 kDa and is insensitive to inhibition with DFP. A histidine residue present at position 58 in thioesterase II has been implicated in the cleavage of the thioester bond. Two thioesterases having striking similarities in their physical properties to thioesterase I and II have also been reported from photosynthetic bacteria, *Rhodopseudomonas sphaeroids*.

The *in vivo* function of the bacterial thioesterases is unknown, and their presence in the organism is an enigma. Several null mutants of both *tesA and tesB* have been made in an attempt to find the role of these thioesterases by Cronan and coworkers. The deletion of *tesA*, *tesB* or *tesAB* gene has no effect on the growth or lipid composition in *E. coli*. However the possibility of another enzyme taking over the function of both enzymes or presence of a third thioesterase in *E. coli* has not been ruled out. The over expression of either the *tesA* or *tesB* to levels that greatly exceed the normal level also has no effect on growth of *E. coli*. Similar result is obtained

when several mammalian thioesterases are expressed in *E. coli*. These observations lead Cronan to conclude that the *E. coli* enzymes, which are assayed as thioesterases *in vitro* are not thioesterases *in vivo*. It has also been shown that *E. coli* thioesterases are unable to cleave acyl-ACPs *in vivo*. In addition to being localized in periplasm, *TesA* also has esterase and chymotrypsin like activity, which lead to the suggestion that it may be a scavenge protein *in vivo*, but nature of substrates could not be clearly identified as neither long chain acyl-CoAs, which are found only as metabolic intermediates nor glycerides, which form micelles at very low concentrations, are likely to be the substrates in this role.

A 22 kDa thioesterase has been isolated in our laboratory from *Alcaligenes faecalis*. Compared to *E. coli* and *Rhodopseudomonas sphaeroids*, each of which contains at least two thioesterases, we could isolate only one thioesterase activity from *A. faecalis*. The enzyme could be extracted from the cells with 1 M NaCl, indicating periplasmic localization of protein within the cells. This was confirmed by electron microscopy using immunogold labelling method. Polyclonal antibodies were raised using either the purified thioesterase (AbTE-N) or gel-pieces containing thioesterase (AbTE-D) excised after SDS-PAGE, as antigen. The specificity of the antibodies was assayed by Western blotting. The titer of antibodies was determined by ELISA and was found to be 20,00,000 and 1,80,000 for AbTE-N and AbTE-D, respectively. Ultra thin sections of *A. faecalis* embedded in LR White resin were incubated with AbTE-N or AbTE-D followed by nanogold labeled secondary antibody. Pre-immune serum was used as control. Gold particles were found exclusively in the periplasmic space. The labelling was much more abundant with AbTE-N than AbTE-D. No labelling occurred with pre-immune serum.

Attempts were made to obtain N-terminal sequence of the thioesterase by Edman's method, but without any success. Thioesterase was resistant to proteolysis by trypsin, therefore, attempts to obtain internal sequences of the protein also met with failure. Attempts at preparation of thioesterase null mutants by N-methyl-N-nitroguanidine (NG) mutagenesis were unsuccessful. No clone could be obtained, which lacked thioesterase activity.

Having failed in attempts to obtain N-terminal sequence and in generating thioesterase null mutants, an indirect approach was attempted at cloning the gene of

A. faecalis. Subcellular localization of the thioesterase of *A. faecalis* and thioesterase I of *E. coli* is same. In addition, substrate specificities and physical properties of these thioesterases showed remarkable resemblance. Since the gene for thioesterase I of *E. coli* (*TesA*) has already been cloned and sequenced, PCR primers based on this sequence were used to amplify gene of *A. faecalis*. Although a fragment corresponding to 550 bp could be amplified, its sequence did not show significant similarity with any of the thioesterase sequence in the data base nor did it appear to be encoding for any protein. The approach turned out to be unsuccessful and was abandoned.

Thioesterase hydrolyzed saturated and unsaturated long-chain acyl-CoAs at physiological concentration with V_{max} 14-37 μmol/min/mg of protein and K_m 11-15 mM. Thioesterase failed to hydrolyze simple aliphatic oxoesters, which rules out glycerides as *in vivo* substrates for this enzyme. The enzyme was able to hydrolyze aromatic oxoesters, but by competition experiments, we were able to demonstrate that long-chain acyl-CoAs are better substrates than aryl esters.

Several evidence are available in the literature that suggests an ancillary role for these thioesterases. A peroxisomal thioesterase, PTE1 with high similarity with bacterial thioesterase *tesB* has been characterized from yeast. PTE1 also has high sequence similarity with a mammalian peroxisomal thioesterase. Deletion of PTE1 gene interferes with the ability of yeast to grow on fatty acids, which suggests an ancillary role for PTE1 in fatty acid utilization. Characterization of several peroxisome proliferators-induced long-chain acyl-CoA thioesterases from mammalian cells further supports the hypothesis that they play a role outside fatty acid synthesis in lipid metabolism. In line with this the ability of bacteria to directly utilize exogenously supplied fatty acids for phospholipids synthesis also suggest a specific function for acyl-CoA thioesterases in the lipid metabolism. In *E. coli,* it has been demonstrated that fatty acid transport is accompanied by an activation of fatty acid to its acyl-CoA form. Boyce *et al.*, therefore, suggested that if exogenous fatty acids are to enter into intracellular acyl-ACP pool, a conversion of acyl-CoA to acyl-ACP must occur and could result from the combined actions of acyl-CoA thioesterase and acyl-ACP synthetase. Whether or not such process occur in *E. coli* is unclear, since *in vitro* studies have demonstrated that this organism's *sn*-glycerol-3-phosphate

acyltransferase can independently utilize both acyl-CoA and acyl-ACP substrates. Such process, however, must occur in case of *Rhodopseudomonas sphaeroids* by a mechanism similar to one proposed because this organism's *sn*-glycerol-3-phosphate acyltransferase has obligate requirement for acyl-ACP substrates as acyl donors for *sn*-glycerol-3-phosphate acylation.

A membrane bound desaturase activity, which converts exogenously supplied palmitic acid to palmitoleic acid, has been reported in *Alcaligenes faecalis*. Activation of palmitic acid to palmitoyl-CoA has been found to be essential for this conversion. Since the concentration of unsaturated fatty acids is regulated in prokaryotes, a thioesterase capable of maintaining palmitic acid to palmitoyl-CoA ratio should, in principle, be able to regulate this conversion. A ^{1}H-NMR spectral method was developed to directly estimate palmitic acid to palmitoelic acid ratios. Partial inhibition of thioesterase with diethylpyrocarbonate resulted in 40% decrease in conversion of palmitic acid to palmitoelic acid. This result can be explained by assuming that the rate of desaturation is directly proportional to the concentration of palmitoyl-CoA, whose concentration in turn is expected to be inversely proportional to thioesterase activity. Thus decreased thioesterase activity should result in increased concentration of palimitoyl-CoA and therefore increase in the rate of reaction. This also implies that thioesterase controls the desaturase activity by regulating the concentration of palimitoyl-CoA.

To provide evidence in support of the proposed role desaturase reaction was studied using strains of *E. coli* which either lacked (HC69) or overproduced (HC63) thioesterase I. Strain HC69, which lacked thioesterase I showed increased rate of conversion as compared to the parent strain LE392. This is in consistent with the result obtained with *A. faecalis* when thioesterase was inhibited. The strain HC63 which overexpressed thioesterase I showed reduced rate of conversion. Thus, for the first time we have able to provide evidence that long-chain acyl-CoAs are the *in vivo* substrates for thioesterases.

CHAPTER 2: Characterization of Thioesterase of *Alcaligenes faecalis*. The Amyloid Formation.

A range of human degenerative conditions including Alzheimer's, poly-glutamine (poly-Q), Parkinson's and Huntington's diseases, prion, encephalopathies and cystic fibrosis and the gelsolin amyloid disease are associated with the deposition of proteinaceous aggregates known as amyloid fibrils in tissues. Understanding molecular basis of amyloid disease require (a) understanding the nature of the pathogenic species and (b) understanding mechanism by which normally soluble protein is transformed into fibrillar form. Designing experiments aimed at probing the nature of aggregate pathogenicity and identification of particular species is hampered by the fact that most *in vitro* aggregation processes leads to heterogeneous mixture of a variety of aggregated species. Finding the *in vitro* conditions that could lead to highly homogeneous population of various types of aggregates are not easy to achieve.

The purified thioesterase of *Alcaligenes faecalis,* previously isolated in our laboratory is a 22 kDa protein, which aggregates to provide a homogenous population of granulated structures, 20-25 nm in diameter. The granular aggregates have β-sheet structure as revealed by their CD spectra, similar to those found in amyloids. We have been able to find several solution conditions, such as pH 2.0, 75% ethanol, 30% trifluoroethanol and high salt concentration to transform this homogenous population of granular aggregates to homogenous population of amyloid fibrils. Fibrillar growth in 75% ethanol was found to be best and has been used routinely for growing thioesterase fibrils used in this study. Under these conditions fibers of ~ 4000 nm length and 17 nm diameter were formed.

Several assay methods were used to confirm the fibrils are indeed amyloids. Congo red dye on binding to these fibers exhibited (i) increase in absorbance, (ii) shift of absorbance maxima from λ_{493} to λ_{505} and (iii) differential spectra with maxima at λ_{535} nm, features characteristic of amyloid fibrils. In addition Congo red stained samples exhibited green birefringence, under cross polarized light. A 2-7 fold increase in fluorescence intensity at λ_{485} occurred in thioflavin binding assay, which further supported the presence of amyloid structures in the samples. The final evidence was provided by transmission electron microscopy.

The phenomenon of seeding is common to aggregation and gelation processes, as it is in crystallization. In the present study a considerable enhancement in the rate of fibrillization occurred, when freshly prepared solution of thioesterase was seeded with pre-formed fibril of the same protein.

While studying transmission electron micrographs obtained after different time periods of incubation of fibrillization solution, several observations were made, which compelled us to propose a mechanism for thioesterase fibril formation. In this case the growth of fibrils seems to have occurred by longitudinal fusion of granular particles. The topography of the fibrils showed a periodicity of 50 nm, which is consistent with the theory that fibrils are composed of a succession of composite units. The tendency of the fibrils to form parallel sheets is quite remarkable and was observed for fibrils grown in 75% ethanol or at pH 2.0.

Formation of amyloid fibrils from thioesterase of *A. faecalis* further support the hypothesis according to which the ability to form amyloid fibrils is a generic property of proteins, and most, if not all proteins have ability to form such structures.

Transmission electron micrographs of fibrils obtained in seeding experiments were quite revealing. The appended fibril, which appears to have grown on preformed fibril by addition of granular particles, had different dimensions than that of the fibril on which it has grown. After rearrangement, which in all probability would involve the formation of a new intermolecular hydrogen bond, these are likely to would lead to a regular fibers of uniform dimension.

We believe that this observation is significant in the context of understanding the exact nature of the species involved in the toxicity of the β-amyloid fibrils. The data presented here suggests that although fibril as a whole is quite inert as no lateral fusion could be seen, but the terminals of fibers appear to be quite reactive and are able to bring requisite conformational changes in the oligomeric units of protein to make them fusogenic. The effect appears to happen in a cascade and the added unit even before its own integration with growing fibril induces fusogenicity in next oligomeric unit. Thus, it would not be out of context to speculate that the toxicity of fibrils may be due to reactive terminal ends. Perhaps this would also explain the slow onset of these diseases, as critical mass of fibrous material would be required to cause toxicity as only a miniscule surface area of whole fiber is available for interaction

with cellular components. But readily concede that we do not have adequate experimental proof at this stage in support of this hypothesis.

CHAPTER 3: Characterization and Applications of a Dehydrogenase from *Penicillium funiculosum*

The pivotal role played by alcohols and amines in stereoselective organic synthesis has driven the search for practical methods for preparing them in optically pure form. Because both dehydrogenases and transaminases are capable of converting a prochiral carbonyl group to an optically pure alcohol or amine, respectively, they have become important additions to the repertoire of organic synthesis. Oxidoreductases are ubiquitous enzymes having wide occurrence in plants, animals and microorganisms. These enzymes can reduce carbonyl groups (ketones and aldehydes) and carbon-carbon double bonds. Depending on the substitution pattern of substrates, both of these reactions offer potential asymmetrization of prochiral substrates, leading to a chiral product. The use of these enzymes as catalysts for large-scale chiral products thus requires efficient procedures for *in situ* regeneration of cofactor for recycling and is the main challenge faced in industrial applications of oxidoreductases. The conversions with whole cells for this purpose is advantageous with respect to saving expensive isolation of the desired enzyme and cofactor recycling; but so far, the products obtained in the whole cell conversions with known microorganisms often lack enantiomeric excess, a pre-requisite for their use in asymmetric synthesis. The need for characterization of the new biocatalysts capable of reducing carbon-oxygen (carbonyls) and carbon-carbon double bonds with high chemo, stereo and enantioselectivity has been well recognized and forms the subject matter of the present study. Here we describe the isolation of a dehydrogenase by microbial screening and its application in the preparation of the chiral side chain of cholesterol-correlating/controlling (Hypercholemia controlling) agents belonging to the class of hydroxymethylglutamyl-CoA (HMG-CoA) reductase inhibitors. The biocatalyst has also been evaluated for general applications in production of chiral alcohols.

Pure cultures of large number of bacterial and fungal strains isolated from soil samples were available in the institute. These were screened for various

oxidoreductase activities. Several fungal and bacterial strains were isolated which could reduce (i) aldehydes to acids, (ii) alcohols to aldehydes and/or acid. No organism could be isolated which could reduce (i) carbonyl group of α-ketoesters or (ii) carbon-carbon double bond. One of the organisms, a fungal strain was selected for specific application in the preparation of ethyl 4-chloro-3-hydroxybutanoate **(4)**, which is a crucial intermediate in the preparation of HMG-CoA reductase inhibitors (statins). **4** was prepared in 90% yield and over 97% e.e. The fungal strain responsible for the activity was identified as *Penicillium funiculosum*. Reduction of ethyl 3-oxobutanoate **(1)** with *P. funiculosum* however proceeded to give <20% e.e., which indicated that substitution of H with Cl can drastically effect the selectivity of enzyme for substrate. Attempt to prepare **13**, an advanced intermediate of statins side chain by reduction of 3,5-dicarbonyl groups of **12** was however not successful.

P. funiculosum was also able to reduce cyclic compound methyl 2,2-dimethyl-5-oxo-1,3-dithiane-4-carboxylate **(14)**. α-substituted β-ketoester **(17)** was also reduced efficiently. The biocatalyst was also able to reduce α-diketone **(23)** and simple ketones such as acetophenone **(26)**, but the e.e. obtained was poor in these cases (50%-75%). 2-Oxo-2-phenylacetonitrile **(19)** on incubation with *P. funiculosum* did not give the expected reduced product, instead hydrolysis of nitrile to amide group occurred.

Dehydrogenases from *P. funiculosum* require $NADP^+$ as cofactor. Presence of NAD^+ in equimolar ratio to $NADP^+$ drastically inhibits the enzyme activity. The dehydrogenase is unstable in solution and loses most of the activity on storage at 4 °C for 72 h. The enzyme was partially purified (3.2 fold) by ammonium sulfate fractionation followed by Q-Sepharose chromatography. Attempts to purify enzyme to homogeneity, however were not successful.