SUMMARY

This thesis describes isolation by microbial screening of two biocatalysts, a thioesterase and a carboxyl esterase for the chemoselective hydrolysis of S-acylsulfanylalkanoates. Thioesterase was found chemoselective for thiol ester group, whereas the carboxylesterase exhibited substrate structure dependent selectivity for thiol ester or oxo ester group. Their applications in the preparation of optically pure ACE inhibitors and NSAIDs have been described. The thesis, for convenience, has been divided into three chapters. Chapter 2 and chapter 3 have been further divided into sections.

Chapter 1: Biotransformations Involving Hydro lytic Enzymes: An Overview

In this chapter, an attempt has been made to briefly review the available literature on the applications of hydrolytic enzymes in organic synthesis. The mechanistic and kinetic aspects involved in the hydrolysis of esters and amides have been discussed. The underlying principles of chiral recognition process and correspondng kinetic implicatons have been discussed in detail. The applications of esterases, lipases and amidases in organic synthesis have been described. The enantiomeric excess, e.e. obtained in the biocatalyzed hydrolysis of many non-natural esters is often in the range of 50-90%. Methods for improvement of selectivity have been discussed.

The applications of hydrolysis in the preparation of chiral drugs and their intermediates have been discussed in details. A brief introduction to the preparation of chiral compounds by chemical methods, especially asymmetric synthesis has also been provided.

Chapter 2

Section I:

Isolation, Purification and Characterization of a Thioesterase for Chemoselective Hydrolysis of S-Acetylsulfanylalkanoate

This section describes isolation, purification and characterization of a thioesterase from *Alcaligenes sp.* ISH-108. Thioesterases are ubiquitous hydrolytic enzymes having wide occurance in plants, animals and microbes, but so far they have not been utilized in organic synthesis.

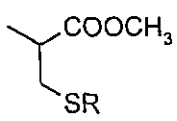
Methyl 3-acetylsulfanyl-2-methylpropanoate was chosen as substrate for screening of microorganisms for isolation of thioesterase for chemoselective hydrolysis of thiol ester group in S-acylsulfanylalkanoates. Preliminary screening was done on plates using alizarin red as indicator. The microorganism giving positive preliminary test were then individually cultured in flasks and assayed for activity. One of the isolate which chemoselectively hydrolyzed methyl 3-acetylsulfanyl-2-methylpropanoate to 3-sulfanyl-2-methylpropanoate was selected. It was identified as bacterium species belonging to genus *Alcaligenes* based on morphological characterstics and biochemical tests. The microorganism was designated as *Alcaligenes sp.* ISH 108.

The enzyme was extracted from the cells by osmotic shock with 1M NaCl. The extracted enzyme appeared to be reasonably pure on SDS-PAGE performed under reducing conditions. It was further purified by size exclusion chromatography on sepharose 4B. The electrophoresis of denatured thioesterase on SDS-PAGE performed under the reducing conditions gave its molecular weight as 22 kDa. The protein moved near the void volume on sephacryl S-300 column. It moved just after the higher molecular weight fraction of blue dextran (2000 kDa) and much before thyroglobulin (MW 669 kDa) on sepharose 4B column. These results indicate that the thioesterase is a large homomeric aggregate having 22 kDa subunits. The average particle size was found to be 21.5 nm by transmission electron microscopy (TEM).

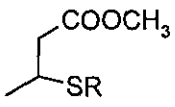
The effect of pH, temperature and metal ions on the activity of thioesterase was studied. The activity of the enzyme was assayed at various pH ranging from 5.5 to 10.5 at 30°. The maximum activity was obtained at pH 10.5. The enzyme was incubated for 20 hr. in different buffers, pH 5.5-10.5. The enzyme retained 100% activity at pH 5.5 to 6.5, 80% activity at pH 7.0 to 7.5 and only 20% activity at pH 10.5. The optimum temperature of thioesterase activity was found to be 65°. After incubation for 3 hr. at different temperatures, the enzyme retained most of the activity at 25-50°, but lost about 30% activity at 70°. Hg^{2+} and Cu^{2+} caused complete inhibition of the enzyme at 1 mM concentration, whereas Zn^{2+} showed 40% inhibition at 10 mM concentration. There was marginal increase in activity by Co^{2+}, Ni^{2+} and Mg^{2+} at 1mM and 10 mM concentration.

Section II: Characterization of the Chemoselectivity of Thioesterase and its Applications

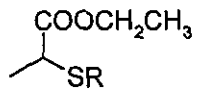
Thioesterase-catalyzed hydrolysis of a variety of substrates was studied to establish the chemoselectivity of thioesterase. The hydrolysis of methyl 3-acetylsulfanyl-2-methylpropanoate (**1**), methyl 3-acetylsulfanylbutanoate (**6**), ethyl 2-acetylsulfanyl-propanoate (**8**), ethyl 2-acetylsulfanyl-2-phenylacetate (**9**) and methyl 3-acetylsulfanylpropanoate (**10**) gave the corresponding thiols **2**, **7**, **11**, **12** and **13**, respectively.



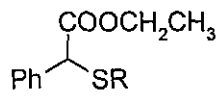
1, R = $COCH_3$, COEt or $COBu^n$
2, R = H



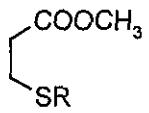
6, R = $COCH_3$
7, R = H



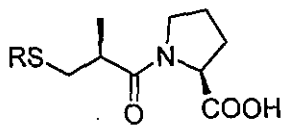
8, R = $COCH_3$
11, R = H



9, R = $COCH_3$
12, R = H



10, R = $COCH_3$
13, R = H



15, R = $COCH_3$
16, R = H

The oxo group remained intact in all the examples studied. It may be argued that there is kinetic bias of biocatalyst in favor of thiol ester and once the thiol is formed, overall affinity of substrate changes, making it a poor substrate for further reaction. The ester group therefore remains unaffected. But biocatalyst failed to hydrolyze the compounds containing only the oxo ester group, such as butyl acetate, butyl phenylacetate and dibutyl succinate, whereas their sulfur analogs were readily hydrolyzed. These results establish the fact that the biocatalyst has no hydrolytic activity towards oxo esters.

The applications of biocatalyst have been demonstrated in racemization free deacylation of (*R*)-ethyl 2-acetylsulfanylpropanoate (*R*-**8**) and (*S*)-ethyl 2-acetylsulfanyl-2-phenylacetate (*S*-**9**). Although these compounds are readily available in optically pure form from chiral pool of lactic acids and amino acids, their racemization free deacylation with a variety of reagents such as alcoholic HCl,

ammonia, 4-chloroaniline, $Ti(OR)_4$ etc. has not been successful. The sensitivity of these compounds to racemization has been attributed to the presence of an electron withdrawing group α-to the sulfur bearing carbon atom. The applications of the biocatalyst has been further demonstrated in the preparation of captopril (**16**), an angiotensin converting enzyme (ACE) inhibitor having considerable pharmaceutical and commercial value.

A study based on the activity of thioesterase was carried out to provide plausible explanation for the observed chemoselectivity. The presence of catalytically important histidine residue was indicated from following observations: (i) Diethylpyrocarbonate completely inhibited the enzyme. (ii) The activity of the enzyme could be recovered to the extent of 60% with hydroxyl amine. (iii) An increase in absorption corresponding to *N*-ethoxycarbonylation of histidine was observed at λ_{245}. That the catalytically important hitidine residue may be present within-in the active site was inferred from the experiments in which substrates thiophenyl propanoate and stearoyl-CoA provided considerable protection against inactivation by diethylpyrocarbonate.

Thus, histidine residue may be acting as base to deprotonate a bound water molecule, which then attacks the thiol ester group. We believe that oxo esters are not hydrolyzed, as in comparison they require higher activation energy. This argument was supported by the fact that activated oxo esters, which require lesser activation energy than normal oxo esters, such as 2,2,2-trifluoroethyl propanoate, p-nitrophenyl propanoate and vinyl acetate were readily hydrolyzed.

The relative rates of thiol/oxo esters offer an interesting study. Although, thiol esters are thermodynamically less stable than corresponding oxo esters, their rate of base catalyzed hydrolysis in aqueous solution are virtually identical. Since thioesterase was able to hydrolyze activated oxo esters, it gave an opportunity to compare the rates of biocatalyzed hydrolysis of thiol/oxo ester pairs. The rates of thioesterase-catalyzed hydrolysis of thiophenyl propanoate/phenyl propanoate and p-nitrophenyl propanoate/p-nitrothiophenyl propanoate pairs were measured. Thiophenyl propanoate reacted 1.4 times faster than phenyl propanoate and p-nitrothiophenyl propanoate reacted 1.7 times faster than p-nitrophenyl propanoate.

Section III: Substrate Specificity of Thioesterase. Proposed Physiological Role

This section describes *in vitro* substrate specificity of the thioesterase. Thioesterase was able to hydrolyze saturated long chain acyl derivatives of CoA with V_{max} 14-37 μmoles/min./mg of protein and K_m 11-15 μM. Stearoyl-CoA ($C_{18:0}$) was the most active substrate. The rate of hydrolysis of palmitoyl-CoA ($C_{16:0}$) and myristoyl-CoA ($C_{14:0}$) were almost similar. The enzyme showed very little activity towards octanoyl-CoA ($C_{8:0}$) and no activity towards acyl-CoAs having chain length smaller than C_8. Unsaturated long chain acyl-CoA derivatives were also hydrolyzed with V_{max} 19-22 μmole/min./mg protein and K_m 9-13 μM.

Thioesterase was able to hydrolyze p-nitrophenyl esters. The effect of chain length on the activity was studied. p-Nitrophenyl propanoate ($C_{3:0}$) was found to be most active substrate, with activity decreasing sharply with increasing and decreasing chain length. p-Nitrophenyl dodecanoate ($C_{12:0}$) showed very less activity and esters having chain length of more than 12 carbons were not hydrolyzed. The shorter chain alkyl and aryl thiol esters were also hydrolyzed. The chain length specificity for these oxo and thiol esters was opposite to that observed for acyl-CoA derivatives.

Thioesterase I and II, isolated from *E.coli* have been extensively studied, but *in vivo* function of these thioesterases is not known, unlike their eukaryotic counter parts in which several of them have a clear cut chain termination function in *de novo* biosynthesis of fatty acids. Two thioesterases of unknown function having striking similarities to thioesterase I and II of *E.coli.* have been isolated from *Rhodopseudomonas sphaeroids.*

The thioesterase reported here-in is different from those reported earlier in terms of one or more of the following properties: molecular weight, subunit structure, catalytic mechanism and turn-over number. The thioesterase appears to be a periplasmic protein as it can be extracted from the cells by osmotic pressure. The usual explanation for the presence of periplasmic hydrolytic enzymes is to allow scavanging of portions of metabolically useful molecules. The acyl-CoAs are metabolic intermediates and are unlikely to be natural substrates in this role. The long chain oxo esters are also unlikely to be substrates as they form micelles at very low concentrations, which may not be able to pass through the outer bacterial membranes

of gram negative bacteria. It is significant that *Alcaligene* sp. ISH 108 thioesterase can hydrolyze shorter chain aryl and alkyl thiol esters and aryl (but not alkyl) oxo esters. In-fact, thioesterase is more active on shorter chain esters of these substrates, has poor activity for $C_{6:0}$ and $C_{8:0}$ esters and no activity for $C_{10:0}$ or longer chain esters. These shorter chain oxo/thiol esters may be the natural substrates for thioesterase, suggesting therefore that this enzyme may be a scavenge enzyme *in vivo*.

Chapter 3

Section I: Isolation of a Biocatalyst for the Preparation of Enantiomerically Pure 3-Acetylsulfanyl-2-methyl-propanoic acid, a Crucial Intermediate for Antihypertensive Drug Captopril. Characterization of its Chemoselectivity

This section describes isolation of a biocatalyst for the preparation of (*S*)-3-acetyl-2-methylsulfanylpropanoic acid (*S*-**6**), a crucial intermediate of cardiovascular drug captopril. The biocatalyst required for the preparation of optically pure *S*-**6** should ideally have the following properties, (i) high chemo and enantioselectivity, (ii) optimum pH, about 7.0 and optimum temperature 30-35°, as substrate is prone to chemical degradation at higher pH and temperature and (iii) tolerance to high substrate concentration.

Screening of microorganisms was done using a three step procedure. Initially tributyrin plates were used to select esterase producing microorganisms, which were then tested for their ability to hydrolyze the desired substrate by plating them on nutrient agar plates containing *R,S*-(±)-methyl 3-acetylsulfanyl-2-methylpropanoate (**5**) and alizarin red at pH of 7.0. The microbial colonies that produced a yellow zone underneath or around the growth were selected. Finally, the selected microorganisms were cultured in individual flask and screened for their ability to produce *S*-(-)-3-acetylsulfanyl-2-methylpropanoic acid (**6**) from *R,S*-(±) methyl 3-acetylsulfanyl-2-methylpropanoate (**5**) in high optical purity. One microorganism having the desired properties was selected. It was identified as *Pseudomonas fluorescens*, based on the morphological features and biochemical tests conducted according to Bergey's Manual.

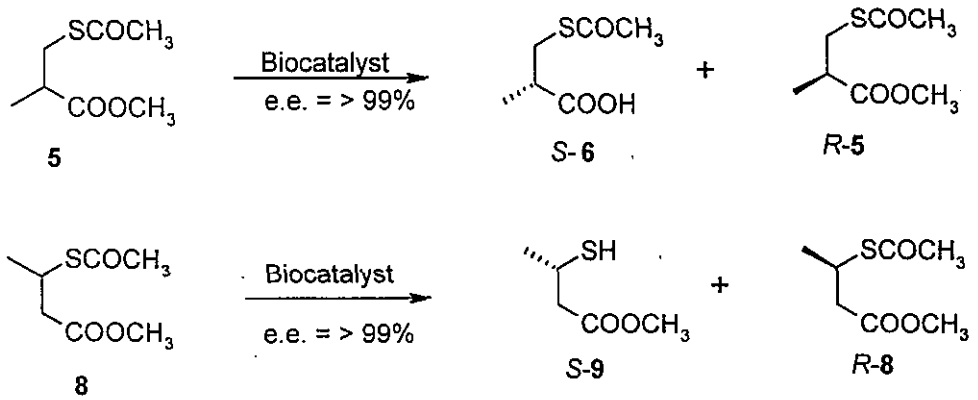
The enzyme responsible for the activity was found intracellular constitutive protein. Its optimum temperature and pH for desired activity were found to be 30° and 6.8, respectively.

For the preparation of 3-acetylsulfanyl-2-methylpropanoic acid (**6**), the reaction was done at scale of 10-60 g, using whole cells of *Pseudomonas fluorescens* as biocatalyst. The reaction was done at pH 7.0 at 30°. The reaction was stopped at 48-49% conversion. The separation of acid and unreacted ester was effected with aqueous sodium bicarbonate. *S*-6 so produced was found to be enantiomerically pure based on e.e. determined by ^{1}HNMR spectral analysis of its diastereomers prepared with (*R*)-2-(naphthyl)ethyl amine.

The biocatalyst (whole cells) was able to tolerate high substrate concentration of upto 352 g (2.0 mole) per liter. The substrate instantaneously formed emulsion on addition to the suspension of biocatalyst in buffer and viscosity of the reaction mixture increased with increasing substrate concentration.

The hydrolysis of a number of substrates was studied to characterize the chemoselectivity of the enzyme. We observed a strong dependence of chemo as well as enantioselectivity on the presence of an α-methyl substituent. The hydrolysis of methyl 3-acetylsulfanylpropanoate (**5**) gave (*S*)-3-acetylsulfanylpropanoic acid (*S*-**6**) (e.e. 99%) and unreacted ester (*R*-**5**) (e.e. 99%; $Eu(hfc)_3$ method). Its isomer, methyl 3-acetylsulfanylbutanoate (**8**) which differed only in the position of a methyl substituent, on enzyme-catalyzed hydrolysis gave optically pure thiol *S*-**9** (e.e. 99%; ^{31}P NMR method) and unreacted ester *R*-**8** (e.e. 99%; $Eu(hfc)_3$ method).

In the light of opposite chemoselectivity observed for isomeric substrates **5** and **8**, we thought it would be interesting to study the hydrolysis of **10**, in which α-methyl group is common to both the oxo ester as well as the thiol ester group. It resulted in the loss of chemoselectivity, although thiol ester group appeared to have reacted faster than oxo ester group. Poor enantioselectivity was obvserved in this reaction.



Finally, hydrolysis of methyl 3-acetylsulfanylpropanoate (**13**), in which both the thiol and the oxo ester group lacked an α-methyl substituent was studied. No chemoselectivity was observed in this case as both the ester groups were hydrolyzed.

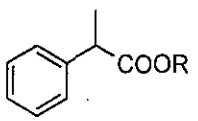
Section II: Preparation of Enantiomerically Pure (*S*)- 2-Arylpropanoic Acids belonging to the Class of NSAIDs

This section describes the applications of *Pseudomonas fluorescens* MTCCB0015 in the preparation of (*S*)-ibuprofen (**17**), (*S*)-ketoprofen (**19**) and (*S*)-flurbiprofen (**20**) in optically pure form. Until recently, all of these drugs have been marketed as racemates.

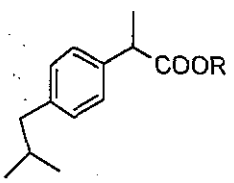
Initially, hydrolysis of methyl 2-phenylpropanoate (**21**) was studied using cell free extract as biocatalyst. In this case the extent of conversion has to be optimized to obtain good e.e. of the acid. Thus, at 20% conversion (*S*)-**22** was produced in optically pure form. The biocatalyzed hydrolysis of methyl esters of ibuprofen (**24**), ketoprofen (**25**) and flurbiprofen (**26**) occurred at much lower rate than that of parent compound **21**. After 20 hr., these compounds were converted to an extent of only 20-25%,

whereas 100% conversion occurred in case of **21**. The e.e. obtained in each case was more than 99%.

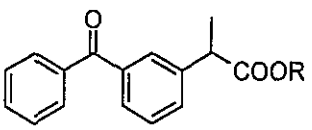
Although high e.e. could be obtained in all the cases, the conversion rate was very low. The rate of conversion was considerably enhanced by use of 2,2,2-trifluoroethyl esters. About 45% conversion occured in 20 hr. for 2,2,2-trifluoroethyl esters of ibuprofen (**28**), ketoprofen (**29**) and flurbiprofen (**30**). More than 99% e.e. was obtained in each case. The profens so produced posessed the desired *S* configuration.



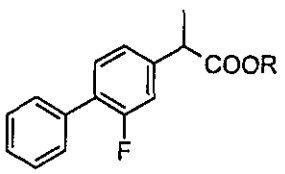
21, R = Me
22, R = H
27, R = CH_2CF_3



17, R = H
24, R = CH_3
28, R = CH_2CF_3



19, R = H
25, R = Me
29, R= CH_2CF_3



20, R = H
26, R = Me
30, R = CH_2CF_3