Summary

It has been emphasized that Exploration and Exploitation of the Microbial Diversity that exists in any country is essential for its Industrial and Economic Progress. During the evolutionary process, we humans have acquired a fair share of their physiology, such as mitochondriafrom the microbes; on the other hand, we have also become susceptible to many deleterious properties of microorganisms. Curiously enough, microorganisms themselves have given us the means to combat many of these diseases. Nevertheless, there are still many diseases, both infectious and metabolic, for which there are no absolute cures currently available, for example tuberculosis, malaria, enteric diseases, various viral infections and metabolic disorders such as Alzheimer's, Parkinson's, Rheumatoid arthritis and cancer to name just a few. Therefore, the exploitation of our microbial diversity for novel and new metabolites is an extremely important and vital endeavor.

Our group in IMTECH has been involved in the exploitation of India's rich microbial diversity for (i) new/novel bioactive molecules and (ii) new/novel enzyme activities for biotransformationsfor the past several years. The work described in this thesis is part of this endeavour. Prior to start of this work, group'sefforts for new bioactive molecules leads it to discover immunosuppressive property of CaeA. In the Part I of this thesis, we have described our efforts at the development of CaeA and its analogs for iron chelation therapy (ICT) for treatment of iron overload condition in mouse model. Work described in Part II of the thesis is one outcome of efforts at discovery of new enzyme activities for possible application in organic synthesis. Accordingly, thesis is presented in two parts

Part I: Evaluation of Caerulomycin A and its analogs for providing relief in secondary iron overload disease in mouse model

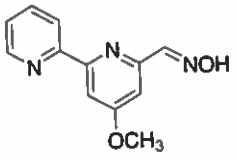
Human has developed highly evolved mechanisms for acquisition, transport, and storage of iron. However, there is no known mechanism for active excretion of iron from the body. This possess a great challenge in treatment of disease states like β-thalassemia (β-TM), sickle cell disease (SCD) and myelodysplastic syndromes (MDS), commonly leading to iron overload conditions in the body. Excess iron accumulation in body results in the saturation of serum transferrin and the development of toxic iron pools in cells and tissues. Non-transferrin bound iron (NTBI) in the plasma and labile cellular iron (LCI)

causes DNA damage and protein dysfunction. Liver, heart, pancreas and other endocrine organs are most commonly damaged and may lead to death if left untreated.Iron chelation therapy (ICT) is used for the treatment of secondary iron overload. Currently, three iron chelators, Deferoxamine (DFO), Deferasirox (DFX) andDeferiprone (DFP) have been approved for treating secondary iron overload due to transfusion.Although, these iron chelators have provided significant relief to patients, but these suffer from one or another drawback on prolonged use. Although several other molecules are currently under various stages of development, there is needto continuously search for an 'ideal' chelator.

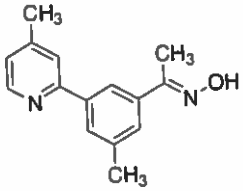
Our group has recently described the use ofCaeAas potent immunosuppressant. CaeAexhibited better immunosuppression than clinically most used drug cyclosporin A. CaeA suppressed both the cell-mediated immunity as well as the humoral immunity, cyclosporin A (CsA) suppresses only the cell mediated immunity. Our group also demonstrated that CaeA exerts its immunosuppressive effect by depleting intracellular iron concentration. Thus, for the first time our group reported that iron chelation is a viable rationale approach to selectively suppress the immune system; because compared to normal cells, rapidly proliferating cells require higher utilization of iron, which is a central regulator for proliferation and function of immune cells. Moreover, CaeA was shown to cause significant decrease in ROS levels in the cells; a property that can provide protection against damage to tissues through generation of ROS by labile iron in iron overload condition.

These properties of CaeA prompted us to initiate study to evaluate CaeA and its analogs for Iron Chelation Therapy (ICT) for the treatment of secondary iron overload in mouse model. Iron overload condition was created in miceby i.p. injection of iron dextran mixed with saline. Treatment of mice with iron overload condition with CaeA or its analogs MeOX or HOX resulted in significant decrease in serum ferritin level and liver iron contents (LIC); parameters that are considered gold standard for estimation of total iron load in the body. CaeA and its analogs were also able to significantly reduce iron level in heart (HIC) and kidney (KIC). A decrease in heart iron content (HIC) by either route of administration of CaeA is quite significant, because cardiac accumulation of iron has been described as primary cause of death in patients suffering from iron overload condition. Histopathology (H&E staining) was performed on heart, liver and spleen tissue. No gross changes in morphology were observed in these tissues.

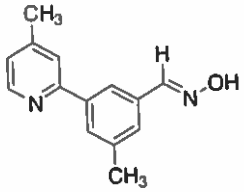
Put together, the results presented in this part of thesis showed that CaeA and its analogs HOX and MeOX have excellent potential of being developed for iron chelation therapy (ICT) for patients with iron overload condition. Based on the *in vitro* and *in vivo* data presented for CaeA and its analogs HOX and MeOX, these molecules may be taken to the next level of clinical studies in the drug development process.



CaeA



MeOX



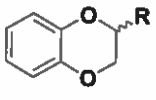
HOX

Part II: Biocatalytic preparation of both the enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid via kinetic resolution with either nitrilase or indole-3-acetamide hydrolase of *Alcaligenes faecalis* subsp. *parafaecalis*. Improving enantioselectivity of nitrilase

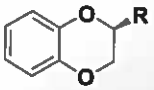
Both the enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid are important chiral building blocks for synthesis of therapeutic agents, most important amongst which is (*S*)-doxazosinmesylate, a primary drug used for the treatment of benign prostatic hyperplasia (BPH). Other examples include WB 4101, an α_1-adrenoreceptor selective antagonist, MKC 242, a potent $5HT_{1A}$ receptor agonist, 2-hydroxymethyl-1,4-benzodioxane, a prostaglandin D_2 receptor antagonist and N-[2,4-oxo-1,3-thiazolidin-3-yl]-2,3-dihydro-1,4-benzodioxane-2-carboxamide, which has antihepatotoxic activity.

Pharmaceutical applications require these enantiomers in optically pure form. In the literature, preparation of both enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**) has been described by a chemical resolution method using (+)-dehydroabiethylamine as chiral base. Using 1:1 equivalent of **1** and resolving agent in methanol, salt was precipitated in about 55% yield from which (*R*)-**1** was isolated in 99.2% *e.e.* Not only this method uses a chiral resolving agent which is rather uncommon and not easily accessible, the efficiency of process was also not very good. An enzymatic kinetic resolution method using esterase from *Serratia marcescens* has also been described for preparation of (*S*)-2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**). However, this method leads to less than optimal optical purity, and further enrichment in

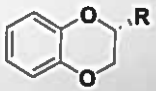
e.e. by crystallization was not efficient because of unfavourable eutectic point of conglomerate. Use of lipases for the kinetic resolution of *rac*-1 has also been attempted. Lipase of *Candida rugosa*, *Candida Antarctica*, *Pseudomonas fluorescens*, *Pseudomonas cepacia* and *Alcaligenes* sp. were tested, but none produced 1 in high *e.e.* The best result obtained was 85% *e.e.* with *Pseudomonas fluorescens*. Therefore, there is need to develop an efficient method for preparation of enantiomerically pure 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (1).



(*rac*)1-3



(*R*)1-3



(*S*)1-3

1, R = COOH; 2, R = CN; 3, R = $CONH_2$

Recently, our group in IMTECH has described a nitrilase activity from *Alcaligenes faecalis* subsp. *parafaecalis* that caused dynamic kinetic hydrolytic resolution of mandelonitrile to produce (*R*)-mandelic acid in 100% *e.e.* at 100% conversion rate. Initially, we explored this nitrilase for preparation of enantiopure 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (1) from corresponding *rac*-nitrile (2). The enzyme proved to be a very efficient hydrolase for this substrate, converting 100% substrate to the product in 4 h. However, we were disappointed to note that in sharp contrast to mandelic acid that was produced in 100% *e.e.*, the *e.e.* of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (1) was 0%.

We proposed a hypothetical working model to explain this difference in selectivity of enzyme and possible means to improve the enantioselectivity of enzyme for substrate **2.** We also proposed that a small alcohol, such as isopropanol if added to the enzyme, would occupy OH-binding cavity of enzyme and prevent polar/larger CN group to enter this cavity, thereby forcing the CN group to occupy second small cavity of the enzyme. Such binding of the substrate enforced by the presence of isopropanol at binding site of the enzyme should lead to enantioselective hydrolysis of the substrate **2.** Accordingly, we carried out *Alcaligenes faecalis* catalyzed hydrolysis of 2 in presence of 0 to 18% (V/V) isopropanol (IPA). No significant improvement in *e.e.* was observed till IPA concentration of 9%. The *e.e.* increased gradually with increase in IPA concentration from 9 to 18% .The *e.e.* was about 60% and 70%, respectively. We were pleased to note

that *e.e.* improved to 100% at IPA concentration of 18%. We termed this approach for improving the enatioselectivity "as enatioselective inhibition approach".

Although, we were able to improve the enatioselctivity of enzyme, the conversion rate was poor and not practical for the preparation of enantiopure **1**. Our attempts to improve conversion rates by addition of various bases lead us to a chance discovery of an amidase activity in *Alcaligenes faecalis* subsp. *parafaecalis*, which catalysed kinetic resolution of benzodioxin-2-carboxamide (**3**) to produce (*R*)-enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**) in >99% *e.e.* The enzyme exhibited excellent selectivity for (*R*)-enantiomer with *E* value of >200. Thus, at about 50% conversion under the optimized reaction conditions, (*R*)-2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**) was obtained in >99% e.e from *rac*-**3**. The remaining amide had (*S*)-configuration and 99% *e.e.* To obtain *S*-amide in 100% *e.e.* the reaction was performed as described above but allowed to proceed to 53% conversion.

The mixture of the (*S*)-amide and (*R*)-acid was separated by aqueous (alkaline)-organic two phase extraction method. The same amidase was able to catalyse, albeit at much lower rate the conversion of (*S*)-amide to (*S*)-acid without loss of *e.e.*It is significant to note that both the enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**) are amenable via *Alcaligenes faecalis* subsp. *parafaecalis* amidase catalysed resolution of amide **3**.

Amidase of *Alcaligenes faecalis* subsp. *parafaecalis* was purified by standard protocols of protein purification. Following proteomics approach, the amidase was identified as indole acetimide hydrolase (GenBank accession number: EKU31356.1). In nature, indole-3-acetamide hydrolase (IaaH) catalyse conversion of indole-3-acetamide (IAM) to indole-3-acetic acid (IAA). IAA is phytohormone of auxin class and is widespread among plants and bacteria that inhabit plant rhizosphere. In plant-bacterial interaction, bacterial IAA is a lead molecule involved in pathogenesis as well as phytostimulation.

The gene encoding for IaaH was cloned and expressed in *E. coli.* Substrate spectrum for the purified recombinant enzyme was analysed using a variety of potential substrates. As expected, IaaH exhibited highest preference for its natural substrate indole-3-acetamide compared to any other substrate tested. It is interesting to note that the activity of IaaH towards 2,3-dihydro-1,4-benzodioxin-2-carboxamide (**3**) was very high (65% compared to indole-3-acetamide). The enzyme exhibited excellent activity for

bicyclic compounds and longer-chain aliphatic diamides and none or poor activity for shorter-chain aliphatic amides and benzamide, suggesting that the enzyme has a wide pocket in the binding site and hydrophobic interactions are probably important for binding. Incidentally, indole-3-acetamide as the natural substrate for IaaH shared, at least in part a similar bicyclic structure with 2,3-dihydro-1,4-benzodioxin-2-carboxamide (**3**), which may account for high activity of IaaH towards this un-natural substrate.

In conclusion, we have demonstrated the production of both (***R***) and (***S***)-enantiomers of 2,3-dihydro-1,4-benzodioxin-2-carboxylic acid (**1**), which are important chiral building blocks for doxazosinmesylate, a primary drug used for the benign prostatic hyperplasia and other pharmaceutically important compounds in >99% *e.e.* by an amidase activity of *Alcaligenes faecalis* subsp. *parafaecalis* catalysed kinetic resolution of benzodioxin-2-carboxamide (**3**). To the best of our knowledge this is the first application of IaaH in production of an industrially important molecule.