Hydantoinases are widely used for the production of natural as well as non natural amino acids which are further utilized for various applications like precursor for antibiotic synthesis, food and feed additives, different agrochemicals etc. In the list of non-natural amino acids, the most important are D-para-hydroxyphenylgycine (hydrolysis product of D,L-5-hyPH) and D-phenylglycine (hydrolysis product of D,L-5-PH) which are used as precursors for the synthesis of the seminsynthetic β-lactam antibiotics viz. amoxicillin, cephadroxyl and ampicillin respectively. Production of amino acids from precursor hydantoin is basically a two step mechanism. In the first step, hydantoin is stereo specifically hydrolyzed to give n-carbamoyl amino acid which is further enzymatically or chemically hydrolyzed to give amino acid. Hydantoinase enzyme is used at the first step of this mechanism. From the industrial application point of view, hydantoinase should be highly thermostable and alkalostable which result in increased productivity (Bommarius *et al.*, 1992). Most of the hydantoinases reported so far have low thermostability and pH optimum near neutrality except bar9HYD which was purified from a mesophillic *Bacillus* sp. AR9. It was found to have half life of 80 min at 70 °C and pH optimum of 9.5 (Sharma & Vohra,1997). This hydantoinase was found to possess all the desired qualities which could make it an attractive target for industry except for its very less substrate specificity for D,L-5-PH and D,L-5-hyPH. The structure of bar9HYD has been recently solved at 2.3 Å (Kishan *et al.*, 2005). Likewise other hydantoinases, bar9HYD was also found to possess a TIM-barrel fold containing active at the C-terminus of β-strands. C-terminus region of bar9HYD was found to form a β-sandwich which had been reported to play no role in the activity of the enzyme but for oligomeric structure only in bsHYD and btHYD (Kim *et al.*, 1998). Interestingly, Lys 150 present in the active site which had been found carboxylated in the crystal structures of all other hydantoinases, was not found carboxylated in bar9HYD and therefore not coordinating with metals also. Besides this, crystal structure of bar9HYD with soaked mercury suggested Cys 317 as a target for mercury while imparting its inhibitory effect on the activity of the enzyme. Studies embodied in the present thesis intends to elucidate the role of different domains as well as residues like Lys 150 and Cys 317 in the activity

as well as stability of bar9HYD along with redesigning the enzyme in order to enhance its activity for industrially important substrates: D,L-5-PH and D,L-5-hyPH.

In an attempt to find out the significance of different domains of bar9HYD, three deletion constructs were made in which either N-terminus (46-460 construct) or C-terminus (1-444 construct) were removed from full length bar9HYD (460 amino acids) or only central portion containing TIM-barrel (amino acid 53 to amino acid 350) was kept intact by removing both N- as well as C-terminal regions. Three constructs were made with N-terminal 6x-His tag in pET28a vector and expressed in BL21(DE3) strains of *E.coli.* TIM-barrel construct formed inclusion bodies from which protein was purified on Ni-NTA agarose column by affinity chromatography after solubilizing inclusion bodies into 0.05 % (v/v) sarkosyl or 8 M urea. Protein purified in the presence of 8 M urea was refolded through on column refolding. Refolding was confirmed by fluorescence as well as CD studies. But protein purified in both manner did not show any activity. CD studies showed that proteins had lesser alpha helical content than natHYD. Other two constructs when expressed gave proteins in soluble fraction but showed significant decrease in the activity. CD studies showed N-terminus region significant for proper folding of the protein as 46-460 construct was found to have almost random coil structure. 1-444 construct also showed decrease in alpha helical content in comparison to natHYD. Trials to enhance the activity in the presence of any metal also could not help. Thus, this study did not substantiate the results of Kim's work which suggested that C-terminus region did not play any role in the activity of enzyme.

In order to find out the role of Lys 150 in bar9HYD, it was mutated into Ala (K150A) and Arg (K150R) through site directed mutagenesis. Proteins with N-terminus 6x-His tag were expressed in BL21 strain of *E.coli* in the presence of $MnCl_2$ and purified on Ni-NTA agarose column through affinity chromatography under native conditions. K150A showed almost negligible activity and substrate binding affinity in comparison to natHYD with D, L-Hyd as substrate. Although, K150R showed higher activity than the K150A, it was significantly lesser than that of natHYD. Substrate binding affinity was also almost half than that of natHYD. None of the metals could restore the loss of activities of these mutants. CD studies in far UV region showed a decrease in alpha helical content in both of the mutations. Interestingly, natHYD as well as K150R both

were found to loose their secondary structure with increase in temperature but K150A did not show any difference. Different fluorescence studies e.g. ANS binding and urea induced unfolding showed almost similar profile in natHYD as well as both the mutations except acrylamide quenching studies which showed lesser accessibility to Trp in K150A than K150R and natHYD. Crystallization attempts were also made for both the mutants in the presence / absence of different substrates. Some small crystals were obtained in K150A with and without hydantoin which could not give sufficient diffraction data because of their small size. Although, crystal structure for these mutants could not be obtained, but different biophysical (fluorescence and CD) and biochemical (steady state kinetics) studies strongly suggest that Lys 150 definitely has some structural as well as functional role.

Similarly, Cys 317 was also studied by site directed mutagenesis. It was mutated into Ser (C317S). C317S showed almost 80 fold lesser activity in comparison to natHYD. However, C317S showed two fold substrate binding affinity with hydantoin in comparison to natHYD. Flourescence studies did not show any significant difference compared to natHYD but CD studies in the far UV region showed an increase in the alpha helical content. Inspite of many differences caused by mutation of Cys into Ser, the characterstic precipitation of enzyme upon introducion of mercury remained unchanged. C317S was precipitated immediately after adding $HgCl_2$ in a manner similar to natHYD. These studies not only substantiate the structural as well as functional significance of Cys 317 but also revealed the possible role of other residue or factor other than the Cys 317 for exerting inhibitory effect of mercury. Thus, some other cysteines or amino acid residues might also be participating in the same phenomenon.

Total 17 point mutations of bar9HYD were generated through site directed mutagenesis after exhaustive rational studies like docking, homology modeling and sequence as well as structure alignment. Out of these 17 point mutations made at different positions (i.e. 63, 64, 65, 94, 158, 159 and 318), two mutations: P318S and L94V showed maximum increased activity with D,L-5-PH and D,L-5-hyPH along with minimum decrease with D, L-Hyd. P318S showed 5.67 fold higher K_{cat} value with D,L-5-PH in comparison to natHYD. Secondary structure studies in the far UV region showed decrease in alpha helical content in almost all of the mutations at all except the mutation

at position 65. The reason for the decrease in helical content could be explained by generating crystallographic symmetry neighbors which showed residues present at the positions 94, 158 and 159 interacting with other molecules. On the other hand, residues 63 and 64 were found to be situated on a β-strand. So, mutations at these 63 and 64 positions resulted in loss of some secondary structure. P318 was located at the beginning of FL8 and therefore restricting its freedom resulted in the introduction of some helical content in this loop which was lost once it was mutated into a smaller residue i.e. Ser.

P318S mutation may be proved to be an excellent choice for commercial application in near future because of having five fold higher activity with D, L-5-PH in comparison to natHYD. P318S is needed to be characterized thoroughly regarding its half life, optimum pH, temp as well as expression conditions followed by its scale up to the fermentor level.