SUMMARY AND CONCLUSIONS

D-amino acids are important chiral synthons for the production of pharmaceuticals, agrochemicals, feed and food additives. D-p-hydroxyphenylglycine is an important precursor for the synthesis of the semisynthetic β-lactam antibiotics viz. amoxicillin, cephadroxyl etc. Demand for D-p-hydroxyphenylglycine is expected to increase in the near future with the introduction of novel semisynthetic antibiotics like cefbuparzone, aspoxicillin and cefpyramide.

D-p-hydroxyphenylglycine can be produced by a two step, chemo-enzymatic or enzymatic, process from DL-p-hydroxyphenylhydantoin (Syldatk C. et.al.,1990). The important first step in these processes utilizes a stereospecific enzyme, D-hydantoinase. This enzyme (dihydropyrimidinase E.C. 3.5.2.2), has been exploited for stereoselective hydrolysis of DL-5-monosubstituted hydantoin derivatives to N-carbamoyl-D-amino acids (Yamada H. et.al.,1978; Yokozeki K. et.al.,1987a) which are further hydrolyzed chemically or enzymatically to D-amino acids (Takahashi S. et.al.,1979; Olivieri R. et.al.,1979). A thermostable D-hydantoinase with a pH optimum in the alkaline range is desirable for two reasons : one, the substrate solubility increases at higher temperatures, two, the process of self-racemization takes place under alkaline conditions (Bommarius A.S. et.al.,1992); thereby resulting in increased productivity.

The D-hydantoinases characterized so far have low thermostability and pH optima near neutrality. Of the five D-hydantoinases isolated from *Pseudomonas sps.* all were stable only up to 45°C (Morin A. et.al.,1990). A D-hydantoinase from *Peptococcus anaerobius* had a half life of 1.0 h at 60°C (Morin A. et.al.,1991). D-hydantoinases reported from

Agrobacterium sps. (Runser S. and Ohleyer E.,1990; Durham D.R. and Weber J.E.,1995) were found to be relatively stable however, little if any data has been provided for their thermostability. A recent study (Lee S.G. et.al.,1995) reports a thermostable D-hydantoinase from a thermophilic *Bacillus stearothermophilus* SD1 which had a half life of 30 min. at 80°C. Although a sizable number of hydantoinase producing organisms have been found and their enzymes characterized, a thermostable, alkalostable enzyme with high specificity towards p-hydroxyphenylhydantoin is still highly desirable and of great commercial importance. It was with this aim in mind that the present study was undertaken.

Two isolates with their ability to convert DL-p-hydroxyphenylhydantoin to N-carbamoyl-D-p-hydroxyphenylglycine were isolated. The isolates were identified as *Pseudomonas sp.* PHA1 and *Bacillus sp.* AR9. On the basis of higher activity, thermo- and alkalostability of D-hydantoinase from *Bacillus sp.* AR9 in comparison to the enzyme from *Pseudomonas sp.* PHA1, the isolate *Bacillus sp.* AR9 was selected for further studies.

D-Hydantoinase production in case of *Bacillus sp.* AR9 was found to be initiated at the onset of stationary phase. A correlation between sporulation and D-hydantoinase was found and maximum activity was achieved in Schaeffer's sporulation medium, in the mid sporulation phase. A correlation between D-hydantoinase production and production of extracellular alkaline protease was also seen. Extracellular alkaline protease was known to be produced during sporulation in case of *Bacillus subtilis.* Yeast extract and malt extract were found to be best nitrogen and carbon source respectively for the production of D-hydantoinase. It was found to be produced constitutively in the stationary phase and none of the substrates and substrate analogs added enhanced the production significantly.

Uracil marginally increased the production by about 20 %. Mn^{2+} was found to increase the production of D-hydantoinase from *Bacillus sp.* AR9 by almost two fold, other metal ions tested had no significant effect on its production.

A three step purification protocol was standardized for the isolation of homogeneous D-hydantoinase from cell lysate. This protocol resulted in a 635 fold purification of D-hydantoinase with a final yield of 18 %. The homogeneous enzyme had a specific activity of 1568 U/mg which is much higher than the specific activity reported for other known D-hydantoinases from various sources.

The D-hydantoinase had a native relative molecular weight of 208 kD as calculated from gel filtration chromatography and a subunit molecular weight of 56 kD as estimated by denaturing SDS-PAGE. These results indicated that D-hydantoinase had a homotetrameric structure. The enzyme had an isoelectric point (pI) of 4.45 which was similar to the D-hydantoinases reported from other *Bacilli.* N-terminal amino acid sequence of D-hydantoinase from *Bacillus sp.* AR9 showed highest similarity with the N-terminal sequence of D-hydantoinases isolated from other Gram's positive organisms indicating their similar origin.

The enzyme had a temperature optimum of 75°C and was found to be quite thermostable. It retained its activity even after two hour at 70°C and had a half life of 75 min at 80°C which makes it the most thermostable D-hydantoinase known till date. The enzyme was found to be more stable in its pure form as compared to the crude lysate and whole cells. D-hydantoinase in whole cells had a half life of 70 min at 70°C and lost complete activity in 15 min at 80°C. The enzyme had a pH optimum of 9.5 and was found to retain more than 90 % of its activity at pH 10 even after 24 h at 25°C. These properties are quite

advantageous for the biotransformations of 5-substituted hydantoin derivatives and give it an edge for commercial utilization. This hydantoinase was found to be strictly D-specific. It cleaved unsubstituted hydantoin as the preferred substrate. It showed a broad substrate specificity and cleaved both aliphatic and aromatic substituted hydantoin derivatives. It cleaved DL-p-hydroxyphenylhydantoin and DL-phenylhydantoin at a relative rate of 19 % and 40 % respectively. It did not cleave any of the disubstituted hydantoin derivatives tested. It had a K_m of 125 mM and a V_{max} of 0.85 μmoles/min when unsubstituted hydantoin was used as the substrate. The enzyme was found to a be true metallo-enzyme as presence of Co^{2+} ions significantly enhanced its activity. Mercuric chloride, p-hydroxymercuribenzoate and N-bromosuccinimide strongly inhibited the D-hydantoinase activity indicating the role of one or more cysteine and tryptophan residues in catalysis. Inhibition by metal chelators O-phenanthroline and EDTA confirmed the role of metal ion in catalysis by D-hydantoinase. A histidine specific chemical inhibitor, diethylpyrocarbonate also inhibited the enzyme to a lesser extent. The enzyme retained more than 50 % of its activity even after four months at 4°C. It was found to be quite stable in the presence of some organic solvents such as hexane and petroleum ether and ionic detergent such as SDS. Presence of tween 80 enhanced D-hydantoinase activity towards DL-p-hydroxyphenylhydantoin.

D-hydantoinase containing *Bacillus sp.* AR9 whole cells were successfully used for about 100 % conversion of 18 g/L of DL-p-hydroxyphenylhydantoin at 37°C at pH 8.5 within 21 h. The product formed was characterized by TLC, ^{1}H NMR and polarimetery and was found to be the D-enantiomer of N-carbamoyl-p-hydroxyphenylglycine. The product was found to be more than 99 % pure as ascertained by polarimetery. ^{1}H NMR analysis of the

product indicated that the product is not further modified by the whole cells to unwanted products.

D-hydantoinase was immobilized on Eupergit C (oxirane beads) by epoxy coupling. An activity of 7.9 U/g (wet weight) was achieved with a binding efficiency of 46 %. Immobilized enzyme had a higher temperature optimum and was also found to be more thermostable in comparison to the native enzyme. It retained more than 80 % of its activity at 80°C after 2.0 h in comparison to 30 % activity retained by native D-hydantoinase under similar conditions. pH optimum of the D-hydantoinase changed to 9.0 and a steep loss in activity was seen above a pH of 9.0 but the stability of the D-hydantoinase in alkaline pH range was found to be better than the native enzyme. Immobilized enzyme had a K_m (app.) of 195 mM and a V_{max} (app.) of 10 μmoles/min which were higher than the K_m and V_{max} of native enzyme. The immobilized enzyme had a longer storage life and could be reused for more than 10 cycles of conversion with only 20 % loss of activity.

D-hydantoinase gene was cloned by preparing a genomic library in *E. coli*. and was found to be expressed under its own promoter. The recombinant plasmid carried an insert of 4.3 kb which was mapped for various restriction endonuclease sites. Unidirectional deletion clones were generated by nested deletions in the insert by Sau 3AI. The smallest region (~1.5 kb) of the insert was ascertained to be producing functional D-hydantoinase with the help of deletion clones. The gene and the flanking regions were partially sequenced with help of deletion clones. A DNA sequence coding for the N-terminal region of the D-hydantoinase was identified and it was found to be similar to the sequence determined by Edman's degradation of the purified enzyme. The sequence obtained was

used for homology searching and found that protein sequence of the D-hydantoinase showed maximum similarity with D-hydantoinase from *Bacillus stearothermophilus.*

This is the first report of a highly thermostable and alkalostable D-hydantoinase from a mesophilic isolate, *Bacillus sp.* AR9. These properties with its strict D-specificity makes it the enzyme of choice for commercial exploitation. Sequencing and overexpression of the D-hydantoinase gene will help in elucidating the mechanism behind its thermostability and add to the pool of information, on what makes enzymes thermostable. Overexpression of the enzyme will also decrease its cost of production for commercial application.