“Bio-chemical and molecular characterization of DDT degrading dehalogenase from *Pseudomonas spp*.,”

Synopsis

Organochlorines have been used extensively around the world as general pesticides which have adversely affected the health of humans and wildlife. The unabated usage of these recalcitrant pesticides has led to their accumulation in soil and water environments. Hence, it is essential that such areas be reclaimed. 1, 1, 1- trichloro- 2,2- bis (*p*- chlorophenyl) ethane (DDT) is one of the most widely used organochlorine pesticides which is most toxic and environmentally persistent organic pollutant. It is one of the recalcitrant pesticides with a long residual action. It is a broad-spectrum pesticide, used extensively in public health programmes (to control insect parasites causing malaria, plague, dengue etc,). It was banned in 1997 but was reintroduced in countries which have failed to control mosquito related diseases. Since DDT residues are lipophillic, they tend to bioaccumulate in the fatty tissues of the ingesting organisms along the food chain. Exposure to DDT in humans can cause nausea, vomiting, dizziness, headache, neurological disorientations and hyperactivities or psychosomatic degenerations such as confusion, excitedness, loss of muscle control and tremors. DDT residues in water and soil are of serious concern as they are carried into the food chain. Mutagenic, carcinogenic and teratogenic natures of these pesticide compounds have been well established.

One method of treating such contaminated soils is bioremediation using microorganisms which is a cost-effective method. Microbial dehalogenases play an important role in the biodegradation of chlorinated pollutants. Dehalogenases eliminate HCl or Cl^- from their halo-organic substrate, leading to the formation of a double bond. DDT-dehydrochlorinase (EC Number 4.5.1.1) is involved in the catalytic degradation of *p, p'*-DDT by eliminating either HCl or Cl^- to form DDD / DDE. Dehalogenases acting on DDT have been studied only with *Musca domestica* (housefly). This enzyme has been shown to work only with *p, p'*-DDT, and not on *o, p'*- DDT.

The study of microbial dehalogenases has been motivated largely by the need to understand and manipulate the biodegradation of chlorinated pollutants such as pentachlorophenol, lindane, DDT, endosulfan and chlorinated phenoxyacetic acids, polychlorinated biphenyls, haloalkanes and haloalkanoic acids. These enzymes are reported to have been recruited in the microorganisms to serve new function in the biodegradation of these novel compounds.

The work on bacterial DDT-dehalogenases was undertaken with the following objectives:

1. Isolation, purification and characterization of DDT- degrading enzymes.
2. Cloning and expression of DDT- dehalogenase gene in suitable hosts.

The research work carried out towards achieving these objectives is brought out in the form of a thesis entitled **"Bio-chemical and molecular characterization of DDT degrading dehalogenase from *Pseudomonas spp*."**. The thesis has been divided into two chapters and contents provided in each chapter are highlighted.

The **introductory chapter** discusses about the discovery of DDT and its insecticidal properties and toxicity. It also deals with the environmental persistence of DDT and its slow migration to non-target areas such as underground water, flora and fauna. The general awareness and the reintroduction of DDT in the recent past for arthropod-borne disease also form a part of the chapter.

The chapter on **Review of literature** discusses about the synthesis of DDT, discovery of its insecticidal properties, production, applications of DDT as pest control chemical, the formulations used, properties of DDT, usage regulations, management and its toxicity has been discussed. Different methods of degradation and pathways adopted by different organisms have also been discussed. Dehalogenase mechanisms, origin and distribution of dehalogenase gene sequences have also been discussed.

CHAPTER 1:

Purification and characterization of DDT- dehydrohalogenase from *Pseudomonas spp.,*

This chapter deals with the isolation, purification and biochemical characterization of DDT dehydrohalogenase from *Pseudomonas putida* T_5. Bacterial isolates used in this study were isolated by long term enrichment of DDT-contaminated soil and sewage. All the isolates were identified based on microscopic, morphological and bio-chemical characteristics using Bergey's Manual of Determinative Bacteriology and Microbact Medvet system. Ten bacterial cultures were identified as *Pseudomonas fluorescens* biovar I (T_1), *Pseudomonas diminuta* (T_2), *Pseudomonas fluorescens* biovar II (T_3), *Burkholderia pseudomallei* (T_4), *Pseudomonas putida* (T_5), *Flavobacterium*

sp (T_6)*, Vibrio alginolyticus* (T_7), *Pseudomonas aeruginosa* (T_8), *Pseudomonas stutzeri* (T_9)*, Pseudomonas fluorescens* biovar V (T_{10}). These bacterial isolates were induced with 10 ppm of DDT and screened for enzyme DDT-dehydrohalogenase activity. Among these, the cell free extract of *Pseudomonas putida* T_5 showed higher DDT- dehydrohalogenase activity and the enzyme was purified by gel exclusion chromatography using Sepharose 6B column to apparent homogeneity with 7.12 fold purification. The relative molecular mass of the enzyme estimated by the SDS PAGE was ~32kDa. Native PAGE revealed the presence of a single band. The purity of the enzyme was confirmed by HPLC and capillary electrophoresis. The enzyme had maximum activity at pH 7.0 and 37°C. The enzyme was stable for 4-5 h at these optimum conditions. The K_m and V_{max}, values for DDT-dehydrohalogenase were 3.7 µM and 6.8 µmoles/min, respectively. The enzyme was a glycoprotein with mannose forming the backbone. A I G R V H N L D I formed the N-terminus chain. Fluorescence spectroscopic studies revealed that amino acids such as tryptophan were in the exposed environment and the enzyme was hydrophilic. AAS studies revealed the presence Zn, Mg, and Ca ions in the enzyme. PMSF inhibition of the enzyme indicated the presence of serine in the active centre of the enzyme. DDT dehydrohalogenases showed very low cross reactivity with other pesticides such as the metabolites of DDT viz., DDA, DDOH and DDE; isomers of HCH, endrin, endosulfan, chlorobenzoic acid and tetrachlorobenzene.

Effect of denaturants on DDT dehydrohalogenase

The effects of denaturing agents such as Urea, Sodium dodecyl sulfate (SDS) and guanidimium hydrochloride (GdmCl) on the structural changes of DDT-dehydrohalogenase enzyme of *Pseudomonas putida* T_5 were studied. There was a progressive loss in catalytic activity of DDT- dehydrochlorinase with increasing concentrations of denaturants, namely urea, SDS and GdmCl. At 10 M urea, 5 % SDS and 1 M GdmCl, the extent of loss in enzyme activity was 98, 78 and 100 % respectively. The emission spectrum of urea denatured enzyme did not show shift, but that of SDS and GdmCl treated enzyme showed very marginal shift. The secondary structure analysis of the enzyme by CD spectrum suggested a predominance of anti-parallel β-pleated sheets in the enzyme than α-helices. Urea denatured enzyme revealed complete loss of α-helix and there was substantial reduction in β-sheets. SDS treated enzyme showed increase in α-helix, turns and random structure. However, there was a marginal loss in β-sheets. GdmCl treated enzyme showed complete absence of α-helix compared to the control enzyme. The β-sheets were also reduced and the number of turns increased. The effects of GdmCl were almost similar to that of urea as observed in CD spectrum.

Effect of metals on DDT dehydrohalogenase

The effect of few metal ions on DDT-dehydrohalogenase activity was studied and it was observed that the enzyme was inactivated by the addition of metal ions. Monovalent and divalent cations (1mM) inhibited the enzyme strongly. The apparent rate constants of inactivation at different metal concentrations and conformational changes in the presence of 100 mM metal

ions were studied. The conformational changes of DDT-dehydrohalogenase were followed by fluorescence spectra and circular dichoism spectra. There was a progressive loss in catalytic activity of DDT-dehydrohalogenase enzyme with increasing concentrations of metals. The fluorescence spectrum of the enzyme exposed to metal ions showed a shift towards the red region. The CD spectrum of this metal treated enzyme showed significant changes such as reduction in α-helices and β- pleated sheets. Alkali metals, alkaline earth metals, heavy metals and transition metals studied, had almost the same changes in the secondary structure of DDT-dehydrohalogenase.

Development of enzyme based biosensor for the detection of DDT

Toxicity of DDT has been well established till date. Residues of DDT have been detected in drinking water, carbonated beverages, milk and milk products, egg, meat, etc., Detection of residues of DDT is of utmost importance to monitor the quality of food before it reaches the market.

DDT can be analyzed by various analytical methods such as TLC, GC, GC-MS and ELISA techniques. Traditional methods are laborious, time-consuming and expensive, require facilities and often fail in rapidity, sensitivity and specificity. In our laboratory, we have developed an enzyme based biosensor for the detection of DDT in water samples. Biosensor development involved application of purified DDT-dehydrohalogenase enzyme isolated from *Pseudomonas putida* T_5, optimization of reaction conditions, fabrication of biosensor system, and optimization of parameters for the detection of DDT using biosensor. Purified DDT-dehydrohalogenase

from *Pseudomonas putida* T_5 was used at 22 U activities. Conditions were optimized for the best stabilizer and cross linking agent that can be used. Among the different stabilizers used, BSA was found to be best. The enzyme was immobilized on to cellophane membrane using glutaraldehyde (cross linking agent) and BSA (stabilizer). The biosensor fabricated consisted of, ion selective electrode, hydrophilic polymer (cellophane membrane) with immobilized DDT dehydrohalogenase enzyme and a signal processing unit which showed elicited signal in terms of mV. Membrane containing immobilized enzyme was secured to the electrode using 'O' ring. Ion selective electrode immersed in reaction cell containing phosphate buffer was connected to signal conditioning unit for processing the signals (current to voltage). DDT was introduced to reaction cell using sample injector and continuously agitated using a magnetic stirrer. The chloride ion released as a result of enzyme activity elicited a biochemical signal. This signal was read by using signal conditioning unit which displayed signals in mV in response to the chloride ion released. Immobilized enzyme showed better activity compared to free enzyme. Analysis of reusability of enzyme revealed that enzyme activity was stable for 5 and 7 cycles for batch type and continuous type of analysis respectively. The biosensor developed was used for testing the environmental water samples for the presence of DDT. The method developed is rapid, specific and easy to execute.

Antibody production and isolation against DDT-dehydrohalogenase enzyme:

Antibodies were raised against DDT dehydrohalogenase in poultry. Purified enzyme was injected to 22 week old Single comb white leg horn poultry at 1 mg /mL concentration and booster doses were given once in 15 days .The antibodies were isolated from egg yolk by PEG 6000 method. The antibody of 8^{th} week had high titres at 1:1 lakh dilution.

CHAPTER 2: CLONING AND EXPRESSION OF DDT DEHYDROHALOGENASE GENE INVOLVED IN DDT DEGRADATION

This chapter deals with the designing of primers for DDT-dehydrohalogenase, amplification, cloning and expression of the gene in *E. coli*. Primers for PCR reaction were designed specifically for DDT-dehydrohalogenase gene using Primer 3.0 software. Genomic and plasmid DNA of all the ten individual isolates of the bacterial consortium were screened for the presence of the gene by gradient PCR between 50°C and 70°C and *Pseudomonas putida* T_5 was chosen for further work. Touch-down PCR was standardized by using optimized annealing temperatures. 30 cycles of optimized PCR conditions obtained were: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 45 sec, annealing at 55°C for 75 sec and extension at 72°C for 45 sec and final extension at 72°C for 10 min. Amplicon size of ~800 bp was obtained when electrophoresis was done on agarose gel (1%). The PCR product of DDT- dehydrohalogenase gene (dhl 1) was purified by gel elution, and ligated to pTZ57R/T vector and cloned in *E. coli* DH5α. The clones were selected on ampicillin Luria agar plates.

The insert from cloned plasmid was removed by double digestion with *Eco*RI and *Xho* I. This was further used for gene sequencing analysis. The purified PCR product was ligated to pET 28a vector and cloned in to *E. coli* BL 21 for expression. The expressed protein was purified using His-Tag purification kit. The purified enzyme was analyzed for activity and was confirmed by western blot, and colony PCR. The sequence was translated using Expasy and compared with the published protein sequence of *Anopheles gambiae* and multiple sequence alignment was done using Multalign software.

The thesis concludes with conclusions and summary.

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