Abstract

1, 1, 1- trichloro- 2,2- bis (*p*- chlorophenyl) ethane (DDT) is one of the most widely used organochlorine pesticides around the globe. It is one of the most toxic and environmentally persistent organic pollutant.

Ten bacterial cultures belonging to DDT degrading microbial consortium were screened for the DDT- dehydrohalogenase activity. Among these, the cell free extract of *Pseudomonas putida* T_5 showed higher DDT-dehydrohalogenase activity and enzyme was purified to apparent homogeneity with 73% overall recovery. The relative molecular mass of the enzyme estimated by the SDS PAGE method 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 was stable for 4-5 h at pH 7.0 at the temperature optima of 37 °C. The K_m and V_{max}, values for DDT- dehydrohalogenase were 3.7 µM and 6.8 µM min^{-1}, respectively. The enzyme was a glycoprotein with mannose forming the backbone. AIG- formed the N-terminus chain. Serine and tryptophan appeared to be involved at the active site. The enzyme appeared to be a metalloprotein containing Zn, Mg, and Ca ions. Monovalent and divalent cations (1mM) inhibited the enzyme strongly. The primary sequence of HPLC purified enzyme was deduced by LC-MS-MALDI-ESI.

Urea, sodium dodecyl sulfate (SDS) and guanidine hydrochloride (GdmCl) are often used as excellent denaturing or “unfolding” agents. The effect of these denaturing agents on the structural changes of DDT-enzyme of *Psuedomonas 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 β-structure in the

untreated enzyme. 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. The urea polyacrylamide gel showed only one band whereas SDS polyacrylamide gel showed closely moving two bands.

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 dichroism 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.

An immobilized dehydrohalogenase based potentiometric biosensor was developed. The chloride ion released as a result of dehalogenation by immobilized dehydrohalogenase during the degradation of DDT was detected by using an ion selective electrode. The voltage response had a direct linear relationship with the concentration of chloride (Cl^-) released with DDT. The immobilization had an advantage in improving the enzyme property and could be used repeatedly without loosing the activity.

Primers for PCR reaction were designed specifically for DDT-dehydrohalogenase gene. 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.