Interaction of gammaxene with site specific mutants of cytochrome P450<sub>cam</sub>

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Cytochrome P450<sub>cam</sub> (CYP101) from soil bacteria <i>Pseudomonas putida</i>, is one of the most well studied heme-b monoxygenase. A large number of X-ray crystal structures of this enzyme and its mutants are now available with different types of substrates that can be used to study the topology of the active site of the enzyme. We have a continuing interest in applying the current knowledge of cytochrome P450<sub>cam</sub>-substrate recognition to rationally design the enzyme for the biotransformation of unnatural substrates, like gammaxene, with the long-term aim of applications in bioremediation of environmental contaminants. Comparison of the structure of target substrate with that of camphor, the natural substrate, led us to engineer the heme active-site and we have found that binding affinities significantly are increased for Y96F, Y96F/L244A and Y96F/T101V mutants. This has shown the way to new functions of the enzymes, which not only has provided a novel approach to the study of the mechanism of this complex super-family of enzymes, but has also led to the discovery of green biocatalysts for environmental applications.

**Keywords:** Bioinorganic chemistry, Gammaxene, Cytochrome P450<sub>cam</sub>, Heme, Oxygenase, Site specific mutagenesis

Polyhalogenated hydrocarbons are used in a vast array of manufactured products as pesticides, herbicides, solvents etc in domestic, agriculture and industrial purposes. However, most of the organohalide compounds are environmentally hazardous, potential carcinogens or mutagens. They are highly insoluble and hydrophobic in nature and therefore get accumulated in fatty tissues of plants, animal food sources<sup>1</sup>. Thus they enter the human body through the food chain and are a potential threat to human life. One of the extensively used and highly contaminating organochlorinated pesticides is Gammaxene<sup>2</sup>. It is classified as the priority pollutant by the US and European Environment Agencies (EPA). Although the developed countries have banned the use of this compound for domestic and agricultural purposes, many developing countries still continue to use it. This compound is also used in small amounts in shampoo and lotions to fight scabies and lice<sup>3,4a</sup>. In humans, gammaxene affects the nervous system and also the liver and kidneys causing carcinogenicity, neurological disorders, etc. High insolubility and inertness have made this compound difficult for biodegradation, resulting in the bioaccumulation in human body<sup>4b</sup>. This compound has also been found to be poisonous for aquatic life and has been indicated to be a potential carcinogen/mutagen in model organisms<sup>4c</sup>. Biodegradation of the highly halogenated compounds have been studied by using certain bacteria and complete dechlorination by reductive dehalogenation was achieved by <i>Shewanella putrefaciens</i> 200 and was proposed to involve reactions of cytochrome c with the pollutants<sup>5</sup>. Reductive dechlorination of certain halogenated hydrocarbons by <i>Methanobacterium thermoautotrophicum</i> was shown to involve the nickel containing enzyme methyl-coenzyme M reductase<sup>6</sup>. However, there have been no detailed reports on the molecular mechanism of biodegradation of this kind of compounds.

Cytochrome P450s, a super-family of b-type heme (Fig. 1A) containing enzymes found in organisms in all domains of life, are involved in C-H activation by molecular oxygen to carry out a large number of metabolic processes like drug metabolism, xenobiotic detoxification as well as in steroid hormone biosynthesis in mammalian tissues<sup>7</sup>. These enzymes are also involved in catalyzing the synthesis of a large number of secondary metabolites in plants and other organisms<sup>8-12</sup>. One of the most extensively studied cytochrome P450, cytochrome P450<sub>cam</sub> from the soil bacterium <i>Pseudomonas putida</i>, is found to be
involved in the metabolism of 1R-camphor to form 5-exo hydroxycamphor regio- and stereospecifically (Fig. 1B and Fig. 2)\textsuperscript{13-16}. Through several research breakthroughs for the last 20-30 years, cytochrome P450\textsubscript{cam} variants have been found to monooxygenate small hydrocarbons, polyhalogenated benzenes, fused and polycyclic aromatic compounds etc. The proposed catalytic cycle of this enzyme has been shown in the Fig. 3. As, cytochrome P450\textsubscript{cam} show very high substrate specificity and product selectivity, it has become a favoured model enzyme to understand the biosynthetic pathways as well as the mechanism of actions for different drugs, hormones, and steroids by mammalian cytochrome P450 enzymes. Introduction of hydrophilic substituents like, hydroxyl group into polychlorinated compounds is expected to increase the solubility and hence, the bioavailability of these compounds for degradation\textsuperscript{17}. Moreover, biodegradation of many organic compounds\textsuperscript{18a} has been shown to involve the oxygenation of the parent compound or the intermediate\textsuperscript{18b,19}. The oxygenation of gammaxene may enhance the solubility as well as reactivity and thus increase the biodegradability of this contaminant. Hence gammaxene was chosen as a representative substrate to test the potential of the cytochrome P450\textsubscript{cam} variants to oxidize the highly chlorinated compounds. In view of developing an efficient method for biodegradation of gammaxene, we have designed different site-specific mutants of cytochrome P450\textsubscript{cam} and carried out detailed spectroscopic studies on the interaction of gammaxene with wild type as well as mutants of cytochrome P450\textsubscript{cam}.

**Materials and Methods**

Restriction enzymes and buffers for molecular biology studies were obtained from New England Biolabs. The organochlorine pesticide (Gammaxene),

![Fig. 1 – (A) Prosthetic group of cytochrome P450\textsubscript{cam}, an iron-(III) protoporphyrin-IX linked with a proximal cysteine-357 residue; (B) Stereospecific hydroxylation of the exo C-H bond at the C\textsubscript{5} position of camphor by cytochrome P450\textsubscript{cam}.

![Fig. 2 – Structure of 1R-camphor bound P450\textsubscript{cam}. [The blank arrow indicates the proposed substrate access channel and the violet sphere indicates the potassium bound enzyme near the B'-helix].

![Fig. 3 – Schematic representation of cytochrome P450 catalytic cycle showing various intermediate states of the heme.]
NADH, 1-(R) Camphor and other usual chemicals were purchased from Sigma-Aldrich company. DEAE Sepharose, Resource Q Sepharose and Sephadex G-25 columns were obtained from GE-Pharmacia Biotech.

**Mutagenesis, expression and purification of WT and mutants of cytochrome P450\textsubscript{cam}**

The recombinant wild type cytochrome P450\textsubscript{cam} was expressed following literature methods in BL21 (DE3) *E.coli* cells using plasmid pCHC\textsubscript{15,20,21}. All the cytochrome P450\textsubscript{cam} mutations described in this work contained the wild type mutation C334A to prevent protein dimerisation via disulfide bond formation\textsuperscript{21}. Site directed mutagenesis was carried out using Stratagene QuikChange Mutagenesis Kit. The primers and the restriction enzymes used for preparing the cytochrome P450\textsubscript{cam} mutants are listed in the Table 1. The mismatches in the primers that were used to introduce the mutations are shown in small letters. All the mutations were also confirmed by sequencing of the mutant plasmids. The physiological electron transfer partners putidaredoxin (PdX) and putidaredoxin reductase (PdR) were expressed and purified using the reported procedures\textsuperscript{22-24}. A schematic electron transfer pathway for the cytochrome P450\textsubscript{cam} enzymes is shown in Fig. 4.

**Docking study**

A theoretical analysis of gammaxene binding to the mutants of cytochrome P450\textsubscript{cam} was performed using the “PatchDock” server\textsuperscript{25-27}. PatchDock generally uses an algorithm that takes as input two molecules and computes three-dimensional transformations of one of the molecules with respect to the other with the goal of maximizing surface shape complementarity while minimizing the number of steric clashes. Complexes may be of the types: protein-protein, protein-small molecule, antibody-antigen, or protein-nucleic acid. The program was tested and shown to successfully predict protein interactions for many examples. PatchDock actually finds docking sites searching for molecular shape complementarity between the protein and small molecule. It first divides their surfaces into patches according to the surface shape (concave, convex, or flat). Then, it applies the Geometric Hashing algorithm to match concave patches with convex patches and flat patches with flat patches and generates a set of candidate transformations. Each candidate transformation is further evaluated by a set of scoring functions that estimate both the shape complementarity and the atomic desolvation energy of the obtained complex. Finally, redundant solutions are discarded by the application of an RMSD clustering. PatchDock is highly efficient, since it utilizes advanced data structures and spatial pattern detection techniques which are based on matching of local patches. These information are then extended and integrated to achieve global solutions. The clustering RMSD for gammaxene-cytochrome P450\textsubscript{cam} complex was kept at 1.5Å for docking experiment.

![Fig. 4 – Electron transfer pathway in cytochrome P450\textsubscript{cam} enzyme system.](image)

**Table 1 – Primers and restriction enzymes used for cytochrome P450\textsubscript{cam} mutants**

| Mutation | Restriction site | Oligonucleotide primers
<table>
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<tr>
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<tbody>
<tr>
<td>F87W</td>
<td>Nco I</td>
<td>5' GCGAGTGCCCaTggATCCCTCCTG 3'</td>
</tr>
<tr>
<td></td>
<td>BamH I</td>
<td>5' CACGAAGGATccAtGGGACTTCGC 3'</td>
</tr>
<tr>
<td>Y96F</td>
<td>Bsam I</td>
<td>5' GCCCGCGAAGCaTCCGGACTTCAT 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' ATGAAAGTCCatGCTTCGCCGCC 3'</td>
</tr>
<tr>
<td>Y96V</td>
<td>Bts I</td>
<td>5' GCCCGCGAAGCtgGACTTCATT 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' GAATGAAGTCCACTGCTTCGCCGCC 3'</td>
</tr>
<tr>
<td>Y96W</td>
<td>Hind III</td>
<td>5' GCCCGCGAAGCtgGACTTCATT 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' ATGAAAGTCCAtGCTTCGCCGCC 3'</td>
</tr>
<tr>
<td>T101V</td>
<td>Cla I</td>
<td>5' CTTCATGCGaTCCGGATCCCG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' CGGATCCATCAgtacGGGATGAA 3'</td>
</tr>
<tr>
<td>Y96P/T101V</td>
<td>Cla I</td>
<td>5' CTTCATGCGaTCCGGATCCCG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' CGGATCCATCAgtacGGGATGAA 3'</td>
</tr>
<tr>
<td>Y96F/L244A</td>
<td>Sfo I</td>
<td>5' GGATGTGTCGccTTACTGCTTGGG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' CCCACCAGTAaggeGCCCACACATCC 3'</td>
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</table>
Spectroscopic characterization

UV-vis spectra of all the enzymes were recorded using Perkin Elmer Lambda 750 spectrophotometer. The gas chromatography-mass spectrometry (GC-MS) studies of the reaction product were carried out using Thermo Electron Corporation spectrometry system. All the circular dichroism (CD) spectra for the secondary and tertiary structure regions were recorded with freshly prepared samples of cytochrome P450\textsubscript{cam} mutants in a quartz cuvette of 1 mm and 1 cm path length respectively using 50 mM Tris buffer (pH 7.4) containing 100 mM KCl on a JASCO-810 spectropolarimeter.

Equilibrium binding of gammaxene to cytochrome P450\textsubscript{cam} variants

The substrate binding induced changes in absorbance of wild type cytochrome P450\textsubscript{cam} and its mutants were determined from UV-visible spectroscopic titrations at \~300 K using wavelength-scan mode\textsuperscript{38}. Binding constants were obtained by analyzing the variation of absorbance at 417 nm and 392 nm on addition of increasing amounts of gammaxene using the following Eq. 1,

$$A = A_0 + \frac{(C_0 + L + K_d)}{\sqrt{(C_0 + L + K_d)^2 - 4C_0 L}} \times d\varepsilon = \cdots (1)$$

where $A_0$ and $A$ are absorbance of the enzyme at 417 nm (or at 392 nm) respectively in absence and in presence of this pesticide of concentration L. The initial concentration of the enzyme is $C_0$ and $d\varepsilon$ is the difference in molar extinction coefficient between the substrate-bound and substrate-free form of the enzyme at the given wavelength and $K_d$ is the substrate dissociation constant. Binding titrations were performed with 4-6 $\mu$M cytochrome P450\textsubscript{cam} variant in 50 mM Tris pH 7.4 and 100 mM KCl.

NADH activity assay

The enzymatic activity of the cytochrome P450\textsubscript{cam} variants was determined from the kinetics of change in absorbance of NADH at 340 nm. The assay solution was made with 1-2 $\mu$M cytochrome P450\textsubscript{cam} variant, 1 $\mu$M putidaredoxin reductase (PdR), 10 $\mu$M putidaredoxin (PdX), 100 mM KCl in 50 mM Tris (pH 7.4) along with 100 $\mu$M of the gammaxene and the reaction was initiated by addition of 300 $\mu$M NADH. All the experiments were performed at room temperature. Concentration of NADH was determined from the molar extinction coefficient ($\varepsilon_{340}$ nm) of 6.22 mM$^{-1}$cm$^{-1}$.

Identification of the enzymatic reaction product

The catalytic mixtures containing 1-2 $\mu$M cytochrome P450\textsubscript{cam} variant, 1 $\mu$M PdR, 10 $\mu$M PdX, 100 mM KCl in 10 mM Tris buffer (pH 7.4) was treated with gammaxene and the catalytic reaction was initiated by addition of 300 $\mu$M NADH. The reaction mixture was incubated at 4 °C for several hours (~5-6 h) till complete consumption of NADH and pesticide and its reaction products were then extracted in ether. The ether extract was mixed with 1:1 methanol for mass spectrometric analyses by GC-MS (Polaris Q, Thermo electron corporation). The diluted extract (1 $\mu$l) was injected into the GC column (DB-5, Thermo-Fisher) at 70 °C. The temperature of the column was kept at 70 °C for 1 min, raised to 170 °C at 10 °C/min, kept at 170 °C for 1 min, raised to 175 °C at 0.3 °C/min, kept at 175 °C for 1 min, raised to 230 °C at 10 °C/min and kept at 230 °C for 2 mins. The column temperature was further raised to 310 °C at 15 °C/min and kept at 310 °C for 2 mins to clean up any residual contaminant. The flow rate of the carrier gas (helium) was kept constant at 1 ml/min throughout the experiment. The MS transfer zone was kept at 280 °C and the recording of the mass spectrum was set to resume 3 mins after the start of the GC elution to avoid huge solvent signals. The mass spectrum was recorded in the m/z range of 100 to 320. The chromatogram was analyzed to determine the amount of gammaxene left in the solutions containing different P450\textsubscript{cam} variants after the reaction.

Results and Discussion

The X-ray crystal structures for substrate-free and camphor-bound forms of cytochrome P450\textsubscript{cam} have been reported earlier (PDB files: 1PHC and 1DZ4)\textsuperscript{16}. The substrate-free enzyme consists of a six-coordinated high-spin ferric heme at the active center with the thiolate of cysteine 357 and a water molecule axially coordinated to the metal center. Binding of the substrate at the active site of the enzyme causes removal of the axial water leading to formation of a five-coordinated high-spin ferric heme at the active center of the substrate-bound enzyme. Analyses of the substrate-bound structure (PDB Code: 1DZ4) of the wild type enzyme shows that the -OH of Tyr 96 forms a hydrogen bond with the carbonyl oxygen of camphor while Val 295, Leu 244 and Phe 87 are involved in hydrophobic interaction with the substrate\textsuperscript{16}. The Thr 101 residue has recently been shown to play an important role in stabilization of the substrate-bound structure of the enzyme and was
shown to undergo subtle conformation change on substrate binding to the enzyme\textsuperscript{20}.

Site-specific mutation of Tyr 96 residue for cytochrome P450\textsubscript{cam} has been extensively studied earlier by Wong and co-workers\textsuperscript{13,29,30}. They showed that the mutations of the Tyr 96 residue significantly improved the activity of cytochrome P450\textsubscript{cam} for the oxidation of a wide range of organic compounds. They proposed that the mutation at Tyr 96 can improve the recognition of hydrophobic compounds by increasing the hydrophobicity of the active site of the enzyme. The mutants of cytochrome P450\textsubscript{cam} containing Y96F have been shown to be extremely efficient in binding aromatic halogenated hydrocarbons.

The UV-visible absorption spectra of those purified mutant enzymes in absence of substrate were almost similar to that of the wild type cytochrome P450\textsubscript{cam} indicating that the electronic properties of the heme in absence of the substrate are not affected by the mutations in the present case. The CD spectra in 195-260 nm region for the substrate-free forms of the WT and mutants of cytochrome P450\textsubscript{cam} matched with each other indicating that mutations at the active site with hydrophobic residues have no significant effect on the secondary structure of the protein. The CD spectra in the tertiary structure region for the WT and the mutants also remained unchanged, except T101V protein indicating subtle changes in the tertiary structure around the metal center on mutation of that enzyme.

Gammaxene is a bulkier and more hydrophobic substrate than the natural substrate of the enzyme, camphor and thus shows weak binding to the wild type enzyme, which is tailor-made for camphor binding. Therefore substrate binding pocket has to be modified to make more space and to increase the hydrophobicity in order to enhance the affinity for gammaxene. In view of the improved binding inside the active site for gammaxene, we created several single and double mutants containing hydrophobic residues like F87W, Y96V, Y96F, Y96W, T101V, Y96F/T101V and Y96F/L244A and thoroughly checked the binding affinities of gammaxene to those mutants.

**Binding of substrates to the enzymes**

Binding of the substrate to the active site of cytochrome P450\textsubscript{cam} causes a change in the UV-visible spectrum of the enzyme, which has been well documented in literatures. The Soret absorption band of the substrate-free enzyme appears at 417 nm and corresponds to the low-spin ferric heme containing axially coordinated water at the active site of the enzyme. Binding of the hydrophobic substrate at the active site of the enzyme causes disruption of the iron-water coordination leading to formation of a five-coordinated high-spin ferric heme species characterized by Soret absorption band at 392 nm in the enzyme. Titration of the enzyme with the substrate thus causes gradual decrease in the absorbance at 417 nm with consequent increase at 392 nm corresponding to binding of the substrate to the enzyme. The variation of the absorbance at 417 nm of the enzyme with increasing concentration of the substrate thus was analysed to determine the binding constant using Eq. 1. The absorbance at 417 (as well as at 392 nm) was fitted to single binding site model as described by Eq. 1 and binding constants for various mutants with gammaxene were calculated. Absorption spectra of Y96F mutant with gammaxene bound is shown in the Fig. 5.

While WT P450\textsubscript{cam} showed no binding to the gammaxene, Y96W and F87W showed very weak binding affinity and spin-transition. On the other hand, the mutants like Y96V, Y96F, T101V, Y96F/T101V and Y96F/L244A were found to significantly enhance the substrate binding constant compared to the wild type (Table 2). Those mutants with either one (e.g., T101V increases hydrophobicity) or both factors like more space or more hydrophobicity (e.g., Y96V, Y96F, Y96F/T101V and

![Fig. 5 – UV-visible absorption spectra of (—, solid line) substrate-free low-spin ferric Y96F mutant of cytochrome P450\textsubscript{cam} (~5 µM), and (---, dashed line) gammaxene (72 µM) bound high-spin ferric Y96F mutant of the enzyme. [Inset: Type I difference spectra for the gammaxene binding (bound – free)].](image-url)
Y96F/L244A) significantly increase the substrate binding affinity and the catalytic activity. However, the introduction of the bulky Trp residue into the active site in the place of the Tyr or Phe in the Y96W and F87W mutants considerably reduces the space in the substrate binding pocket, which does not help the binding of the gammaxene molecule at the active site of the enzyme.

Docking study\(^{25-27}\) provided us the information about the orientations of gammaxene inside heme active site of the mutants of cytochrome P450\(_{\text{cam}}\). Top 10 solutions\(^{25}\) were obtained for each of the mutants like Y96F, Y96F/L244A etc. Then, the distances between the heme and the nearest carbon atom of the gammaxene docked in the active site were compared in each case, with the distance between C\(_5\)-camphor (the hydroxylated carbon atom of camphor) and the heme iron in the wild type cytochrome P450\(_{\text{cam}}\). The average distance was found to be 4.5-6 Å. As Y96F mutant showed high binding affinity towards gammaxene, top four solutions of Y96F mutant docked with gammaxene are shown in the Fig. 6.

<table>
<thead>
<tr>
<th>P450(_{\text{cam}}) variant</th>
<th>(K_b) (µM(^{-1}))</th>
<th>NADH consumption rate</th>
<th>Gammaxene consumption rate</th>
<th>Coupling (%)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>0.04</td>
<td>26</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>Y96W</td>
<td>0.05</td>
<td>20</td>
<td>NA</td>
<td>-</td>
</tr>
<tr>
<td>F87W</td>
<td>0.08</td>
<td>28</td>
<td>NA</td>
<td>-</td>
</tr>
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<td>5</td>
</tr>
<tr>
<td>Y96F</td>
<td>0.5</td>
<td>996</td>
<td>150</td>
<td>15</td>
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<tr>
<td>Y96F/L244A</td>
<td>0.8</td>
<td>138</td>
<td>18</td>
<td>13</td>
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<tr>
<td>T101V</td>
<td>0.24</td>
<td>95</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Y96F/T101V</td>
<td>0.7</td>
<td>135</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 6 – The first four solutions (A-D) of docking of gammaxene to the active site of Y96F mutant of cytochrome P450\(_{\text{cam}}\) obtained by PATCHDOCK calculations. [The substrate (gammaxene) is shown in cyan colour at the top of the heme ring and the F96 residue is shown in golden colour above the substrate].
Kinetics of NADH consumption

The enzymatic cycle of cytochrome P450$_{cam}$ involves two equivalents of electron transfer from NADH to the enzyme through putidaredoxin (PdR) and putidaredoxin reductase (PdX). Reduction of the enzyme by sodium dithionite cannot be used for determination of the enzymatic activity of cytochrome P450$_{cam}$ as the electron transfer process in the enzyme has been shown to be highly vectorial in nature. The oxygenase activity of the enzyme is measured from the rate of consumption of NADH by the enzymatic system consisting of cytochrome P450$_{cam}$ along with PdX and PdR in presence of the substrate. The decrease in absorbance at 340 nm corresponding to NADH is used to monitor the consumption of NADH. Earlier studies have shown that the mixture of cytochrome P450$_{cam}$ with PdX and PdR consumes NADH even in absence of the substrate owing to the aerial oxidation of NADH, as well as oxidation of the reduced forms of PdX and PdR. This background rate of electron transfer is used as a control containing all the components of the enzyme reaction except the substrate and the observed rates of NADH consumption was obtained by subtracting the control from the rate in presence of excess substrate. The NADH consumption rate by the enzyme has also been shown to depend on the presence of KCl. The wild type as well as Y96W and F87W mutants did not show any significant decrease in the gammaxene concentration in the catalytic mixture. The NADH consumption rates were very small and no detectable oxidation of gammaxene was observed for these mutants. On the other hand, the mutants like Y96V, Y96F, T101V, Y96F/T101V and Y96F/L244A were found to enhance the NADH consumption rate as well as the gammaxene consumption rate, as determined from spectroscopic and GC-MS studies.

Detection of the product of enzymatic reaction

The amount of substrate consumed during catalysis was calculated from the difference between area under the substrate peaks in the chromatogram of control and catalytic reaction mixture using the standard calibration plot. The rate of substrate consumption was calculated there from and divided by the rate of NADH consumption to get the coupling in percentage, i.e., Coupling = 100 × (substrate consumption rate/NADH consumption rate). Area analysis of the GC chromatogram of the gammaxene peak that showed the retention time of 15.64 min in total ion chromatograph (TIC) and a base peak at m/z = 183 (Fig. 7) under the stipulated temperature gradient programme, was performed using stipulated temperature gradient software. A standard calibration

![Fig. 7](image_url)
plot (Fig. 8) of area under the peak against gammaxene concentration with standard gammaxene solutions in CHCl₃ was made using the same temperature programme with characteristic base peak of the compound at $m/z = 183$. The final concentrations of gammaxene in the solution after reaction in presence of different P₄₅₀ₐ₅ variants were determined with the help of the calibration plot. Consequently the rate of the gammaxene consumption and the coupling of the NADH consumption with gammaxene oxidation by P₄₅₀ₐ₅ variants were determined. Unfortunately, we could not detect any new peak in the TIC that could be assigned to any of the reaction products of gammaxene. Hence, the possibility of formation of some charged products with low solubility in ether or dehalogenation of this pesticide in the catalytic reaction cannot be ruled out. These enzymes thus increase the coupling of the electron transfers with the substrate oxidation (Table 2). However, it must be conceded that, even the highest coupling efficiency (15% for Y₉₆F) is still much smaller than that in case of camphor hydroxylation by the wild type enzyme (~100%).

The architecture of the substrate binding pocket of the wild type enzyme is such that it can anchor the natural substrate, camphor extremely efficiently and small variations in the substrate structure can alter the substrate specificity and also product selectivity of the enzyme for the substrate. Our study shows that the protein engineering of cytochrome P₄₅₀ₐ₅ can give a possibly cleaner method for the degradation of pesticides. Based on the above GC-MS and equilibrium binding titration results, it also can be concluded that it is indeed possible to engineer the active site of an enzyme to make a more efficient ‘green’ catalytic system for organochloro pesticide like gammaxene following the basic principles of modulating the enzyme-substrate interactions.

Conclusions
The toxicity of halogenated pesticides arises mainly because of their low-solubility in water leading to accumulation of these compounds in the cell membrane. The catalytic reaction of cytochrome P₄₅₀ₐ₅ mutants with gammaxene observed in the present study, thus, offers a method of solubilisation of these halogenated toxic compounds from water but suitably designed biocatalytic systems. These results also led to creation of new uses of the enzyme, which supported the rational approach of design of the active site of cytochrome P₄₅₀ₐ₅ to achieve substrate specificity in making a green biocatalyst.

Acknowledgement
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References