Chapter III
Metabolic Engineering: 
Expression of Opd and MfphF in 
*Pseudomonas aeruginosa* PAO1161 
to achieve complete mineralization of methyl parathion
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Results:

In preceding chapters the author has attempted to establish the role of MfphF (Orf243) in degradation of organophosphorus compounds (op-compounds). Though there was no direct evidence to implicate MfphF in degradation of op-compounds such as methyl parathion, its involvement in degradation of meta-fission products is established beyond any doubt. As the \( p \)-nitrophenol (PNP) concentration in the medium containing induced cultures of \( E. coli \) having expression plasmid encoding MfphF got depleted, its involvement in degradation of PNP cannot be ruled out. However the present study has failed to gather direct evidence to implicate the involvement of MfphF in degradation of PNP despite of noticing a clear decrease in PNP concentrations only in the cultures having the expression plasmid encoding MfphF. In the light of these observations the author has attempted to engineer \( E. coli \) and \( Pseudomonas \ aeruginosa \) PAO1161 to mineralize op-pesticides such as parathion and methyl parathion by introducing both \( opd \) gene and \( mfphF \) into these strains. The present chapter describes the strategies used in engineering these strains and the results obtained pertaining to the degradation of methyl parathion.

1. Expression of \( opd \) and \( mfphF \) in \( E. coli \) JM109 (DE3):

In the present study the author has used expression plasmids that are available in his laboratory. One of them, pSM4 encodes MfphF as a native protein whereas the pSM5 encodes parathion hydrolase (PH) with C-terminal his-tag (Siddavattam et al., 2003). The pSM4 is a derivative of pET15b and hence it contains ColE1 replicon. Similarly the pSM5 is a derivative of the broad host range vector pMMB206, which contains RSF1010 replicon. Therefore these two plasmids are found to be compatible and are expected to
replicate and segregate in the E.coli. If an E.coli strain is transformed with these two plasmids the strain should be able to synthesize both parathion hydrolase and MfphF. These two enzymes together are expected to contribute for mineralization of parathion and methyl parathion. Based on these sound theoretical assumptions the author has transformed E.coli JM109 (DE3) with expression plasmids pSM4 and pSM5 and induced the cultures following the procedures described in materials and methods section. When the total proteins were analyzed it was clearly found that these two proteins were found in the induced cultures. One of them corresponding to the size of parathion hydrolase whereas the size of the other protein matched the size (27 kDa) of MfphF indicating that these two proteins were successfully co-expressed in E.coli JM109 (DE3) (Fig.4.1). After ascertaining the successful co-expression of these two proteins further studies were conducted to assess the ability of engineered strains in mineralization of methyl parathion. The E.coli JM109 (DE3) expressing both parathion hydrolase and MfphF showed both methyl parathion hydrolyzing ability and the ability to degrade PNP. However, the rate of PNP degradation was very low and it was found to be comparable to the in vivo PNP degradation studies described in chapter-I. Interestingly in the cultures where the PH and MfphF were co-expressed the amount of MfphF was found to be very high when compared to the expression levels in the cultures where the MfphF was expressed (Fig.4.1). Such an observation prompts to think of possible structural stabilization of MfphF in presence of parathion hydrolase probably thorough protein-protein interactions. Hence the author conducted further studies to understand such interactions, if any, by co-expressing these two proteins in Pseudomonas aeruginosa PAO1161 as MfphF is found to be properly folded in P. aeruginosa PAO1161.
Fig.4.1. Expression of *mfphF* (*orf243*) in *E.coli* JM109 (DE3). Lanes: 1, molecular mass markers; 2 and 3, protein extracts prepared from induced (lane 2) and uninduced (lane 3) cultures of *E.coli* JM109 (DE3) cells containing both the expression plasmids pSM4 (*mfphF*) and pSM5 (*opd*); 4 and 5, show protein extracts prepared from the induced (lane 4) and uninduced (lane 5) cultures of *E.coli* JM109 (DE3) cells carrying only pSM4 (*mfphF*). Expression of parathion hydrolase (Opd), MfphF, and vector-encoded chloramphenicol acetyltransferase (Cat) are indicated with arrows.
Fig. 4.2. Schematic diagram showing the structure of opd-operon.
2. Co-expression of Parathion Hydrolase (PH) and MfphF in *Pseudomonas aeruginosa* PAO1161:

Co-expression of both parathion hydrolase (PH) and MfphF in *Pseudomonas aeruginosa* PAO1161 require unique strategies. One of the expression plasmid encoding MfphF (pSM4) is unstable in *Pseudomonas aeruginosa* PAO1161 as it contains ColE1 type of replicon. Therefore, the author followed elegant strategies to construct an expression plasmid by bringing both the *opd* gene and *mfphF* (*orf2*43) under the control of *lacUV5* promoter of broad host range plasmid pMMB206 (Morales, 1991). In this construct the open reading frames of both *opd* and *mfphF* are brought under the transcriptional control of vector specific *lavUW*5 promoter and terminator elements and hence appear as if these two genes constitute a single operon (Fig.4.2). The detailed strategies involved in the construction of *opd*-operon are described below.

3. Construction of *opd*-operon:

While constructing expression plasmid to co-express both parathion hydrolase and MfphF the plasmid pSM5 (Siddavattam et al., 2003) is taken as a source plasmid. In plasmid pSM5 the *opd* gene is cloned under the transcriptional control of *lacUV5* promoter of the expression vector pMMB206. In plasmid pSM5 the 3' end of *opd* gene is modified in such a way that the stop codon is mutated to fuse with the vector encoded sequence that specifies histidine residues. To facilitate cloning of *mfphF* downstream of *opd* gene the 3' end of the *opd* gene need to be replaced with the similar region of the wild type *opd* gene particularly to reestablish the translational stop codon of the *opd* gene. The wild type *opd* gene can be precisely taken as 1.3 kb *PstI* fragment (Mulbry et al., 1987). To obtain the 3' end of *opd* gene the complete gene was cloned in pUC18 as
PstI fragment (pSM13) (Fig. 4.3 A & B). After cloning opd gene as PstI fragment, the 3' region of the gene was taken as 1 kb SalI fragment as there is one internal SalI site in opd gene and the second one in the multiple cloning site of the vector (Fig. 4.3A). This 1 kb SalI fragment was used to replace the 3' region of the opd gene found in the expression plasmid pSM5 by digesting with SalI (Fig. 4.4). Such replacement brings back its own transcriptional terminator sequence to the opd gene along with its translational stop codon. There is an unique BamHI site upstream of the transcription terminator sequence of the opd gene and another unique PstI site downstream of the putative transcription terminator sequence of the opd gene. These two sites are used to insert mfphF as BglII-PstI fragment (pSM16) by eliminating the transcription terminator sequence of the opd gene (Fig. 4.5 A & B). The BamHI site is precisely located downstream of the translational stop codon of the opd gene. As BamHI site is used to clone mfphF it automatically places the 5' end of mfphF immediately downstream of the translational stop codon of opd gene (Fig. 4.5 A). Therefore, if transcription is initiated from the lacUV5 promoter under which the opd gene is cloned this transcript synthesis extends beyond opd gene as there is no transcriptional terminator sequence. Such an extension of transcription leads to the synthesis of a polycistronic RNA consisting of both opd and mfphF specific sequences. The vector specific transcription terminator sequence is available downstream of PstI site. Hence it would prevent further elongation of transcription process leading to the synthesis of a precise mRNA molecule containing only opd and mfphF specific sequences. In order to clone mfphF as BglII-PstI fragment it was amplified using expression plasmid pSM12, which encodes MfphF as a his-tagged protein, as a template by following the strategy described in chapter-I. The expression
Fig. 4.3A. Schematic diagram representing the strategy followed to clone wild type 
*opd* gene in pUC18 vector. Figures were not drawn to scale.
Fig. 4.4. Schematic diagram representing the strategy followed to replace 3' end of \textit{opd} gene from pSM5 with the 3' end of wild type \textit{opd} gene. Figures were not drawn to scale.
Fig. 4.5.A. Schematic diagram representing the strategy followed to clone \textit{mfphF} (\textit{orf243}) in pSM15 in order to construct \textit{opd}-operon.
Fig. 4.3.B. Cloning of *opd* gene in pUC18. Lanes: 1, Kilo base ladder; 2 & 3, undigested pUC18 & pSM13; 4 & 5, pUC18 & pSM13 digested with *PstI*; 6, pSM13 digested with *SalI*. The 1 kb *SalI* fragment is used to replace the 3' end of expression plasmid pSM5 borne *opd* gene is shown with arrow mark.
Fig. 4.5.B. Construction of opd-operon: Cloning of \textit{mfphF} in pSM15. Lane 1 represents Kilo base ladder; Lane 2 & 3 represent pSM15 undigested and digested with \textit{BamHI} and \textit{PstI}; Lane 4 & 5 represent undigested and digested recombinant plasmid with \textit{BamHI} and \textit{PstI}. The resulting recombinant plasmid is designated as pSM16. The arrow marks indicates the release of \textit{mfphF} upon \textit{BamHI} and \textit{PstI} digestion.
plasmid (pSM16) constructed in this manner contains both opd and mfphF under the control of lacUV5 promoter of the broad host range vector pMMB206 (Fig.4.2). Therefore, these two genes can be mobilized and expressed both in E.coli as well as in Pseudomonas aeruginosa PAO1161. As mfphF is found to be in soluble form when expressed in P. aeruginosa PAO1161 mobilization of this construct into Pseudomonas aeruginosa PAO1161 is expected to synthesize MfphF in active form along with parathion hydrolase. Such a situation would be ideal for conducting studies to elucidate interaction between these two proteins and to establish their role in complete mineralization of op-compounds such as methyl parathion and parathion.

4. Mobilization of pSM16 into Pseudomonas aeruginosa PAO1161:

The construct pSM16 was mobilized into P. aeruginosa PAO1161 by non-quantitative triparental plate mating method (Goldberg and Ohman, 1984) by taking E.coli DH5α strain carrying the pSM16 as donor, P. aeruginosa as recipient and E.coli (pRK2013) as helper strain. The conjugation experiment were performed following the protocols described in materials and methods section. Donor, recipient and helper strains, which were in log phase, were taken in the ratio of 1:2:1 and plated on LB plate and mating was proceeded for 24 h. Simultaneously donor and recipient and helper strains were also plated separately on LB plates as control and incubated for 24 h. Later the cells grown on these plates were carefully plated on chloramphenicol (30µg/ml) and ampicillin (100µg/ml) plates before incubating the plates for 24 h for colony development. Donor, recipient and helper strains were failed to grow on the selective plates whereas the exconjugants have grown indicating the success of conjugation experiment. Then exconjugants which were Amp' and Cm' were retained and analyzed
for the presence of plasmid by KADO method as described in materials and methods section. The strain having the plasmid was selected and used for further studies.

5. Expression and localization of MfphF and Parathion hydrolase in *P. aeruginosa* PAO1161:

Overnight cultures of *P. aeruginosa* with and without expression plasmid pSM16 were sub cultured into 10 ml of LB medium and overexpression of Opd and mFphF were carried out as described in materials and methods. After induction, protein extracts prepared from both induced and uninduced cultures was analyzed on 12.5% SDS-PAGE (Fig.4.6). A 39 and 30 kD protein bands correspond to the size of the parathion hydrolase and MfphF was noticed in induced cultures. Similar protein band was absent in uninduced cultures as well as in protein extracts prepared from the control *Pseudomonas aeruginosa* PAO1161 cells (Fig.4.6). After successful expression of these two proteins further experiments were carried out to assess the ability of *Pseudomonas aeruginosa* PAO1161 (pSM16) in degradation of methyl parathion. The cultures of *Pseudomonas aeruginosa* PAO1161 strains with expression plasmids pSM5 and pSM16 that express, parathion hydrolase and both parathion hydrolase and *meta*-fission product hydrolase (MfphF) respectively were grown to log phase in minimal salts medium. After the culture has reached to the log phase they were induced to produce these two enzymes. Simultaneously 60 µM of pure methyl parathion was added and the concentration of methyl parathion was monitored at different time intervals following the procedures described elsewhere (Zhongli et al., 2001). The concentration of methyl parathion as shown in Fig.4.7 disappeared constantly from the cultures of *Pseudomonas aeruginosa* having pSM16. However in similar cultures having pSM5, that encode only parathion
Fig. 4.6. Expression and localization of MfphF and Parathion hydrolase (PH) in *Pseudomonas aeruginosa* PAO1161. Lane 1 represents molecular weight markers, Lane 2, 3 and 4 represent total cell proteins extracts prepared from *Pseudomonas aeruginosa* PAO1161, *P. aeruginosa* PAO1161 (pSM16) uninduced and induced cultures. Lane 5 represents particulate fraction of induced culture of *P. aeruginosa* PAO1161 (pSM16), Lanes 6 and 7 show proteins found in 20 and 35% ammonium sulphate fractions prepared from soluble proteins of *P. aeruginosa* PAO1161 (pSM16). The MfphF and Parathion hydrolase (PH) proteins were indicated by arrows.
Fig. 4.7. Mineralization of methyl parathion. A. *Pseudomonas aeruginosa* PAO1161
B. *Pseudomonas aeruginosa* PAO1161 (pSM5) uninduced (●) and induced (■) cultures, triangles indicate the accumulation in of p-nitrophenol in induced culture. C. *Pseudomonas aeruginosa* PAO1161 (pSM16) uninduced (●) and induced (■) cultures; triangles indicate the accumulation of p-nitrophenol in induced culture. The decrease of methyl parathion was recorded at A273 nm (Zhongli et al., 2001) and the PNP concentration was calculated as described in materials and methods.
hydrolase the PNP has accumulated (Fig. 4.7). There was no disappearance of methyl parathion added to the control cultures of *Pseudomonas aeruginosa* PAO1161 having neither pSM5 nor pSM16. This clearly indicating the methyl parathion degradation is plasmid (pSM16) encoded property.

6. Studies on Parathion hydrolase (PH) and MfphF interactions:

Further studies were carried out to study the interactions between PH and MfphF using the *Pseudomonas aeruginosa* PAO1161 cultures having expression plasmid pSM16. In pSM16 the genes that encode these two enzymes have been kept under the control of transcriptional and translational signals of the vector. Further more these two proteins are translated using the same polycistronic mRNA. Hence the intracellular concentrations of these two proteins are expected to be similar and probably would provide ideal situation to assess the protein-protein interactions, if any, between these two proteins. Before proceeding to conduct experiments to determine protein-protein interactions a detailed study were undertaken to assess the sub-cellular localization of both PH and MfphF. When the induced cultures were fractionated into particulate and soluble fractions sizable portion of these two proteins was found in soluble fractions (Fig. 4.6). Hence the soluble portion is taken for studying the possible interactions between PH and MfphF.

7. Purification of MfphF and Parathion hydrolase (PH) complex from *Pseudomonas aeruginosa* PAO1161:

After establishing the sub-cellular localization of MfphF and parathion hydrolase (PH) in *P. aeruginosa* (pSM16), further experiments were carried out to purify the MfphF using Ni-affinity column as it contains his-tag at the N-terminal end. If PH
interacts with MfphF it is expected to be co-purified along with MfphF. The soluble fraction prepared from the lysate of *Pseudomonas aeruginosa* PAO1161 (pSM16) was applied on to Hi-Trap Ni-column and purification of the his-tagged MfphF was performed as described in materials and methods section. The eluted fractions were assayed for MfphF and Parathion hydrolase activities. The eluted have only showed MfphF activity and PH activity was not observed. In contrast, the flow-through collected from the fractions showed activity for both PH and MfphF. This is rather surprising result. In this study the author has successfully purified MfphF using Hi-trap nickel column by independently expressing it in *Pseudomonas aeruginosa* PAO1161. Its failure to bind to nickel column in presence of PH could be due to masking of his-tag of MfphF upon complexing with PH. In order to gain further insights on possible interactions between these two proteins, the conventional protein purification protocols have been used. The soluble fraction was subjected to ammonium sulphate fractionation. When these fractions were analyzed, most of the MfphF activity was found in the fraction that was obtained at 20% concentration (Fig.4.6). However, in the fractions that were obtained at 35% ammonium sulphate concentration the activity of both MfphF and PH were obtained (Fig.4.6). The ammonium sulphate (35%) precipitated fraction containing MfphF and PH were passed though the Sephacryl S 300 HR column (80 x 1.5 cm) to know if these two proteins are together in the form of a complex. Those fractions that showed both MfphF and PH activity were pooled and concentrated through lyophilisation. The lyophilized fraction was analyzed on 12.5% SDS-PAGE (Fig.4.8). A 39 and 30 kD protein bands corresponding to the size of PH and MfphF was observed on
Fig. 4.8. Purification of parathion hydrolase (PH) and MfphF complex from *Pseudomonas aeruginosa* PAO1161 (pSM16). Lanes 1 indicates molecular weight markers. Lanes 2 indicates presence of PH and MfphF in pure complex. Lane 3 represents pure MfphF. The PH and MfphF are indicated with arrows marks.
gel. These results clearly demonstrate that MfphF and PH form complex when co-expressed in *Pseudomonas aeruginosa* PAO1161.

8. **Kinetic properties of Parathion Hydrolase (PH) and MfphF complex:**

Purified complex of MfphF and Parathion Hydrolase (PH) from *Pseudomonas aeruginosa* PAO1161 (pSM16) was used to assay for MfphF and PH activity. The specific activities were found to be half when HOHD and methyl parathion were used as assay substrates, when compared to the specific activities obtained with independently purified enzymes (Table 4.1). This data has further strengthened our earlier description of PH forming complex with MfphF and shows an additional evidence to claim possible interactions between these two proteins.

9. **Determination of the molecular mass of PH: MfphF complex:**

After establishing possible interactions between PH and MfphF and determining the apparent activity towards methyl parathion and *meta*-fission products an attempt was made to determine the size of the complex. The native molecular weight of MfphF and Parathion hydrolase complex was determined by gel filtration chromatography using Sephacryl-300 HR (Sigma) as described in materials and methods. The column void volume (V₀) and the elution volumes of standard proteins as well as the pure complex were shown in Table 4.2. The Kₘ value was calculated as described in material and methods and a graph was plotted by taking the Kₘ values on x-axis and molecular weights of standard proteins on y-axis (Fig 4.9). A straight line was drawn with best fit the points on the graph. The molecular weight of PH:MfphF complex was calculated using the graph. The Kₘ value of the complex touched the line on y-axis corresponding the size of ~150 kDa.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Protein fraction</th>
<th>μ mole of HOHD/min/mg of protein</th>
<th>μ mole of PNP formed/min/mg of protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MfphF</td>
<td>23 (± 0.23)</td>
<td>ND</td>
<td>This work</td>
</tr>
<tr>
<td>2.</td>
<td>Parathion hydrolase</td>
<td>ND</td>
<td>24.4 (± 6.9)</td>
<td>Brown et al., 1980</td>
</tr>
<tr>
<td>3.</td>
<td>Complex</td>
<td>10 (± 0.45)</td>
<td>11 (± 0.92)</td>
<td>This work</td>
</tr>
</tbody>
</table>

ND=Not Detected.

Table 4.1. Comparison of specific activities of Parathion Hydrolase and MfphF with PH:MfphF complex.
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the Protein</th>
<th>Mw (kDa)</th>
<th>Ve (ml)</th>
<th>Kav</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Amylase</td>
<td>200</td>
<td>20</td>
<td>0.1379</td>
</tr>
<tr>
<td>2.</td>
<td>Alcohol dehydrogenase</td>
<td>150</td>
<td>26</td>
<td>0.3448</td>
</tr>
<tr>
<td>3.</td>
<td>Galactosidase</td>
<td>116</td>
<td>32</td>
<td>0.54</td>
</tr>
<tr>
<td>4.</td>
<td>Albumin</td>
<td>67</td>
<td>39</td>
<td>0.76</td>
</tr>
<tr>
<td>5.</td>
<td>PH:MfphF complex</td>
<td>?</td>
<td>25</td>
<td>0.319</td>
</tr>
</tbody>
</table>

Table 4.2. Elution volume and $K_{av}$ values of standard proteins and PH:MfphF complex.
Fig. 4.9. Determination of native molecular weight of PH:MfphF Complex. The $K_{av}$ value of PH:MfphF complex is indicated with a square (□) mark. Similar values of molecular weight standards are shown with circles (•).
Discussion:

Metabolic engineering is considered as a byproduct of recombinant DNA technology. Through recombinant DNA technology it is possible to clone genes from any source and these cloned genes can be used to transform microbes, plants and animals to create novel metabolic pathways leading to the production of novel products useful to the mankind. In the field of environmental biotechnology several incidents of degrading the recalcitrant molecules from the environment have been reported through metabolic engineering (Shimazu et al., 2001; Keneva et al., 1998; Richins et al., 1997; Timmis et al., 1994; Mulchandani and Chen, 2005). The present study describes the genetics of op-pesticide degradation in *Flavobacterium* sp. ATCC27551 isolated from the agricultural soils (Sethunathan and Yoshida, 1973). The op-pesticide degrading gene (opd) encodes for a tri-esterase which hydrolases the triester linkage found in parathion and methyl parathion. The *Flavobacterium* sp. ATCC27551, from where the plasmid pPDL2 borne opd gene was cloned, parathion and methyl parathion is degraded through a process of co-metabolism and it does not have the capability of using either of these neurotoxicants as source of carbon. Further more the p-nitrophenol (PNP), the hydrolytic product of methyl parathion or parathion considered to be the potential toxicant for microbes is not degraded. These two aspects are considered to be the negative points if *Flavobacterium* sp. ATCC27551 has to be used in bioremediation operation. The present study clearly demonstrates the potential of MfphF in degradation of PNP under in vivo conditions. Therefore, the author has constructed an expression plasmid (pSM16) by bringing the opd gene and mfphF under the control of lacUV5 promoter. In native *Flavobacterium* sp. these two genes have opposite transcription orientation. The opd promoter is not
recognized by the transcriptional machinery of *E.coli* (Mulbry and Karns, 1989). Therefore, the *opd* expression in heterologous hosts especially in *E.coli* using its indigenous promoter is not possible. Similarly there is absolutely no information about the transcription of *mfphF*. The information gathered through nucleotide sequence can only suggest that the *mfphF* is translationally coupled to *tnpA* gene of transposon Tn3. Under such conditions it is rather difficult task to eliminate toxic residues of op-compounds using wild type *Flavobacterium* sp. ATCC27551 despite of having genetic information capable of degrading these toxic molecules. In the present chapter the author constructed an expression plasmid by bringing them into same transcriptional orientation in plasmid pSM16. These two genes were further kept under the control of an inducible *lacUV5* promoter. If the structure of the construct is examined these two genes appear as part of an operon being transcribed from a *lacUV5* promoter. This artificially constructed *opd*-operon found in the broad host range expression plasmid pMMB206 has successfully expressed parathion hydrolase and MfphF both in *E.coli* and *Pseudomonas aeruginosa* PAO1161. The *Pseudomonas aeruginosa* PAO1161 (pSM16) has shown the ability to degrade both methyl parathion and its degradation product PNP. The results presented in the Fig.4.7 stands as testimony to show that the engineered *Pseudomonas aeruginosa* PAO1161 is capable of degrading both methyl parathion and PNP. In the previous chapters presented in this study it is notices that the MfphF has shown more stability in presence of PH. Infact this was the observation which prompted to undertake this study leading to the construction of ‘*opd*-operon’ to actually use these constructs for studying the possible interactions between these two proteins. As discussed before engineered strains of *Pseudomonas aeruginosa* PAO1161 (pSM16) has shown the ability
to degraded both methyl parathion and PNP. It is rather clear indication to show that these two proteins encoded by plasmid pSM16 are active in *Pseudomonas aeruginosa* PAO1161. Therefore this expression system was also used to study protein-protein interactions. The MfphF when independently expressed bound to the nickel column and was successfully purified by affinity chromatography. However, when MfphF is expressed with PH, it has failed to bind the nickel column. Hence the conventional protein purification techniques such as ammonium sulphate fractionation and gel filtration techniques were used to show if the fractions that showed MfphF activity possessed PH activity as well. As expected among the fractions collected only very few of them showed MfphF activity. Interestingly the fractions that were tested positive for MfphF activity were also found to be positive for PH activity. These active fractions when analyzed on SDS-PAGE have showed the protein bands corresponding to the size of PH and MfphF (Fig.4.8). This is rather direct evidence which shows that these two functionally related proteins form a complex. Further studies describing the molecular mass of the complex clearly suggest that these two proteins probably exist in equimolar concentrations in the complex. Since the size of the complex, as determined by gel-filtration chromatography, is \(~150\) kDa then it appears that the complex is made by taking two molecules from each protein. The protein content present in these two bands supports this prediction (Fig.4.8). The apparent specific activity of the complex when compared with the specific activities of the pure MfphF is exactly half when HOHD is used as an assay substrate (Table.4.1). This data rather serves as supporting evidence to claim that these two proteins form complex and are in equimolar concentration.
Enzymes are considered as catalytic units of the living organism. They perform a specific task and play a major role in virtually every aspect of cell function. Evolution of enzymes for the degradation of various xenobiotic compounds imparts an advantage for microorganisms to thrive hostile environments. The toxicity of xenobiotic compounds to humans led the identification and characterization of various microorganisms for safe removal of these toxic substances. However, to match the exceeding concentration of pollutant's it has become evident to generate more potent organisms with high degradable abilities. The one possibility is to equip the organisms by genetic manipulation of enzymes with enhance substrate specificity and the association of enzymes into multifunctional enzymes (Timmis et al., 1994). Association of enzymes has an advantage of efficient substrate channeling in the crowded milieu of cell.

Catabolic pathways consist of multiple enzymatic reactions; for each reaction, the relevant enzyme must bind the corresponding intermediate-its substrate-and convert it to the next intermediate-its product. The release of a reaction product into the cellular milieu by one enzyme results in its dilution, which can only be partly offset by a low \( K_m \) of the next enzyme in the pathway. Circumvention of the dilution of intermediates, accompanied by an improved substrate flux through the pathway, might be achieved by physically linking enzymes acting in sequence, so that intermediates are passed more or less directly from one active center to the next. The two component monooxygenases and dioxygenase and three-component dioxygenases in the biodegradation of aromatic compounds were well known examples of multifunctional enzymes (Vandermeet et al., 1992). The two-component monooxygenases of \( p \)-nitrophenol degradation perform both reduction and oxidation reaction (Kadiyala and Spain, 1998; Kitagawa et al., 2004).
Three component-dioxygenases involved in the addition of two adjacent hydroxyl group on the aromatic ring of (chloro) benzene, toluene, naphthalene, (chloro) biphenyl, and (chloro) benzoates were characterized from various bacterial strains (Ensley et al., 1983; Furukawa and Miyazaki, 1986; Gibson and Subramanian, 1984; Harayama et al., 1986; Irie et al., 1987; Mondello et al., 1989; Neidle et al., 1989; Stephens et al., 1989; Vandermeer et al., 1991). The enzyme complex is generally formed from three different components, a terminal oxygenase (also called iron-sulfur-protein or hydroxylase protein) which consists of two different subunits (alpha and beta), a ferredoxin, and an NADH-ferredoxin reductase (Gibson et al., 1990). In the light of the above-cited literature, considering the unique organization of \textit{opd} gene and \textit{mfphF (orf243)} the author attempts to propose that Parathion hydrolase and MfphF form a multi enzyme complex to mineralize organophosphates like parathion and methyl parathion. The present study has clearly demonstrated the role of these two enzymes in degradation of organophosphates especially parathion and methyl parathion besides leaving critical questions unsolved especially pertaining to the involvement of MfphF in degradation of \textit{p}-nitrophenol.