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In silico Approaches for the detection of organophosphate pesticides by OPH-Enzyme based biosensors

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Abstract
Among the various groups of pesticides that are being used world over, *p*-Nitro phenyl (*p*NP)-substituent Organophosphates (OPs) are acutely toxic and more extensively used group of pesticides. Organophosphorus hydrolase (OPH) is an enzyme involved in the hydrolysis of aromatic group of OPs pesticides. Thus, OPH is an extremely attractive biological component used in the biosensors for the detection of *p*NP substituent OPs concentration. The difference in the structural organisation of the OPH enzyme from different microbe will influences the catalytic activity of these enzymes. So, the rationale behind choosing specific microbial OPH enzyme as a biocomponent in the biosensors for the detection of *p*NP substituent OPs is not yet reported. The current study has been performed to predict the biocomponent based on sequence and structural analysis as well as modelling and docking studies of OPH enzymes. The correlation of binding energy with experimentally determined binding affinities is used for the validation of the docking approach. Based on the current *in silico* docking study, two microbial OPH enzymes from two different species are predicted to show high specificity for *p*NP substituent OPs group of pesticides. Thus, it may be recommended to use them as a biocomponent for the biosensors after experimentally validating them through *in vitro* and *in vivo* analysis.

Keywords: biocomponent, docking approach

Introduction
Pesticides are indispensable to modern agriculture. Pesticides are a major group of environmental pollutants (Kanekar, 2004) [18]. The estimated annual application of pesticides is more than 4 million tons, but only 1% of this reaches the target pests (Gavrilescu, 2005) [12]. In India, consumption of pesticides is increasing at the rate of 2 to 5% per annum. Currently among the various groups of pesticides that are being used world over, organophosphates (OPs) form a major and most widely used group accounting for more than 36% of the total world market (Aprea., 2000; Barr., 2004; Curl., 2003) [3, 4, 9]. In India, organophosphates are the highest consumed pesticide and it constitutes about 50% of the overall pesticide consumption. The World Health Organization estimates that every year 3 million people experience acute poisoning by OPs. (WHO, 1990). The most widely used group of Organophosphorus (OPs) pesticides are Organophosphorus aromatic nitro pesticides which includes parathion and methyl parathion (Thomson, 1976; Willoughby and Morgan, 1982; Stolyarov, 1998, Peter *et al*., 2010) [27, 29, 25, 22]. The World Health Organization (WHO) classified methyl parathion as extremely and highly hazardous compound (Peter, 2010) [22]. In India, acute poisoning and death by Organophosphorous (OPs) pesticides have reached alarming epidemic proportions due to the continuous usage of all three WHO hazard classes of organophosphates (OPs) by the farming community.
The toxicity of *p*-Nitro phenyl (*p*NP)-substituent Organophosphates (OPs) involves irreversible inhibition of the enzyme acetyl cholinesterase (AChE), causing neurotoxicity and other potent health hazards towards unintended target such as human beings and wild-life. OP cholinesterase inhibitors block the function of acetylcholinesterase, causing the accumulation of excessive acetylcholine in the synaptic cleft. This causes neurotoxic effects such as neuromuscular paralysis (i.e. continuous muscle contraction) throughout the entire body. The adverse short-term effects of exposure to OPs have been studied mostly in the nervous system, which is their primary target (Gupta at al., 2001) [14].

Chronic occupational exposure to OPs have been linked to increased risk for cancer development such as non-Hodgkin lymphoma (Waddell *et al*., 2001) [28] and some types of leukaemia (Brown *et al*., 1990) [6].

Detection of the *p*NP substituent OPs pesticide residue levels in Indian soil is essential, as these pesticides have been reported to be present in levels far beyond the levels permitted. Organophosphorous hydrolase (OPH) enzyme (E.C. 3.1.8.1) is known to be capable of hydrolyzing the phosphotriester P-O bond of *p*NP substituent Ops pesticides at higher rates. The successful use of currently researched technologies for detoxification of organophosphate will require sensors for monitoring and control of the process (Mulchandani *et al*., 1998) [21]. The very first step to control the "environmental pollution" is "environmental monitoring" *ie*., to detect the concentration of pollutant in the polluted environment. The tolerance limit for total pesticide is 0.5g/L (Buonasera *et al*., 2009) [7].

Cholinesterases and Phosphotriesterases are the two OP mitigation enzymes. Cholinesterases are generally non-catalytic and the limitations for biosensors based on cholinesterases are unstable due to incomplete regeneration of the enzyme activity as a result of strong irreversible binding of certain inhibitors (Rosa *et al.,* 1994; Marty *et al*., 1992; Dzyadevich *et al*., 1994) [24, 20, 10]. Phosphotriesterases are categorized based on their bond type hydrolysis. The phosphotriesterases are given the E.C. 3.1.8 designation and are further divided as Aryl dialkyl phosphatases (or) Organophosphorous hydrolase (OPH) (E.C. 3.1.8.1) and DF Pase or Organophosphorous Acid Anhydrolase OPAA) (E.C. 3.1.8.2). Although, OPAA is well documented for its ability to hydrolyze the P-F bonds of Diisppropylflurophosphate, sarin and soman, it has slow P-O or P-CN and no P-S, hydrolysis activity (Cheng *et al*., 1996) [8]. However, OPH is known to be capable of hydrolyzing the phosphotriester P-O bond of Organophosphorous (OPs) aromatic pesticides such as paraoxon (Harel *et al*., 2004, Mackness *et al*., 1997 and Teiber *et al*., 2004) [15, 19]. Thus, Organophosphorus Hydrolase (OPH, E.C. 3.1.8.1) is extremely attractive for biosensing of Organophosphorous (OPs) nitro aromatic pesticides (Rainina *et al*., 1996 and Mulchandani *et al*., 1998) [23, 21].

OPH from the above listed organism is a homodimer and an $(\alpha\beta)_8$ member of the TIM Barrel-fold family of hydrolases. A carboxylated lysine and four histidine residues which coordinate the two divalent cations are required in the active-centre, for catalytic activity. The metals in turn coordinate an activated hydroxyl that functions as the nucleophile in the OP hydrolysis reaction. The activity and substrate specificity of the OPH enzymes differ between different microbial cells due to sequence and structural differences between them (Yang *et* al., 2003) [30]. Hence the present study has been designed to screen the microbial OPH enzyme that shows high specificity for *p*NP substituent OPs group of pesticide using molecular docking studies, which could be an ideal choice of biocomponent for biosensors. Also, to analyse the docked conformation for the identification of functionally critical residues which support the binding of *p*NP substituent OPs with OPH in the energetically favorable conformation.

Materials and Methods

OPH proteins (E C.3.1.8.1) were analysed by finding their sequence homologs from microbial proteins employing BLASTP search and generated a non-redundant dataset. A multiple sequence alignment (MSA) was constructed from the said dataset using ClustalW and percent sequence identity was calculated for all the entries. Based on the percent identity, distantly related set of microbial OPH proteins were chosen as the OPH - representatives. The conservation of active site residues were examined on the multiple sequence alignment of representative proteins. The structural domain for the representatives was retrieved from PDB (Berman *et al*., 2000) [5]. The domain information was analysed using pfam, Fold and superfamily information was retrieved using SCOP database (Andreeva *et al*., 2020) [2].

The correlation of binding energy with experimentally determined binding affinities was performed to overcome the inherent limitations in reproducing accurate docking poses from computer algorithms and the heuristic approximations (such as rigid ligand docking and the absence of lipids and solvents), this correlation was merely to obtain confidence in our docking protocol. The experimentally determined binding affinity of paraoxon with each microbial OPH protein representatives was obtained from the literature (Hawwa *et al*., 2009, Yang *et al*., 2003, Elias *et al*., 2008) [16, 11, 30]. The binding energy for paraoxon with each microbial OPH protein representatives was obtained using autodock 4.2, was plotted along with the experimentally determined binding affinity and the correlation factor (correlation coefficient) for the two different datasets ie., binding energy (Kcal/mol) and binding affinity (mM) was calculated and analysed using scatter plot.

The crystal structure of the six different microbial OPH representatives (PDB entry code 1PSC, 2D2G, 2ZC1, 3F4D, 2VC5 and 1P9E) was retrieved from the protein data bank (PDB) (Table 1).

Table 1: List of representative proteins from various microbial species

S. No.	PDB-ID	Species source
1.	1PSC	*Pseudomonas diminuta (Pd*OPH*)*
2.	2D2G	*Agrobacterium radiobacter* (*Ar*OPH)
3.	2VC5	*Sulfolobus sulfataricus (Ss*OPH*)*
4.	2ZC1	*Deinococcus radiodurans (Dr*OPH*)*
5.	3F4D	*Geobaciluus stearothermophilus* strain 10 (*Gs*OPH)
6.	1P9E	*Pseudomonas* sp. WBC3 (*P* sp. WBC3OPH)

Five of the ligand molecules chosen were documented in the NCBI – Pub Chem Compound database and two of them at Chemspider database (Table 2).

Table 2: List of ligand molecules and their IDs**.** Listed molecules from 1-5 were chosen from NCBI-Pubchem and 6-7 from Chemspider database

S. No.	Molecule name	Molecule ID
1.	Paraoxon	CID 9395
2.	Parathion	CID 991
3.	Methyl paraoxon	CID 13708
4.	Methyl parathion	CID 4130
5.	Nitrophenylhalon	CID 67554
6.	Bisparaoxon	CSID 12902
7.	Bisparathion	CSID 22560

Canonical smiles of all the seven ligands were retrieved from the corresponding chemical databases, in which they are documented. The canonical smiles was then accessed in CORINA server (Sadowski *et al*., 2003) [17], which is used to generate the 3D – chemical structure for docking and saved in pdb format. The resulting ligand structure was optimised in PRODRG2 server (Schuttelkopf and Aalten, 2004) [1]. The receptor and ligands were pre-processed, grid was generated around the active site and docking was performed as per the standard procedure.

Results and Discussion

The sequence analysis of the PTE domain and Beta lactamase domain possesses microbial OPH protein (E.C.3.1.8.1) yields one beta lactamase possesses OPH protein from *Pseudomonas* sp. WBC3 and five distantly related PTE domain possessing OPH proteins from other species as the microbial OPH protein representatives. Presence of these domains was verified through SCOP domain definition. The active site residue annotation of PTE domain possessing microbial OPH protein representatives performed using ClustalW, revealed that the metal coordinating residues were conserved but the substrate binding pocket residues were non-conserved in all the representatives. Non – conserved substrate binding pocket residues includes the active site residues which were reported to be involved in the stabilization of the metal co-ordinating residues, proton – relay mechanism and hydrogen bonding with the substrate

```
1PSC            MQTRRVVLKSAAAAGTLLGGLAGCASVAGSIGTGDRINTVRGPITISEAGFTLTHEHICG 60
2D2G            --------------------------------TGDLINTVRGPIPVSEAGFTLTHEHICG 28
2VC5            ---------------------------------MRIPLVGKDSIESKDIGFTLIHEHLRV 27
2ZC1            ------------------------SSGLVPRGSHMTAQTVTGAVAAAQLGATLPHEHVIF 36
3F4D            --------------------------------MAKTVETVLGPVPVEQLGKTLIHEHFLF 28
                                                      .  ..:   : * ** ***.

1PSC            SSAGFLRAWPEFFGSRKALAEKAVRGLRRARAAGVRTIVDVSTFDIGRDVSLLAEVSRAA 120
2D2G            SSAGFLRAWPEFFGSRKALAEKAVRGLRHARAAGVQTIVDVSTFDIGRDVRLLAEVSRAA 88
2VC5            FSEAVRQQWPHLYNEDEEFRN-AVNEVKRAMQFGVKTIVDPTVMGLGRDIRFMEKVVKAT 86
2ZC1            GYPGYAGDVTLGPFDHAAALASCTETARALLARGIQTVVDATPNDCGRNPAFLREVSEAT 96
3F4D            GYPGFQGDVTRGTFREDEALRVAVEAAEKMKRHGIQTVVDPTPNDCGRNPAFLRRVAEET 88
                   .     .            ...  .     *::*:** :  . **:  :: .* . :

1PSC            DVHIVAATG--LWFDPP--------LSMRLRSVEELTQFFLREIQYGIEDTGIRAGIIKV 170
2D2G            DVHIVAATG--LWFDPP--------LSMRMRSVEELTQFFLREIQHGIEDTGIRAGIIKV 138
2VC5            GINLVAGTGIYIYIDLP--------FYFLNRSIDEIADLFIHDIKEGIQGTLNKAGFVKI 138
2ZC1            GLQILCATG--FYYEGEGATTYFKFRASLGDAESEIYEMMRTEVTEGIAGTGIRAGVIKL 154
3F4D            GLNIICATG--YYYEGEGAPPYFQFRRLLGTAEDDIYDMFMAELTEGIADTGIKAGVIKL 146
                .::::..**   : :                : .:: :::  ::  ** .*  :**.:*:

1PSC            ATTG-KATPFQELVLKAAARASLATGVPVTTHTAASQRDGEQQAAIFESEGLSPSRVCIG 229
2D2G            ATTG-KATPFQELVLKAAARASLATGVPVTTHTSASQRDGEQQAAIFESEGLSPSRVCIG 197
2VC5            AADEPGITKDVEKVIRAAAIANKETKVPIITHSNAHNNTGLEQQRILTEEGVDPGKILIG 198
2ZC1            ASSRDAITPYEQLFFRAAARVQRETGVPIITHTQEGQQ-GPQQAELLTSLGADPARIMIG 213
3F4D            ASSKGRITEYEKMFFRAAARAQKETGAVIITHTQEGTM-GPEQAAYLLEHGADPKKIVIG 205
                *:     *   : .::*** ..  * . : **:      * :*   : . * .* :: **

1PSC            HSDDTDDLSYLTALAARGYLIGLDHIPHSAIGLEDNASASALLGIRSWQTRALLIKALID 289
2D2G            HSDDTDDLSYLTGLAARGYLVGLDRMPYSAIGLEGNASALALFGTRSWQTRALLIKALID 257
2VC5            HLGDTDNIDYIKKIADKGSFIGLDRYGLDLFLPVDKRNETTLR--------------LIK 244
2ZC1            HMDGNTDPAYHRETLRHGVSIAFDRIGLQGMVGTPTDAERLSV-----------LTTLLG 262
3F4D            HMCGNTDPDYHRKTLAYGVYIAFDRFGIQGMVGAPTDEERVRT-----------LLALLR 254
                *  .. :  *       *  :.:*:   . :    .                     *:

1PSC            QGYMKQILVSNDWLFGFS-SYVTNIMDVMDRVNPDGMAFIPLRVIPFLREKGVPQETLAG 348
2D2G            RGYKDRILVSHDWLFGFS-SYVTNIMDVMDRINPDGMAFVPLRVIPFLREKGVPPETLAG 316
2VC5            DGYSDKIMISHDYCCTID-WGTAKPEYKPKLAPRWSITLIFEDTIPFLKRNGVNEEVIAT 303
2ZC1            EGYADRLLLSHDSIWHWLGRPPAIPEAALPAVKDWHPLHISDDILPDLRRRGITEEQVGQ 322
3F4D            DGYEKQIMLSHDTVNVWLGRPFTLPEPFAEMMKNWHVEHLFVNIIPALKNEGIRDEVLEQ 314
                 ** .::::*:*          :                :    :* *:..*:  * :

1PSC            ITVTNPARFLSPTLRAS- 365
2D2G            VTVANPARFLSPT----- 329
2VC5            IFKENPKKFFS------- 314
2ZC1            MTVGNPARLFG------- 333
3F4D            MFIGNPAALFSAHHHHHH 332
                :   **  ::.
```

Fig 1: Multiple sequence alignment of representative proteins with PTE domain

Docking of each protein representative with each of the seven ligands, results in the generation of 4200 set of docked conformations The best docked pose for each of the representative protein verses each ligand molecule was chosen based on the literature supported molecular interactions. Based on the analysis of docked conformation of OPH enzymes with ligand molecules, SsOPH protein found to have high binding affinity for all the seven ligands. The list of residues found to be involved in final docked- conformation (pose) of *Ss*OPH (Table 4.5), it was inferred that the Tyr97 residue was found to be oriented towards the leaving group of the ligands. In *Pd*OPH, Trp131 & Phe132 was reported to be "second shell" residue. Phe132, has been referred to as a critical residue in the "gateway" into the active site of wild-type OPH Phe132 is positioned within the leaving group subsite and forms stacking interactions with Trp131 (Goodspeed *et al.*, 2001) [13] which supports the catalysis of OPH. Tyr97 and Ile98 residue of *Ss*OPH was replaced by Trp131 & Phe132 residues in *Pd*OPH and *Ar*OPH, whereas in case of *Dr*OPH and *Gs*OPH, Tyr97 of the *Ss*OPH is conserved but the Ile98 is replaced by Tyr residues. In *P*sp.WBC3OPH, F119 and F196 was reported to be positioned ideally to anchor the leaving group of the ligands (Yang *et al.*, 2003) [30].

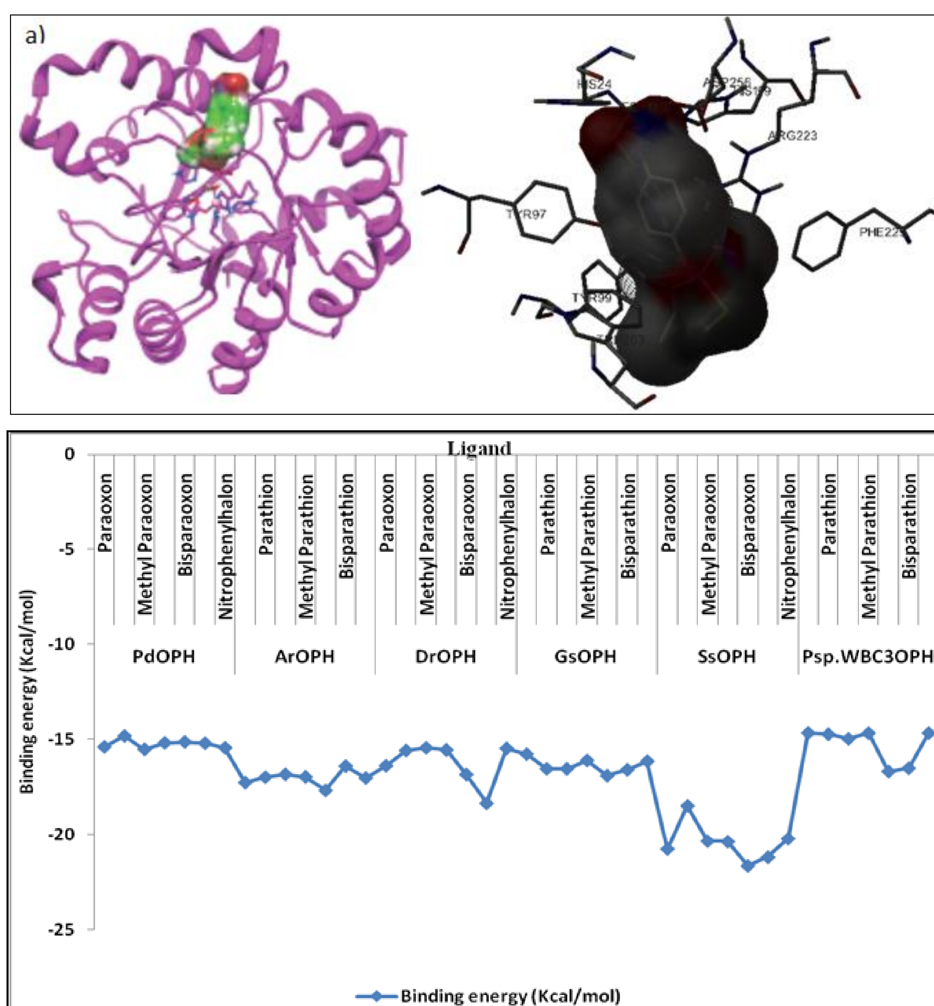

Fig 2: Docked pose and binding energy chart of all docked molecules. a) docked conformation of parathion with *Ss*OPH.b) Binding energy of all ligand docked molecules.

In OPH of $(\alpha\beta)_8$ member of the TIM Barrel-fold family of hydrolases, functionally important residues, like Histidines at positions 254 and 257, were found to support catalysis by participating in an extensive series of interaction with other residues within the active site. These interactions sustain and/or support the OPH-catalysed reaction and indirectly define the structural characteristics affecting the substrate-binding site. In addition to H254 and H257, another "second shell" residue, Phe132, has been referred to as a critical residue in the "gateway" into the active site of wild-type OPH Phe132 is positioned within the leaving group subsite (Table 3) and forms stacking interactions with Trp131 (Goodspeed *et al.*, 2001) [13]. In OPH from *Pseudomonas* sp. WBC-3, functionally important residues like Phe119 and Phe196 are found to be ideally placed to anchor the phenyl group of methyl parathion (Yang *et* al., 2003). [30]

Table 3: Representative proteins and their corresponding residues leaving group subsite.

S.no	Protein	Residues – leaving group subsite
1.	*Pd*OPH	W131 & F132
2.	*Ar*OPH	W131 & F132
3.	*Ss*OPH	Y97 & Y98
4	*Dr*OPH	Y99 & Y100
5.	*Gs*OPH	Y98 & I99
6.	*P* sp.BC3OPH	F119 & F196

In all OPH proteins, except *S*sOPH, both of the second shell residues are found to be bulky (aromatic) - hydrophobic residue, whereas in *S*sOPH one of the second shell residue is

found to non-bulky (aliphatic) hydrophobic residue. It may be possible that the presence of bulky residues in the active site may not give a sufficient three dimensional space for the substrate to bind with the receptor in a energetically favourable way. Thus, *Ss*OPH protein with non-bulky residue in the leaving group subsite shows high binding affinity for the ligands and it might be recommended as the microbial OPH protein which shows high substrate specificities for nitro aromatic group of OPs pesticides.

The binding energy obtained for paraoxon with each microbial OPH protein representatives was found to correlate positively with the experimentally determined binding affinities. The plots show a linear trend with the correlation coefficient of 0.4129 (Table 4 & Figure 3), which is used for the validation of the docking approach.

Table 4: Binding energy of paraoxon compared with binding affinity.

S. No	Protein	Substrate	Km value (mM) – (Reference - Review of Litt.)	Binding energy (Kcal/mol) – Docking studies
1	*Pseudomonas diminuta* – OPH (*Pd*OPH)	Paraoxon	0.078±0.008	-15.40
2	*Agrobacterium radiobacter* - OPH (*Ar*OPH)	Paraoxon	0.190±0.01	-17.27
3	*Deinococcus radiodurans* – OPH (*Dr*OPH)	Paraoxon	3±0.2	-16.39
4	*Geobacillus stearothermophilus* strain 10 - OPH (*Gs*OPH)	Paraoxon	2.1±0.32	-15.78
5	*Sulfolobus solfataricus* – OPH (*Ss*OPH)	Paraoxon	0.060±0.009	-20.77

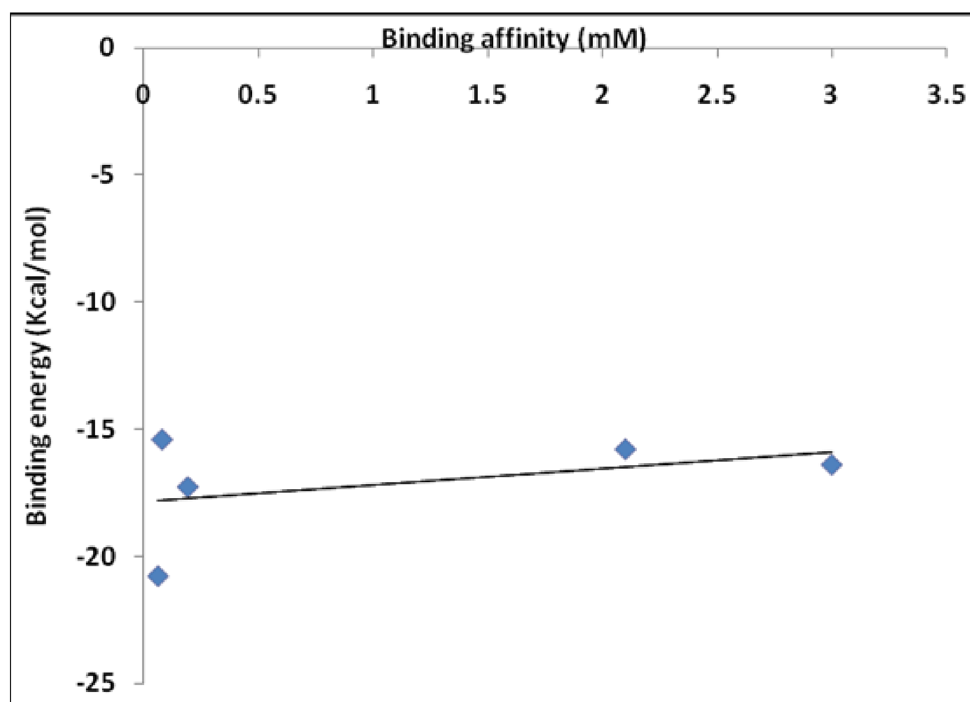

Fig 3: Binding energy of paraoxon compared with binding affinity (experimental value).

Conclusion

Using sequence analysis, five set of PTE domain possessing microbial OPH proteins (*Pd*OPH, *Ar*OPH, *Dr*OPH, *Gs*OPH and *Ss*OPH) and 1 β–lactamase domain (*P*sp.WBC3OPH) possessing microbial OPH protein were identified. Sequence analysis revealed that the metal coordinating residues were conserved but the substrate binding pocket residues were non-conserved in all the representatives. *In silico* analysis reveal that OPH protein from *Sulfolobus sulfataricus* (*Ss*OPH) was identified to have low binding energy followed by OPH from *Agrobacterium radiobacter* for the ligands chosen in our study. Thus, it may be recommended to use *Ss*OPH as a biocomponent for the biosensors used in the detection of OPs aromatic nitro group of pesticides. However, the results of the present study have to be validated at the wet lab using strains of sulfolobus sulfatricus. It is also important to establish the enzyme kinetics of the OPH enzyme of the strain before exploring its potential use as a biocomponent for biosensors that could detect organophosphorus aromatic nitro pesticides with high specificity.

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