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In silico modeling, docking of ThPON1-like protein, and *in vitro* validation of pesticide tolerance in *Trichoderma harzianum*

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ABSTRACT

Overuse of pesticides in agriculture has extremely detrimental effects on the environment and various life forms. Agricultural soils containing residual pesticides can be decontaminated economically and effectively using microbes that produce pesticide degrading enzymes. Before onsite bioremediation, *in silico* studies can identify the potential microbes that can degrade pesticides. This study aims to identify the organophosphate (OP) pesticide degrading potential of beneficial fungi *Trichoderma harzianum* using *in silico* tools. Among various types of OP hydrolyzing enzymes (PTE/PON1/SsoPox), only a *Trichoderma atroviride* paraoxonase 1 like (TaPON1-like) enzyme was found in *T. harzianum*. Pfam domain searches revealed an arylesterase domain that has a role in OP degradation. Phylogenetic analyses and multiple alignments of the sequences of various OP hydrolyzing enzymes revealed their diversity. Homology modeling of TaPON1-like protein in *T. harzianum* was done using MODELER 10. Model evaluation and validation led to the choice of the best model with the lowest DOPE score. Modeled *T. harzianum* paraoxonase 1 like protein was docked with six selected hazardous OP pesticides/intermediate. This study helped to identify the OP pesticide degrading enzyme in *T. harzianum*. *In vitro* and *in silico* studies showed similarities, suggesting that *in silico* screening for pesticide degrading microbes might be feasible before cumbersome onsite remediation.

1. INTRODUCTION

Crop protection is increasingly reliant on pesticides to increase agricultural production. Easily accessible and effective, these chemical tools are being used for pest and disease control without much thought of their effects on the environment [1]. Unregulated use of pesticides leads to their accumulation in different ecosystems including agricultural soils [2]. The maximum residue levels of commonly used pesticides are between 0.2 parts per million (ppm) and 1.2 ppm; foods contaminated with pesticides above these levels may pose a health risk over time due to their bioaccumulation and biomagnifications [3,4]. The presence of pesticides above maximum residue limits has been reported by researchers worldwide in various foodstuffs [5-7].

Microbial bioremediation is an innovative and potentially safer and economically viable solution to clean up agricultural soils. Pesticide degrading enzymes present in these microorganisms enable them to break down the toxic pesticide molecules by cleaving their specific bonds such as P-O, P-F, P-S, and P-C bonds [8]. Based on experimental results and theoretical studies, multiple pathways have been identified for the degradation of organophosphorus pesticides in various microorganisms. These pathways involve one or the other pesticide degradative enzymes [9,10]. However, among all, organophosphate (OP) hydrolyzing enzymes are the most specific and extensively studied enzymes in terms of OP pesticide degradation [11]. Thakur *et al.*, 2019 [12] list six types of OP hydrolyzing enzymes: OPDA, PTE, DFPase, SsoPox, OPAA, and PON 1 found in different bacteria and fungi.

Thousands of pesticides or agrochemicals are manufactured and being applied in agriculture worldwide. Each of these pesticides has a different chemical structure, toxicity, and environmental fate [4,13]. Remarkable research has been undertaken and reported over recent decades for the bioremediation of various pesticides [14-17]. However, studying each of them using conventional bioremediation techniques is next to impossible. The conventional bioremediation approach is cumbersome, time-consuming, and costly. Useful insights can be derived from the available genomes and transcriptome sequences in the databases.

In the last decade, many genes and enzymes involved in pesticide degradation have been identified and their sequences are now available in the public domain [18]. Here, a newly developed *in silico* bioremediation technology can be utilized for obtaining quick, low-cost, and timely information before proceeding with onsite remediation of toxic pollutants [19]. Protein modeling, molecular docking, and

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other high throughput techniques can be employed to predict the biodegradation potential of an organism. *In silico* bioremediation can address the research gaps and predict the customized degradative enzymes for instantaneous removal of toxic pesticides [20-23]. *In silico* analysis of pesticide degradation potential of some bacterial enzymes has been done, but studies with fungi (especially *Trichoderma*) are very few. *Trichoderma*, multicomponent plant growth-promoting fungi, is a key microbial partner in achieving sustainable agriculture. Since long it is being used as a biocontrol agent and biofertilizer in agriculture [24]. In recent years, its role in the bioremediation of toxic pollutants has also been established, but there is still a lot to explore. To date, only one or two research papers describe the probable molecular mechanism of OP pesticide degradation in *Trichoderma harzianum.* In a study by Sun *et al.*, 2018 [25], a *T. atroviride* paraoxonase 1 like (TaPON1-like) protein was found in *T. atroviride* strain T23, which showed 27% query coverage and 34% sequence identity with HuPON1 protein. TaPON1-like is homologous to HuPON1, which is reported to be involved in the degradation of OP pesticides, esters, and lactones. Deletion of the TaPON1-like coding gene reduced P-O bond breakdown efficiency/OP pesticide degradation potential in *T. atroviride*.

The present study aims to investigate the mechanisms of pesticide degradation in a plant-friendly fungus *T. harzianum* using *in silico* protein modeling and molecular docking studies. The efficacies of binding of *T. harzianum* enzyme with the toxic pesticide ligands were calculated. In addition, a comparison of the results of both *in silico*, and *in vitro* seed germination studies was performed to check if the results are comparable. This study shed light on the probable molecular mechanism of OP degradation in *T. harzianum* and also demonstrates that *in silico* methods can be utilized to screen potential microorganisms before onsite bioremediation.

2. MATERIALS AND METHODS

2.1. Selection of Pesticides

Systematic literature search on OP pesticides using the online database PubMed (www.ncbi.nlm.nih.gov/pubmed) advanced search was performed using different combinations of appropriate search terms. Information on total registered pesticides, banned pesticides, and pesticide management bill 2020 were collected from government statistical databases and websites. Five-year compound annual growth rate (CAGR) (2019-2024) and market analysis reports were also considered to choose the pesticides that will be used more frequently in coming times.

2.2. Pesticide Degrading Enzyme Sequence Retrieval and Analysis

A list of OP pesticide degrading enzymes (OP hydrolases) was retrieved from various databases such as Uniprot, NCBI, BRENDA, ExPASY, KEGG, Swiss Prot, and literature survey. These enzymes were, further, searched in the *T. harzianum* genome using the NCBI blast server. The test sequence was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/). Analysis of selected sequence was done by domain search and phylogenetic analysis using Pfam domain search and MEGA software, respectively. The subcellular localization of the target protein was predicted using the Target P-2.0 server (https://services.healthtech.dtu.dk/service.php?TargetP-2.0).

2.3. Homology Modeling and Validation

T. harzianum hypothetical protein was modeled using MODELLER version 10. MODELLER is used for homology or comparative modeling of protein three-dimensional structures [26]. The query sequence was BLAST against the PDB database to get a similar structure to the query and the template was downloaded from PDB (https://www.rcsb.org/search). Once the PDB files are obtained, the most matching template was selected, and the query was aligned with the template using the python script of MODELLER. The model for the query was generated using the MODELLER python script. Phyre2 tool was used to predict and analyze protein 3D structure (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The final model was evaluated based on DOPE profiles and the model with the lowest DOPE score was selected. Model validation was done by creating and analyzing the Ramachandran plot using the Ramachandran Plot Server of Zhiping Weng's lab (https://zlab.umassmed.edu/bu/rama/).

2.4. Molecular Docking with Pesticide

Before docking the molecules, the binding site of the receptor protein was identified using an online tool called BiteNet (https://sites.skoltech.ru/imolecule/tools/bitenet/). Open babel version 2.4.1 was used for conversion of SDF file format of pesticide ligands to PDB format. Structures of the chemical compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) [27]. The docking was performed using Autodock Vina [28]. The electrostatic potential calculation, model visualization, and image generation were performed using the PyMOL software (www.pymol.org). Discovery Studio Visualizer v20.1.0.19295 was used for visualization of a docked complex, 2D, 3D interaction, and other PDB files (https://discover.3ds.com/discovery-studio-visualizer-download). UCSF Chimera, candidate version 1.15, was also used for visualization of surface view of docked complex (https://www.cgl.ucsf.edu/chimera/).

2.5. Pesticide Tolerance and Seed Germination Restoration by *T. harzianum*

T. harzianum was exposed to Dimethoate (DM) and Monocrotophos (MCP) in the range of 0 to 2100 ppm and biomass was recorded to assess its tolerance to pesticide. Host plant (*Sorghum bicolor*) seeds were treated with two pesticides, namely, DM and MCP at the concentration of 300 ppm and the germination% was recorded after 48 h of incubation in the plant growth room. All the experiments were placed in triplicates. Seed germination restoration on *T. harzianum* treatment was calculated and compared [Figure 8]. Comparison of *in silico* docking scores and *in vitro* work was done to check the similarity of results.

3. RESULTS AND DISCUSSION

3.1. Selection of Pesticides

Total 30 OP pesticides were registered in India in 1968 for various agricultural purposes such as insecticide, weedicide, nematicide, and mosquito control [29]. Out of 30, 13 pesticides are banned and ten pesticides are used for other non-agricultural purposes. Acephate, Chlorpyrifos, Chlorpyriphos-methyl, Diazinon, Dichlorvos, Fenthion, Malathion, Parathion-methyl, Phorate, Phosphamidon, Quinalphos, Triazophos, and Trichlorfon are banned [30]. This study included Ethion, Profenofos, Anilofos, DM, MCP pesticides and one of the common pesticide degradation intermediate products, Dimethyl phosphate, based on the data collected from published literature, statistical reports, pesticide management bill 2020, CAGR, and market analysis reports. In addition, all the information about the application of these selected pesticides was collected and compared [Table 1].

Structural details such as bond structure and the presence of halogens were also studied to identify their potential environmental hazard [Figure 1]. Pesticides chosen for this study are readily available in the market and are frequently used by farmers.

3.2. Pesticide Degrading Enzyme Sequence Retrieval and Analysis

Varieties of microbial enzymes have evolved in nature with the capability of degrading OP compounds [31]. OP hydrolyzing enzymes have been extensively studied for their ability to degrade toxic OP

Table 1: Current status of selected organophosphate pesticides in India.

Pesticide	Rate (Kg/ha)	Crops	Toxicity class	CAGR (2019-24)	Market availability	Production (2019-20)	Consumption (2019-20)	Environmental residues	Biodegradation
1. Monocrotophos	0.5-1.5	Cotton, Maize, potato, fruits, sugar beet *Banned in vegetables	Class IB	−8.10%	Readily available	5817 MT	552 MT	Found>MRL	*Bacillus*, *Pseudomonas*, *Streptomyces sp.Starkeya novella, Aspergillus, Macrophomina etc.*
2. Dimethoate	0.2-2	Horticultural crops, vegetables. cereals	Class II	0.16%	Readily available	1446 MT	366 MT	Found>MRL	*Paracoccus* sp., *Raoultella* sp, *Pseudomonas*, *Lactobacillus*, *Cyanobacteria*
3. Profenofos	1.7	Cotton and vegetables maize, potato, soybean, and sugar beet	Class II	11.64%	Readily available	12631 MT	426 MT	Found>MRL	*Pseudomonas putida Burkholderia Trichoderma*
4. Ethion	0.25-1	Tea, cotton, maize, cucurbits	Class III	3.31%	Readily available	2127 MT	37 MT	Found>MRL	*Pseudomonas* and *Azospirillum*
5. Anilofos	0.4	Transplanted paddy, soybean	Class II		Available	Weedicide (commonly given as weedicide)	138 MT	Comparatively less reports on above MRL range	*Rhodanobacter xiangquanii sp* (Novel)

MRL: Maximum residue limits, CAGR: Compound annual growth rate

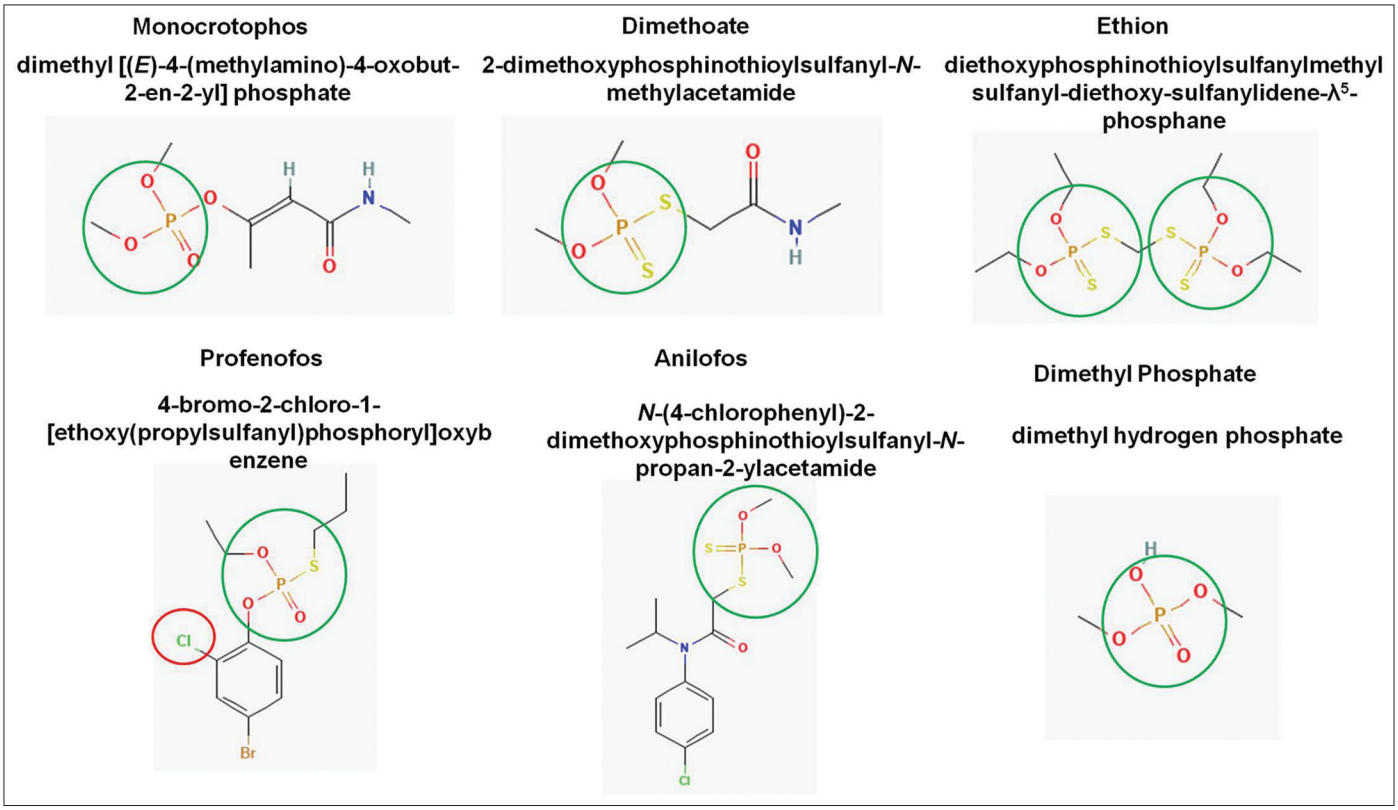


Figure 1: Chemical structure of selected organophosphate pesticides.

pesticides [12,32,33]. OP hydrolyzing enzymes can be classified into six different types, each having its structure and origin [Table 2]. Comprehensive lists of OP pesticide degrading enzymes were prepared and their sequences were retrieved from various databases such as Uniprot, NCBI, BRENDA, ExPASY, KEGG, and Swiss Prot. These enzymes were, further, searched in the *T. harzianum* genome using NCBI blast. Among OP hydrolyzing enzymes, only PON1 type (TaPON1-like) of enzyme was found in *T. harzianum* (>PNP56599.1:1-437 hypothetical protein THARTR1_03295) and showed 65.23% identity with TaPON1-like enzymes of *T. atroviride* [25]. In the Pfam database, protein sequences are categorized into domains and families. Protein domains provide insight into the function of that protein. The probable biological function of a hypothetical protein can be deduced based on its domain structure [34]. Pfam domain search analysis revealed the presence of characteristic arylesterase (paraoxonases) domain in the test sequence that has a characteristic role in OP pesticide degradation. Subcellular localization using Target P server revealed peripheral likelihood = 6.74 (at 419). Moreover, multiple sequence alignment and phylogenetic analysis of various OP hydrolyzing enzymes revealed diversity in sequences. Bacterial OP hydrolase with PTE domain was clustered together, while those of higher organism (PON1 type) with arylesterase domain were clustered separately to them. The *in silico* protein modeling work done in this manuscript has the basis from pesticide degradative protein reported as TaPON1-like in *T. atroviride* [25]. The author of the manuscript proposes nomenclature as *T. harzianum* paraoxonase 1 like (ThPON1-like) as the work was conducted on *T. harzianum*. The TaPON1-like enzyme in *T. harzianum* (ThPON1-like) was closer to the protein of higher eukaryotic organisms [Figure 2]. A family of OP hydrolyzing enzymes hydrolyze and detoxifies a wide range of toxic

Table 2: Enzymes involved in pesticide biodegradation.

Enzymes	Enzyme (kDa)	Gene	Organism	Source
1. *Alkaline phosphatase*	86 kDa	Alpl/phoA	Bacteria, Fungi	*NCBI*
2. *Carboxyl esterase*	56.5 kDa	*CE/carE1*	Bacteria, Fungi	*UNIPROT*
3. *OP hydrolyzing enzymes*				*PDB*
				KEGG
OPH	72 kDa	*opd*	Bacteria	*BRENDA*
PTE	19 kDa	*hocA*	Bacteria	
DFPase	35.21 kDa	dfpase	Sea squid	
TaPON1-like	43 kDa	*tapon1*	*T. atroviride*	
SsoPox	144 kDa	*php (S)*	*Archaea*	
	178.28 kDa	*opaa*	*Alteromonas Proteobacteria*	
4. Monooxygenase	41 kDa	*Cyp450*	Bacteria, Fungi	

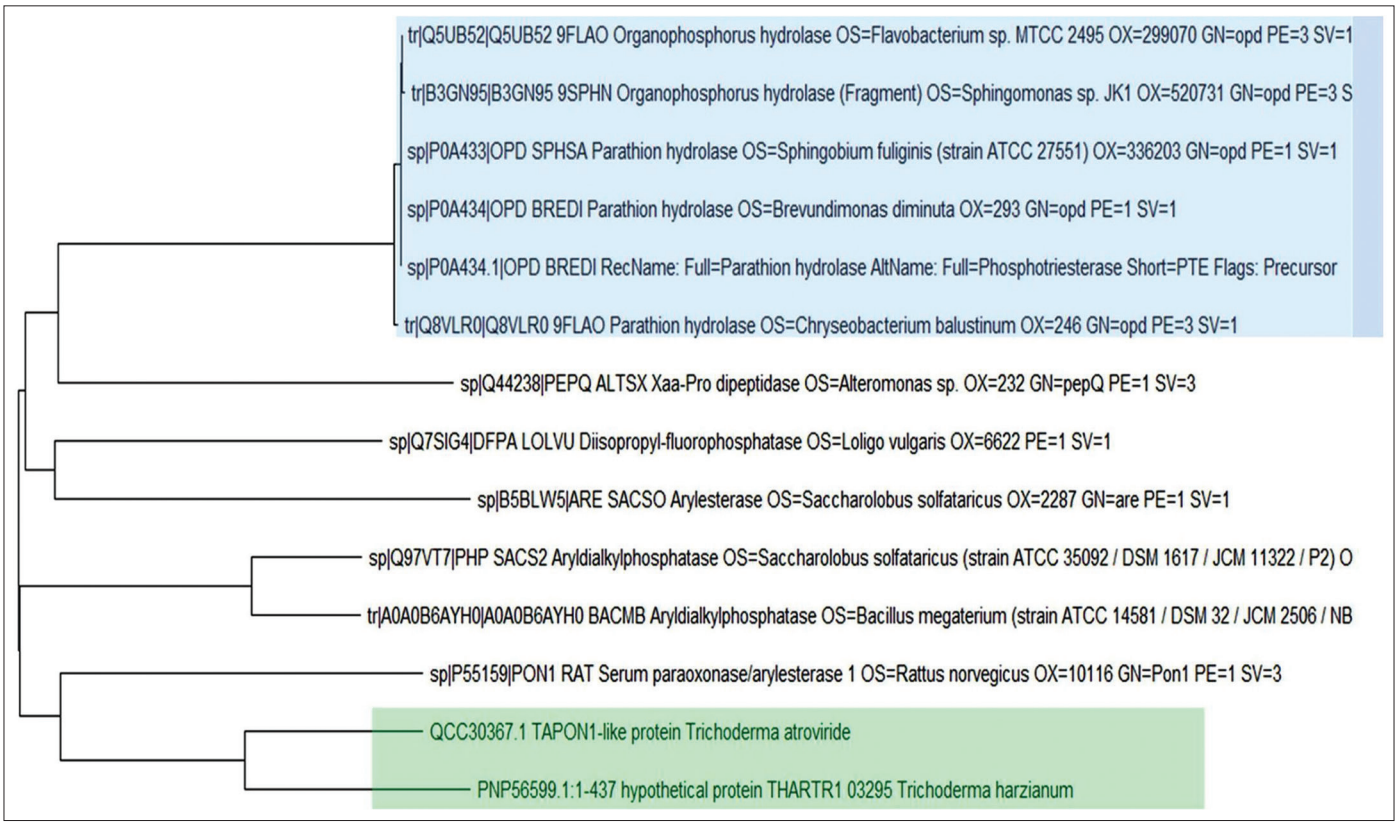


Figure 2: Phylogenetic analysis of organophosphate hydrolyzing enzymes in different species.

OP pesticides by cleaving the various phosphorus-ester bonds (P-O, P-F, P-CN, and P-S) and converts them into less toxic intermediates and end products [12,31-33]. Figure 3 depicts a general mechanism of pesticide degradation pathway by OP hydrolyzing enzymes.

3.2.1. Arylesterase domain - (NDLFAESPTSFFVTNDHYYTEGFM RAVEDLLPRATWTNVLH)

The query sequence was BLAST against the PDB database to find the closest structure. The best template was *Serum paraoxonase/ arylesterase* 1 (PDB ID: 1V04). The hypothetical protein >PNP56599 when aligned with arylesterases 1 shared 21.0% identity and 33% query coverage with 0.48E-09 E value. MODELLER guidelines suggest that even around 25% identity indicates a potential target unless it is <100 residues and this query sequence is more than 467 residues and it has 33% query coverage to the template. Dutta *et al*., 2013 [35], constructed a similar protein model for docking studies when query template identity was around 25%, but the domain responsible for the function was conserved. Protein domains are the fundamental units of proteins, which can fold, function, and evolve independently. The domains of proteins fold separately from the rest of the protein and serve a specific function. Protein domains with the same functions might be found in diverse groups of organisms [36]. In this study, also the comparative sequence alignment of query and template displayed conserved amino acids. The target sequence had maximum conserved residue in the coverage of the *arylesterase domain* that is specifically required for interaction with OP pesticide [Figure 4]. Arylesterase domain is mainly found in serum paraoxonases/arylesterases; some reports also mention their presence in *Trichoderma atroviride* [25]. The entire arylesterase domain was present both in the template and query proteins and is highly conserved. Even though there is less sequence identity between query and template sequences, a sound model was made, because the arylesterase domain was retained. As per Sun *et al*., 2019 [25], despite the low identity (34%) between PON1 and TaPON1-like, due to the presence of conserved motifs belonging to the same six-blade β-propeller hydrolase subfamily, PON1 protein is a valid template for modeling the structure of TaPON1-like. In another study, the query template identity was around 25%, but a sound model was built as the PDZ1 domain of the protease chain was conserved [35].

3.3. Homology Modeling and Validation

Homology modeling is one of the prerequisites for *in silico* bioremediation as molecular docking studies relies on the protein model and its specific three-dimensional structural properties (α-helix, beta-sheet, loop, etc.) [19]. The comparative modeling of >PNP56599-ThPON1 was performed using a restrained-based approach implemented in MODELLER10. A set of five models for the target protein was constructed. The resulting three-dimensional models of ThPON1-like were sorted according to scores calculated from the discrete optimized protein energy (DOPE) scoring function. The final model that shared the lowest Root Mean Square Deviation (RMSD), relative to the trace (Cα atoms) of the crystal structure was selected. The plotted DOPE score profile (below) shows regions of relatively high energy for the long active site loop between residues 90 and 200. Ramachandran plot is a plot to visualize energetically allowed regions for a polypeptide backbone torsion angles psi (ψ) against (phi) ϕ of amino acid residues present in a protein structure. In our model, most of the amino acids fall on the highly preferred region (>93%), 4% in preferred except for a few (2%) indicates a good model. A graphical illustration of protein's three-dimensional structure, DOPE score profile, and Ramachandran plot is given in Figure 5. Dutta *et al.*, 2013 [35], accepted the model when the amino acid in allowed region was around 82.7%.

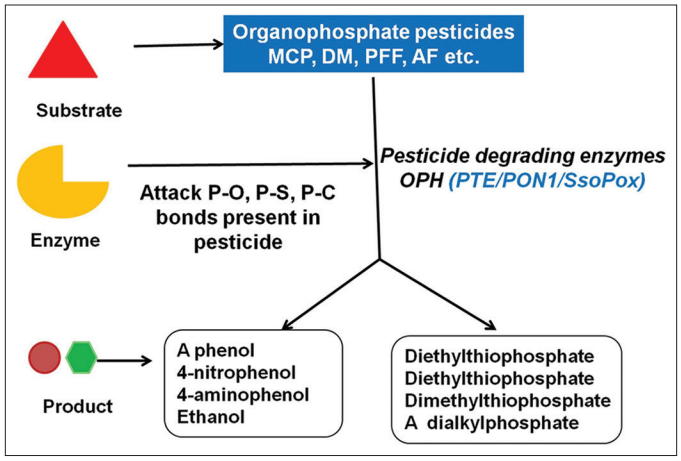


Figure 3: Molecular mechanism of organophosphate pesticide degradation.

3.4. Molecular Docking with Pesticide

Molecular docking has effectively been used over the past few years to perform the biodegradation process of environmental contaminants remediation. It is an extremely relevant and low-cost approach to understanding ligands reaction mechanisms with high accuracy of proteins or enzyme binding [37]. The purpose of docking is to produce and evaluate plausible intermolecular complex structures. Docking assay allows examining how protein molecules (enzymes) and ligands (pollutants) interact to form a stable docked complex. The compound strongly binds to the active site of the target molecule with minimum binding energy (ΔG) serves as a potential molecule. Before performing *in vitro* or on-site bioremediation, docking studies can predict the binding efficiencies of toxic pollutants with potent detoxifying enzymes present in microorganisms. Several researchers screened microbial proteins and enzymes for their ability to bind and form stable complexes with toxic pollutants using a similar approach [20,21,38,39]. The active site or binding site of particular enzymes needed to be explored for understanding its structural features and functions towards ligand binding. Before docking, the binding site of the hypothetical protein was predicted using BiteNet. The site with highest score (0.82) was chosen for the study. Molecular docking of ThPON1-like with 5 OP pesticides and 1 common degradation intermediate revealed their interaction with reasonable binding energy score [Table 3]. The binding free energy of the pesticides was in the order Anilofos (−5.8) > Profenofos (−5.6)> MCP (−5.1)> Ethion (−4.8)> Dimethylphosphate (−4.4)> DM (−4.1). Anilofos showed the best interaction and DM had the lowest interaction with ThPON1-like which is due to differences in their chemical structure, bond, and size [Figures 6 and 7].

3.5. Comparative Analysis of *In silico* and Plant-based Studies with Selected Pesticides

Some studies compared results from *in silico* and *in vitro* experiments. For instance, Aamir *et al.*, 2018 [40], have identified the fungal SDR as novel targets for fungicides. They observed similar results between *in silico* analysis and *in vitro* assessment of the fungicide on the pathogen. In a separate study, Singh *et al*., 2021 [41], investigated how carbamate pesticide intermediates affected membrane architecture in *Escherichia coli* using both *in vitro* and *in silico* techniques. In the above-

Figure 4: Sequence alignment of >PNP56599 with *Serum paraoxonase/arylesterase 1*. *Indicate the conserved amino acids; Sequence of Arylesterase domain was highly conserved.

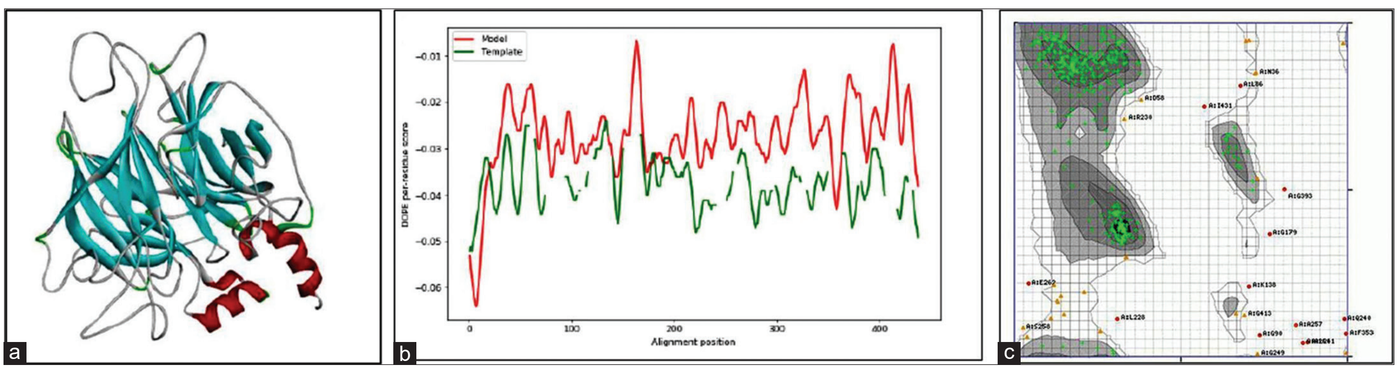


Figure 5: Protein model construction and validation (a) modeled ThPON1-like, (b) DOPE profile (c) Ramachandran Plot

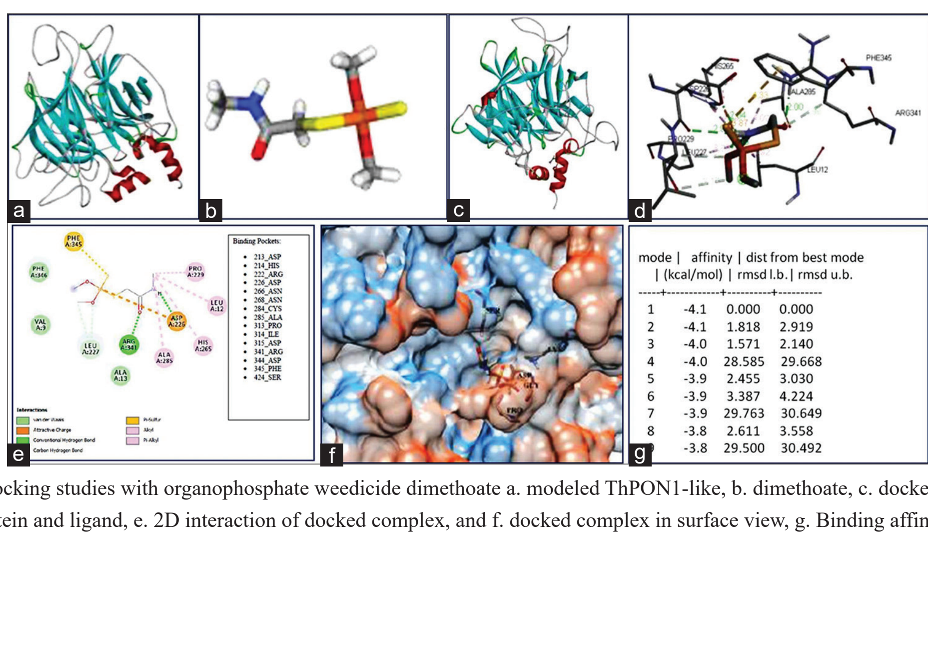


Figure 6: Molecular docking studies with organophosphate weedicide dimethoate a. modeled ThPON1-like, b. dimethoate, c. docked complex, d. interaction between protein and ligand, e. 2D interaction of docked complex, and f. docked complex in surface view, g. Binding affinity (Kcal/mol)

Table 3: Molecular docking of ThPON1-like with selected pesticides and degradation intermediates.

Pesticide	Bonds	PubChem ID	Structure	Binding energy (kcal/mol)
Monocrotophos	P-O, P=0	5371562		−5.1
Dimethoate	P-O, P-S, P=S	3082		−4.1
Profenofos	P-O, P-S, P=S-Cl, -Br	38779		−5.6
Ethion	P-O, P-S, P=S	3286		−-4.8
Anilofos	P-O, P-S, P=S	91687		−5.8
Dimethyl phosphate	P-O, P=O	13134		−4.4

ThPON1-like: *Trichoderma harzianum* paraoxonase 1 like

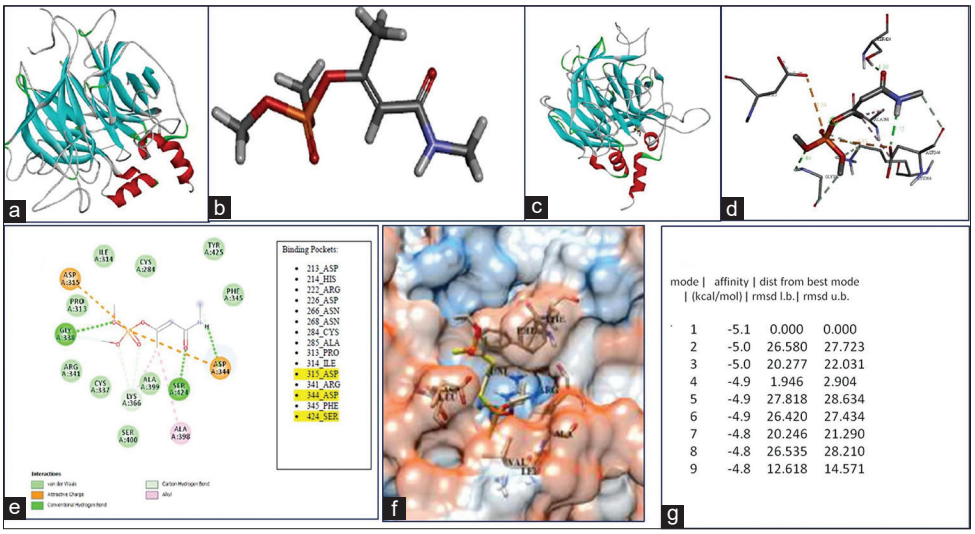
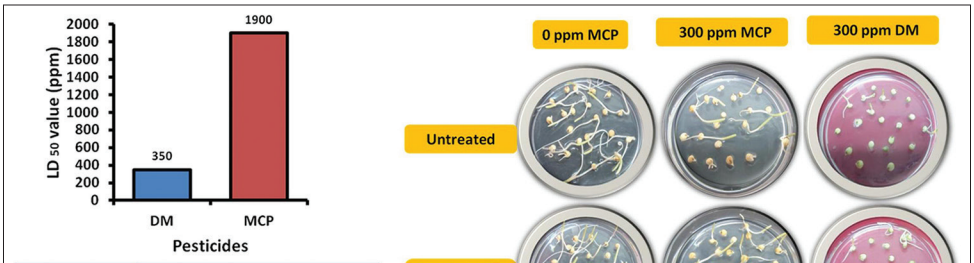


Figure 7: Molecular docking studies with organophosphate insecticide monocrotophos. (a) modeled ThPON1-like, (b) monocrotophos, (c) docked complex, (d) interaction between protein and ligand, (e) 2D interaction of docked complex, and (f) docked complex in surface view, g. Binding affinity (Kcal/mol)

mentioned studies, the results obtained from *in silico* and *in vitro* studies were consistent. As part of the current study, the researchers compared results of *in vitro* tolerance assay, seed germination experiment, and *in silico* analysis using two selected pesticides, MCP and DM, to see if the results were comparable. *T. harzianum* isolate showed higher tolerance to MCP (lethal dose [LD_{50}] > 1900) compared to DM (LD_{50} > 300). *In silico* studies also displayed the same pattern as binding with MCP (Binding energy −5.1 Kcal/mol) was more stable than DM (Binding energy −4.1 Kcal/mol). The lower the binding energy the better is the interaction between enzyme and pesticide ligand [37].



Pesticides	Binding energy (Kcal/mol)
DM	-4.1
MCP	-5.1

Figure 8: Comparative analysis of *in silico* and plant-based studies with selected pesticides**.**

Seed germination assays with MCP and DM pesticides revealed that both pesticides are toxic to plants, but better seed germination was observed in *T. harzianum* treated seeds compared to respective pesticide controls. At 300 ppm concentration of MCP, the germination percentage of untreated seeds was 45% while *T. harzianum* treated seeds had 80% germination. When seeds were treated with 300 ppm DM, they had a 25% germination rate, whereas *T. harzianum* treated seeds showed a 40% germination rate [Figure 8]. Germination study results were similar to those from *in silico* studies. Under MCP and DM stress conditions, the *T. harzianum* treatment increased seed germination by 35% and 15%, respectively. The germination restoration activity of *T. harzianum* is better in the presence of MCP than DM, because *T.harzianum* contains an enzyme that binds more efficiently to MCP than DM (as evidenced by *in silico* studies).

4. CONCLUSION

Multicompetent plant-friendly fungus *T. harzianum* is a key component in sustainable agriculture, but its bioremediation potential is not well explored, especially in the case of OP pesticides. This study suggests that PON1 type of OP pesticide hydrolyzing enzyme (ThPON1-like) is present in *T. harzianum*. In addition, this study proves that results obtained from and *in silico* and *in vitro* studies are comparable. *In silico* bioremediation could reliably help to identify the potential microbial detoxifying enzymes for detoxification of pesticides before going for onsite bioremediation.

5. ACKNOWLEDGMENT

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

There are no animal or human subjects involved in this study.

10. DATA AVAILABILITY

The gene and proteins sequences are available online in the NCBI database (Accession numbers mentioned in the article).

11. PUBLISHER'S NOTE

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