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Regular paper

In silico explorations of bacterial mercuric reductase as an ecofriendly bioremediator for noxious mercuric intoxications

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Mercury is a major pollutant in the environment due to its high concentration in the soil. In this study, a mercuric reductase was extracted from *Pseudomonas aeruginosa*. The sequence of the enzyme was retrieved from the literature and structural homologs were identified. The protein bonded with Mercuric compounds and their interaction was briefly studied. Autodock Vina was used to perform a molecular docking with the target protein. Results showed that the sequence consists of most of the random coil 44.74% followed by α-helix and B-turns. Moreover, the protein was predicted to have a FAD/NAD(P)-binding domain. The virulence factor prediction using different approaches of Virulentpred and VICMpred suggested that P00392 is non-toxic. Next, the mutational analyses were performed to predict the active site residues in the resulting models and to determine mutants. The results show that the enzyme is involved in the bioremediation of mercury by using in-silico techniques. Finally, molecular docking studies were conducted on the best-selected model to find the active site residues and to generate a pattern of interaction to understand the mode of action of the substrate and its catalytic activity which refers to the binding with mercury.

Keywords: Mercury, bioremediation, Mercuric reductase, heavy metals, molecular docking

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Abbreviations: CASTp, Computed Atlas of Surface Topography of proteins; FAD, Flavin-Adenine Dinucleotide; MSA, Multiple Sequence Alignment; NADPH, Nicotinamide adenine dinucleotide phosphate

INTRODUCTION

The proliferation of industrial waste into the environment has led to the grave predicament of heavy metal contamination which has proved to be a peril to both the biosphere and humankind. These heavy metals can accumulate in the environment from various sources (Gworek *et al*., 2020). Soil, water and especially air can get contaminated with these heavy metal compounds and hence, it is causing disastrous impacts on our environment and health. Even at low concentrations, heavy metals including mercury, cadmium, copper, lead and chromium are cytotoxic, carcinogenic and mutagenic in nature. The main cause of the release of such heavy metals into the environment is the utilization of non-biodegradable materials which directly affect the biosphere (Mandal & Mishra, 2023). The World Health Organization has classified four of the ten heavy metals as being of significant public health concern which includes: cadmium, arsenic, lead, and mercury (Budnik & Casteleyn, 2019).

Mercury can be produced in the atmosphere due to natural eruptions i.e., volcanoes, forest fires and weathering of rocks. Mercury can be found in a variety of forms in the inorganic state; metallic mercury, mercury vapor, mercuric salts, and mercury dioxide (Xu *et al*., 2022). The organic state includes the mercuric compounds in which the mercury is bonded to a structure containing carbon atoms, and inorganic mercury in the form of compounds like methyl, ethyl, phenyl, or similar groups. Although the concentration of mercuric compounds in the air is less concerning, it is causing more damage to the soil. Human mercury exposure has adverse health consequences, such as headaches, insomnia, neuromuscular effects, difficulty in breathing, irritability, chest pain, stomachaches and cognitive or motor dysfunction. Mercury poisoning may also occur if the blood mercury levels exceed 100 ng/mL which will result in the malfunctioning of muscles (Duan *et al*., 2020).

There are several conventional methods to decontaminate an environment containing mercuric compounds. An alternative method to distinctively remove this heavy metal is by utilizing a biological technique based on the use of a biological enzyme *mercuric reductase*, extracted from *Pseudomonas stutzeri* with an ecofriendly approach (Al-Ansari, 2022). Bioremediation is a safe approach that can be utilized to decontaminate our environment from these heavy metal pollutants by the use of microorganisms. Microorganisms are considered best for these types of mechanisms as they are well-known for their resistance against heavy metals. They have the potential to adopt various detoxifying mechanisms which include biomineralization, bioaccumulation and biosorption (Al-Ansari, 2022). Therefore, the aim behind opting for this topic is to use a mercury-resistant bacterium thriving in a mercuric environment and study the enzyme which is involved in the bioremediation of mercury by using in-silico techniques. Mercury-resistant *Pseudomonas stutzeri* is found in highly contaminated soil enriched with mercuric compounds. It is a gram-negative, rod-shaped, motile bacterium with greater metabolic diversity in the nature (Wan *et al*., 2020).

In this study, *mercuric reductase* from *P. stutzeri* was selected as a candidate for further investigations (Zheng *et al.*, 2018). The sequence of mercuric reductase retrieved from literature and structural homologs was identified. Structural analysis via using bioinformatic tools was performed and validated to get good-quality protein structures and models. The protein bonded with mercuric compounds and their interaction was briefly studied. The models obtained from these strategies were further refined to remove steric clashes with other compounds. The main residue which is involved in ligand binding was determined based on the active sites and binding pockets identified in the structural homology (Bianchi *et al.*, 2012). Finally, docking studies were conducted on the best-selected model to find the active site residues and generate a pattern of interaction to understand the mode of action of the substrate and its catalytic activity which refers to the binding with mercury. Lastly, the mutational analyses were performed to predict the active site residues in the resulting models and to determine mutants. According to the study conducted, *P. stutzeri* mercuric reductase is a non-virulent protein that may be used for cheap and environmentally acceptable bioremediation of mercury (Sodhi *et al.*, 2019).

MATERIALS AND METHODS

Sequence Analysis

Sequence Retrieval

The FASTA sequence of Mercuric Reductase protein was retrieved from UniProt KB (https://www.uniprot.org) with the specifically allocated UniProt ID: P00392. The sequence contained a total of 561 amino acids and the annotation score 5/5 which confirmed it as a sequence of good quality and useful for all kinds of bioinformatics analysis.

Sequence Characterization

The sequence characterization was done by the prediction of Physiochemical Parameters. The computing for the physiochemical parameters of the Mercuric Reductase protein under study was performed by ExPAsy-ProtParam Tool (https://web.expasy.org/protparam) which is an open-access online server by the Swiss Bioinformatics Resource Portal. The sequence was submitted in the FASTA format and various Physiochemical annotations like Molecular Weight, Theoretical pI, Aliphatic Index and GRAVY were calculated.

Sequence Comparison & Multiple Sequence Alignment

MSA or multiple sequence alignment is a crucial tool in understanding the interrelationships among sequences and identifying functionally important conserved regions. To detect conserved regions in the sequences, the ClustalW and MEGA version X MSA tools were utilized. Furthermore, to determine sequence homology and infer evolutionary relationships, phylogenetic analysis was performed using MEGA version X with the neighbor-joining algorithm and Poisson substitution method.

Function Prediction

The role of protein domains in protein function is essential as they often determine the protein's overall function, specificity, and interaction with other molecules. Protein domains can carry out various functions, such as binding to DNA, RNA, or other proteins, catalyzing chemical reactions, and transporting molecules across cell membranes. By containing specific sequence motifs, domains can also be responsible for post-translational modifications such as phosphorylation, glycosylation or ubiquitination, which can alter protein activity, stability or localization. Therefore, the protein domain prediction was performed by CATH DB (http://www.cathdb.info) which is an online bioinformatics tool for protein domain prediction. The protein sequence was inputted in FASTA format, and the domains were predicted.

Virulence Factors Identification

Keeping in view that the protein is planned to be used as a Bioremediator, it is necessary to make sure that the protein should not be toxic or virulent (Muhammad Naveed *et al.*, 2023). Therefore, the identification of virulence factors was performed with VirulentPred (http://bioinfo.icgeb.res.in/virulent), that is an online server for virulent factors identification. The protein sequence was used as input and the results were obtained.

Secondary structure prediction

Secondary structure prediction was performed by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) which uses position-specific matrices score that is produced by PSI-BLAST to employ neural network methods (M Naveed, I Ali, *et al.*, 2023). It predicts the secondary structure of the protein sequence. In addition to identifying protein characteristics and recognizing folds, secondary structure prediction is an intermediate step in predicting three-dimensional structures (Mohamadi *et al.*, 2022).

The analysis of the secondary structure sequence was also performed by the SOPMA online tool (https://npsa.lyon.inserm.fr/cgi-bin/secpred_sopma.pl) which analyzes the number of features of secondary structure such as b-turns, a-helix and coil etc. In addition to analyzing the amino acid sequence of a given protein, this tool offers information about protein secondary structures (Buchan & Jones, 2019).

Structure Prediction

Template Recognition

The PSI-BLAST program was used to carry out a sequence similarity search against the PDB database to provide a list of results that were most similar to the query sequence to identify the template. The sequence with the highest sequence identity, the largest query coverage, and the score with the lowest e-value was chosen as the reference template. Based on the outcomes returned by BLAST, the coordinates of the template structure were obtained from the Protein Data Bank (Bekker *et al.*, 2022). The motif and domain analysis were identified by Pfam and InterProScan. InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search) is an online tool used to obtain the functional analysis and classify the protein sequences into the families and domains as well as binding sites (Blum *et al.*, 2021). The Pfam (https://pfam.xfam.org/) is a database that contains a large collection of families of proteins each represented by specific Markov models (HMMs) and modeling by multiple sequence alignment (Mistry *et al.*, 2021).

Model Generation

The homology modeling for the three-dimensional structure was performed by SWISS-MODEL (https://

swissmodel.expasy.org/templates/) which is a tool for automatic homology modeling and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) which is a tool that creates 3D structures and remote homology service as well as finds binding sites in the query structure to predict the 3D structure of the mercury reductase protein (Naveed *et al.*, 2023). The best-predicted structure from each tool was chosen and was further validated for experimental analysis (Komari *et al.*, 2020; Pasaribu *et al.*, 2021).

Model Validation

The predicted structure from the two tools was further validated by performing Ramachandran plot analysis by using the PROCHECK (http://services.mbi.ucla.edu/PROCHECK/) *via* the platform of SAVES v5.0 (http://servicesn.mbi.ucla.edu/SAVES/) (M Naveed, N Ain, *et al.*, 2023), which outlines the stereochemical characteristics of the structures (Reddy & Rao, 2020).

Docking Studies

Interaction Analysis

Autodock Vina, a free docking engine, allows for molecular docking. This is a collection of automated docking technologies that are integrated for the prediction of the interaction of small molecules with the protein (Eberhardt *et al.*, 2021). The docking analysis of a chosen Mercuric compound was presented by Autodock Vina. Protein and ligand were first prepared, then active sites were identified, and finally a grid box was set up (by default) (Naveed *et al.*, 2023). The mercurial compound with the lowest binding energies was chosen for docking with the target protein.

Site Directed Mutagenesis

Mutant Identification

For mutation identification, four different tools were used. The I-Mutant (http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0) online tool was used to observe the impact of single nucleotide polymorphisms on the stability of the protein. It predicts the effect of SNPs on the tertiary structure of the protein value based on the free energy change. PHD-SNP (http://snps.biofold.org/phd-snp/phd-snp.html) is based on support vector machines that predict whether point mutation links with a genetic disorder or is a neutral polymorphism. It predicts the human deleterious SNPs (Mustafa *et al.*, 2020). The other mutation identification tool is SIFT (Sorting Intolerant from Tolerant) (https://sift.bii.a-star.edu.sg/), which employs sequence homology to predict the impact of amino acid substitution and whether it has a damaging impact on protein structure or not. It gives the probability score less than or equal to 0.05 if it is deleterious or a prediction value greater than 0.05 when it is tolerant. Another tool MuPro (http://mupro.proteomics.ics.uci.edu/) predicts the stability of protein decreases or increases and its effect on the protein structure (Naveed *et al.*, 2019).

Model Generation

The 3D structure of mutant mercury reductase protein was generated by using SWISS-MODEL (https://swissmodel.expasy.org). It is an automated tool that predicts the 3D structure of protein and generates the model based on the homology modeling (Pasaribu *et al.*, 2021; Komari *et al.*, 2020).

Model Validation

The tertiary structure was validated by the PROCHECK through which Ramachandran Plot was created for Mercuric Reductase P00391. PROCHECK checks the stereochemical property of protein and analyzes the geometry of the residues present in the protein structure. The Ramachandran Plot was analyzed based on Rama favored regions present in the plots (Reddy & Rao, 2020).

Mutant Docking Studies

Prediction Of Binding Sites

Computed Atlas of Surface Topography of proteins (CASTp) (http://cast.engr.uic.edu) is an online bioinformatic tool that locates, measures and delineates concave surface regions on the 3D structure of the protein. It finds out the binding pockets of protein that locate or void buried in the internal of the protein surface. It includes a flexible interactive, interface, visualization as well as on the fly calculations for the input structure (Chandran *et al.*, 2022).

Molecular Docking of the Mutants

After validating the best 3D mutant model, Autodock Vina was run to perform docking upon the protein receptor. The selective structures as ligand were taken from PubChem and were accounted for docking studies with Mercuric (Hg) compounds and complexes. Through this, the intramolecular interactions were evaluated in the final docked complex. To put it simply, PubChem is a repository for information about chemical compounds and their performance in various biological experiments (Eberhardt *et al.*, 2021; Chandran *et al.*, 2022).

RESULTS

Sequence Analysis

The protein sequence of Mercuric Reductase from *Pseudomonas aeruginosa* was obtained from UniProtKB with the UniProt ID P00392. Analysis of protein domains and motifs revealed that its function is in the

Table 1. Physiochemical characterization of the Mercuric reductase protein P00392

Number of amino acids	561
Molecular weight	58728.03
Theoretical pI	5.60
Negatively charged residues	60
Positively charged residues	48
Formula	$C_{2571}H_{4171}N_{733}O_{796}S_{20}$
Total No. of atoms	8291
Ext. coefficient	23420
Estimated half-life	30 hours (mammalian reticulocytes, *in vitro*) >20 hours (yeast, *in vivo*) >10 hours (E*scherichia coli, in vivo*)
Instability index	30.67
Aliphatic index	95.40
GRAVY	0.092

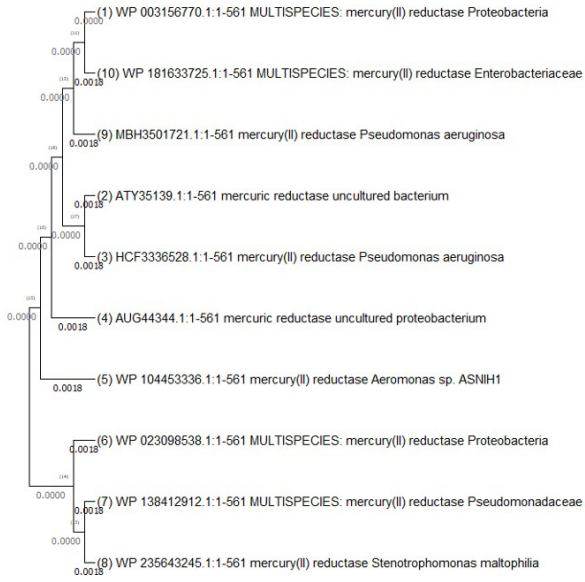

Figure 1. Phylogenetic tree of the Mercuric reductase P00392 generated by MEGA-X Neighborhood-Joining Method.

reduction of mercury and that the protein family is predicted to have a FAD/NAD(P)-binding domain. Physio-chemical properties of the sequence were computed and are presented in Table 1. The instability index for P00392 was found to be 30.67, indicating its higher stability in the test tube compared to the other protein, which had a slightly higher instability index. Virulence factor prediction using different approaches of Virulentpred and VICMpred suggested that P00392 is non-toxic. Homologous sequences for P00392 were identified using BLASTp, and 10 sequences were retrieved. These sequences were suitable for multiple sequence alignment (MSA) to determine conserved regions and phylogenetic analysis to infer the evolutionary relationship. The phylogenetic tree constructed by Neighborhood-Joining Method is given below in Fig. 1. A sequence similarity search was performed to search for crystal structures of the closest homologs available in the Protein Data Bank (PDB).

Table 2. Secondary structure analysis by the SOPMA tool

Alpha-helix	Extended strands	Beta turn	Random coil
35.29	19.96	0.0	44.74

Structure Predication

Secondary structure prediction

As shown in Fig. 2, the predicted secondary structure of mercury reductase protein consists of a majority of the coils as viewed by using PSI-PRED followed by a-helix. The secondary structure of a protein plays an important role in determining its stability, function, and interactions with other molecules. In the case of mercury reductase, the predominance of coils in its secondary structure suggests that it is a flexible protein that can undergo conformational changes to interact with its substrate and catalyze the reduction of mercury. The presence of alpha helices in the secondary structure also indicates that the protein may have structural stability and rigidity in certain regions. It is important to note that the predicted secondary structure is based on computational methods and may not precisely reflect the actual structure of the protein. Further experimental studies such as X-ray crystallography or NMR spectroscopy would be needed to confirm the actual secondary structure of mercuric reductase. The analysis of secondary structure by SOPMA is given in Table 2.

Secondary structure analysis was performed by SOPMA. Results show that the mercury reductase sequence

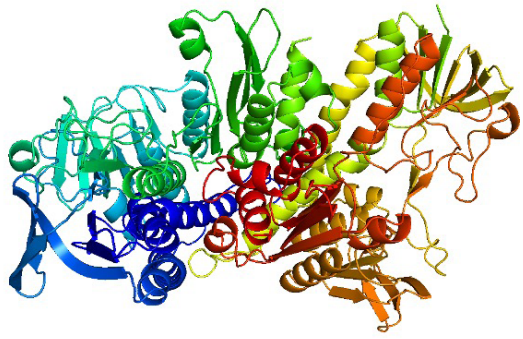

Figure 3. Predicted 3D Model of Mercuric reductase P00391 by Swiss-Model

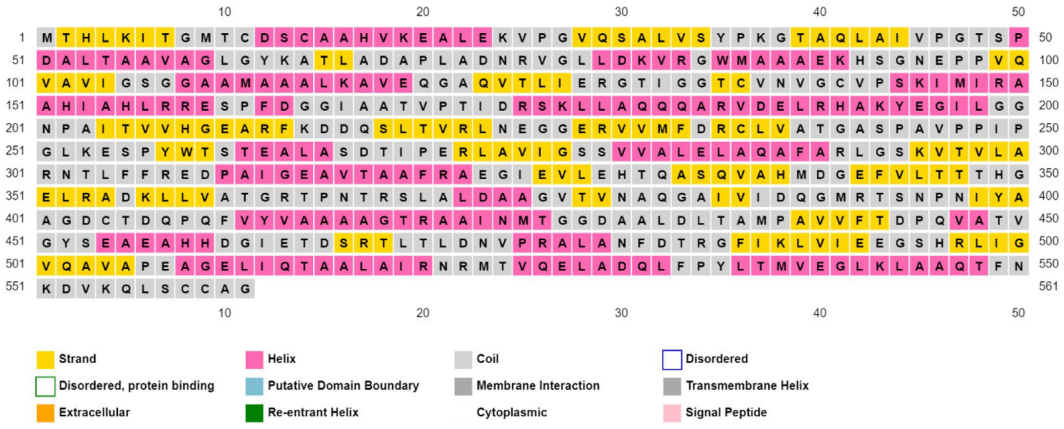

Figure 2. The predicted secondary structure of Mercuric reductase by PsiPred

Table 3. Identified mutations and classification of mutants by *in-silico* approaches

Species	Amino Acid Substituted	Mutation Position	Amino Acid Replaced	I-Mutant Results	MU-PRO Results	PHD-SNP Results	SIFT Results
Uncultured proteobacterium	L	346	M	Decrease	Decrease	Neutral	Neutral
Mercury(II) reductase (*P. aeruginosa*)	V	137	I	Decrease	Decrease	Neutral	Neutral
Aeromonas sp. ASNIH1	E	91	D	Decrease		Deleterious	Neutral
S. maltophilia	A	154	T	Decrease	Decrease	Neutral	Neutral
Mercury(II) reductase (*P. aeruginosa*)	A	114	V	Decrease	Decrease	Deleterious	Deleterious
Mercury(II) reductase (*C. freundii*)	A	192	V	Decrease	Decrease	Deleterious	Neutral
Mercury(II) reductase (*Burkholderia sp.* EMB26)	P	98	S	Decrease	Decrease	Neutral	Neutral
Mercury(II) reductase (*D. lacustris*)	L	32	M	Decrease	Decrease	Neutral	Neutral
Mercury(II) reductase (*S. maltophilia*)	V	442	A	Decrease	Decrease	Neutral	Neutral
Mercury(II) reductase (Proteobacteria)	T	240	A	Decrease	Decrease	Deleterious	Deleterious

consists of most of the random coil 44.74% followed by α-helix and B-turns as shown in Table 2.

Generation of 3D Structure

The best model generated by Swiss-Model with the highest scoring was selected for further analysis. The generated 3D structure is given below in Fig. 3. The predicted 3D structure was further validated, and the Ramachandran assessment predicted the validation score of the predicted protein model. The Ramafavoured regions were observed to be 92.6% that possess the protein model as stable and having good quality. Therefore, the model was valid enough to be used for various kind of bioinformatic analysis.

Site Directed Mutagenesis

The FASTA sequence of the Mercuric Reductase protein was analyzed by aligning it with the sequences of other species. The local alignment was performed by BlastP tool by NCBI, and the alignments tab accessed all the present mutations. Top 10 mutations were selected, and their effects were studied by I-Mutant, MuPro, PHD-SNP and SIFT tolls. The mutants having negative effects on the structure and function of protein were further selected for the mutagenesis into the actual structure of protein so the effects could be accessed. The selected SNPs are given below in Table 3.

SNPs with negative effects were selected and using the PyMol mutagenesis was introduced into the protein structure. The mutated structure of the Mercuric Reductase protein is given below in Fig. 4. It was further utilized for interaction studies to analyze the effects of the mutated structure of protein.

Docking Studies

Interaction Analysis

Autodock Vina was used to perform molecular docking between the chosen mercuric compound and the target protein. The docked model which was selected based on the lowest binding energy retained the binding energy of −7.9 kcal/mol, predicting the more efficient binding

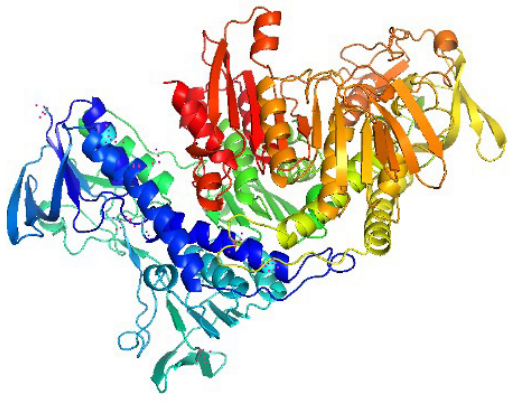

Figure 4. Mutated protein structure with the highlighted mutant areas.

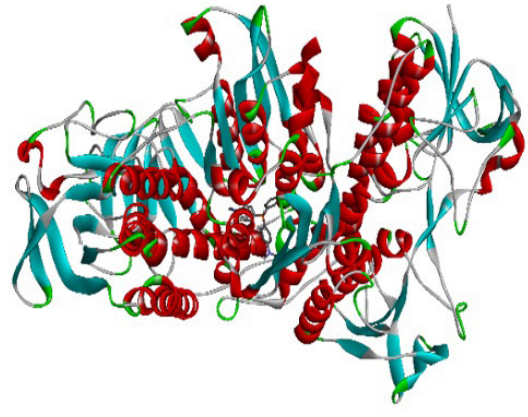

Figure 5. A docked complex of Bis[tris(p-dimethylaminophenyl) phosphine] mercuric chloride complex with targeted protein

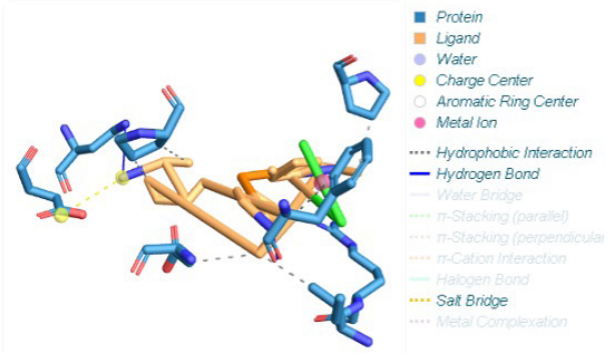

Figure 6. Interaction complex of the Mercuric reductase with Mercury compound.

with the protein. The docked Complex of Bis[tris(p-dimethylaminophenyl) phosphine] mercuric chloride complex compound with targeted protein is shown in Fig. 5.

Validation of docked complex:

Protein-ligand interaction profiler (PLIP) provided interpretation of the interaction data by estimating the bond length. As the range of hydrogen bond is between 2.7-3.3 angstroms, the three hydrogen bonds detected as 2.73, 3.19, 2.91 angstroms. Due to the slightly greater distance of bond length of van der Waals forces (3.3-4.0 angstroms) it was predicted that two van der Waals or hydrophobic bonds were present, with a length of 3.69 and 3.54 angstroms, respectively. The interaction predicted by PLIP (Protein-ligand interaction profiler) is shown in Fig. 6.

Prediction of binding sites

The analysis of active sites was performed by using CASTp online tool. This tool analyzes the proteins surface topography and measures the available surface area of the protein as a potential active site. The 3D model highlights the active sites as seen in Fig. 7.

Interaction Studies of the Mutated Mercuric Reductase

Autodock Vina was utilized to produce a mutant protein docked complex, among which the best one was chosen to make a docking complex with the maximum binding affinity of -6.6. After this, a visualizer is used to study the interactions among the ligand (*S-Mercuric-N-dansylcysteine*) and the protein (Studio, 2008). For example; hydrogen bonds, hydrophobicity, aromaticity, charge distribution and ionizability etc., which confirmed the stability of the complex as shown in Fig. 8.

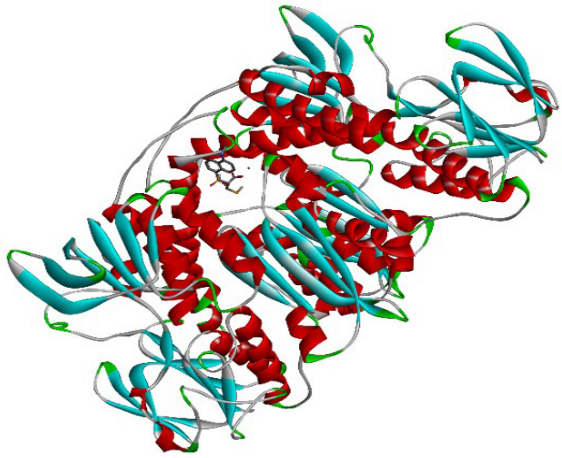

Figure 8. The docked complex of S-Mercuric-N-dansylcysteine with the targeted mutant protein.

Validation of mutated docked complex:

Protein-ligand interaction profiler (PLIP) was used for the further interpretation of the interaction between the mutant protein and the ligand by measuring the bond length. According to the range, two hydrogen bonds were detected with bond length of 2.53 and 2.48 angstroms between the protein and the ligand, respectively. Moreover, one van der wall force was detected between them with value falling in between the range; 3.99 angstroms. The interaction predicted by PLIP (Protein-ligand interaction profiler) is shown in Fig. 9.

DISCUSSION

Mercury toxicity (Hg) mainly depends on the route of exposure and the chemical form of Hg. Its most toxic form is Hg^{+2} and its non-toxic form is Hg^{0}. Its accumulation in the food chain causes deleterious effects on human health (Gworek *et al.*, 2020). Lohren *et al.*, revealed the toxic effect of Hg accumulation on the central nervous system that harms the blood-brain barrier and facilitates the entrance of other toxicants to penetrate into

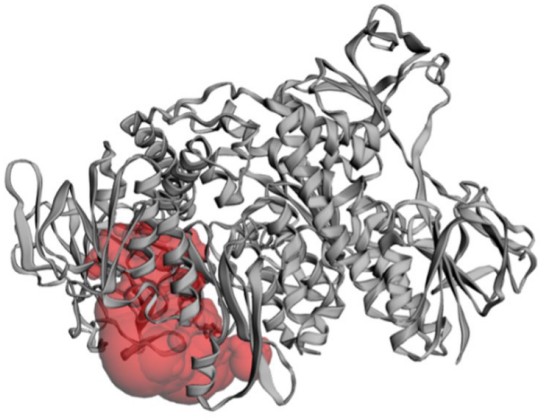

Figure 7. The 3-D structure and its active site.
The red area depicts the active site, while the grey area depicts the rest of the structure as represented in cartoon style.

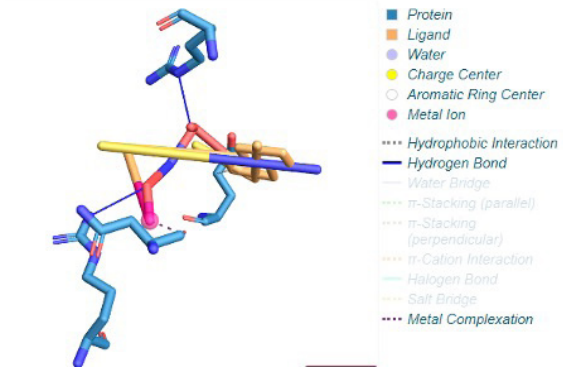

Figure 9. Interaction complex of the mutated Mercuric reductase with Mercury compound

the brain (Gworek *et al*., 2020; Al-Ansari, 2022; Singh & Kumar, 2020).

Nowadays, the prime focus of different industries is to abolish the effect of Hg toxicity by remediation of Hg-polluted soil or water. Bacteria harbor an extensively studied Hg-detoxification mechanism, Mer operon which encodes the functional proteins for transportation (merT, merP and/or merC, merF), lysis of organomercurial compounds (*mer*B), reduction of mercuric ion reductase (merA) and a secondary regulatory protein (merD) to modify toxic Hg (II) to nontoxic elemental state Hg (0) (Paria *et al*., 2022; Nivetha *et al*., 2023). Structural studies determined a unique fold of protein, *MerB,* which is a significant conformational transformation that occurred on the binding of the substrates as organomercurial compounds. Structural as well as computational studies revealed that aspartic acid and two cysteine residues in the active site are responsible for cleaving the carbon–mercury bond. The second enzyme, mercuric reductase (*Mer*A) encoded by the *mer*A gene is directly involved in the reduction of the reactive ionic form Hg (II) to the volatile form Hg (0) (Rahayu *et al*., 2021; Somayaji *et al*., 2022). *Mer*T, a membrane-bound protein is responsible for the uptake of Hg (II) and is arranged on the *mer* operon under the control of *Mer*R, a unique responsive regulator of metal. Silver and Hobman *et al*., reveal the mercury reduction process by mercury reductase (Naguib *et al*., 2019). The binding of Hg^{+2} to the carboxyl-terminal subunit part of Cys 557- Cys 558 of mercury reductase then quickly move towards the thiol-thiol exchange to the monomer pain of Cys 135-Cys 140. The Cys 135-Cys 140 pair is the active site that causes the reduction of Hg^{+2} into Hg^{0} by the FAD cofactor that helps in the electron transport (Meyer *et al*., 2023).

Conventional methods such as bioreactors have been used for the mercury cleanup of wastewater by up to 90%. Mercury-resistant bacteria have a major advantage as conventional practices produce a large amount of mercury load biomass (Duan *et al*., 2020). A recent study on a non-pathogenic bacterial isolate of Pseudomonas putida SP-1, which volatilizes mercury by 89%, has shown the efficiency of mercury-resistant bacteria in the bioremediation process. However, case studies act as a bridge to some extent to fill the gap between field application and laboratory research. In-silico studies identify the efficacy and potential of mercury-resistant bacteria in the bioremediation of mercury toxication. The current study shows that mercury reductase is the best candidate as a bio-remediator and mercury accumulator (Gupta *et al*., 2022; Priyadarshanee *et al*., 2022).

In a recent study, the screening of the mercury reductase calcium complex compound that results in highly competent attachment to the target protein of mercury reductase isolated from the bacteria source was done through computational analysis. Protein stability depends on the instability index. The instability index of the protein was less than 40 as shown in Table 1. Our results are consistent with the statement given by Mirzaei *et al*. where protein having a stability index of less than 40 is stable and when greater than 40 shows that protein may be unstable (Gamage *et al*., 2019). The aliphatic index was predicted to be where a higher value of the aliphatic index shows the thermal stability of the protein. This is in line with the statement given by Sahay *et al*., that the aliphatic index is considered a positive factor for the increase of thermostability of globular proteins. The GRAVY score was found to be positive with a 0.092 value, which indicated that mercury reductase is generally a hydrophobic protein. The reason for hydrophobicity may be due to the presence of large numbers of non-polar amino acids. Another study conducted by Zhou and Pang supported our results by showing that protein folding stability was mainly attributed to the hydrophobic interactions among non-polar amino acid residues. Thus, based on a few parameters of the Expasy ProtParam, the mercury reductase could be considered a stable protein (Sharma *et al*., 2022; Naveed *et al*., 2022).

The mutational analysis revealed that it affects the binding sites of mercury reductase protein. The mutations identified from five tools as shown in Table 3. Docking studies have been found helpful to understand the protein-ligand interactions (Eberhardt *et al*., 2021). The model was then generated and docking analysis of both the non-mutated protein and mutant protein shows that binding affinity energy reduces from –7.9 to 6.6 KJ/mol. The 3D structure of the mutant model generated from SWISS-MODEL shows the removal of two ligands of FAD (Flavin Adenine Dinucleotide) that were present in the non-mutated protein. As mutation T240A was introduced it acts as an active binding site as shown in the active site prediction Figure 7. The binding sites and motifs for NADPH and FAD in different MerA proteins vary among residues ALA, CYS, TYR, LEU, THR, PRO, SER, ASN, VAL, GLY, ALA, ASP, LYS, PHE, GLU and ARG. As the distance among binding residues of FAD and NADPH varies in microorganisms, no such correlation was observed between the protein-ligand binding affinity and binding residues (Chandran *et al*., 2022). As a result of folding into a specific three-dimensional structure, the amino acids undergo conformational changes to perform the function they are designed to perform. Thus, the binding affinity of mutant protein reduces due to the conformational change of amino acid residues and binding active sites (Afroz *et al*., 2023).

Engineered proteins, particularly enzymes, are being used more frequently in various industries due to their selective ligand binding, capabilities and catalytic ability as food additives. The urge to engineer or generate proteins with higher specificity, activity and stability has increased along with the number of possible applications for engineered proteins (Gupta *et al*., 2022). As the application of protein technology develops, exploiting the potential advantages of modulating remote regions will become imperative. The present study reveals that the mutation affects the binding pockets of mercury reductase, and it acts as a mercury accumulator and candidate for bioremediation of mercury in the field of application. It is a stable protein with an applicable Ramachandran plot which validates its stability, and virulent predictions show that it is non-virulent and causes no toxicity. Protein engineering bacterial mercury reductases can produce enzymes that reduce mercury more efficiently with no toxicity, and that will function with additional contaminants (Wan *et al*., 2020; Meyer *et al*., 2023).

CONCLUSIONS

In conclusion, this study provides insights into the molecular and structural features of mercuric reductase. The results suggest that the protein has a stable structure and a specific function of reducing mercury. The information obtained from this study could be useful in developing strategies to bioremediate mercury-contaminated sites. Additionally, the methodology used in this study, such as homology modeling, molecular docking, and virulence factor prediction, could be applied to other proteins and enzymes to further understand their

properties and functions. Overall, this study contributes to our understanding of the biochemical and molecular mechanisms involved in the bioremediation of mercury pollution in the environment.

Declarations

Author Contributions. Conceptualization, M..A, M.N., R.N, A.S and T.A; methodology, M..A, M.N., R.N, A.S and T.A; software, M.A; validation, A.A.S; formal analysis, T.U.; investigation, M..A, M.N., S.R, N.U resources, M.A and A.A.S.; data curation, T.A.; writing—original draft preparation, T.A and M.N.; writing—review and editing, M..A, M.N., R.N, A.S; visualization, F.A, N.U; supervision, T.A and A.A.K.; project administration, A.A.S and M.A; funding acquisition, T.A

Conflicts of Interest. The authors declare no conflict of interest.

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