INSIGHT INTO THE MESOPHILIC BACTERIAL CHROMATE REDUCTASE: AN INSILICO STUDY TOWARDS REMEDIATION OF CHROMIUM POLLUTION THROUGH MICROBES

Abstract

Industrial use of chromium leads to the contamination of natural environment with chromium pollution and the hexavalent form is very harmful to the living system compared to the trivalent and other forms. The most sophisticated way of chromium reduction is the use of an enzyme chromate reductase exclusively produced by the bacteria. An insilico sequence and structure analysis of chromate reductase enzyme from three mesophilic bacteria *Acetobacter aceti, Escherichia coli,* and *Pseudomonas putida* has been studied. Amino acid variation study indicates a highest number of charged and uncharged residues in *P. putida* and *A. aceti* comparedto *E. coli*. Secondary structure analysis indicates that protein from *E. coli* contains additional beta-hairpin and beta bugle. Higher number of salt bridges, aromatic-aromatic interactions, cation-pi interactions in *P. putida* chromate reductase provides its more stability then the other two. Molecular dynamics simulation studies through RMSD and RMSF indicates less fluctuation in case of *P. putida* chromate reductase while lowest Rg indicates tightly packed nature and lower SASA indicates better folding of the said protein. This is the first reporting on structural and sequence analysis of chromate reductase of the three mesophilic bacteria and among them *P. putida*proved to be the most sable one to be used in the industrial level for better chromate alleviation.

Keywords: Chromate reductase, bacteria, homology modelling, protein- protein interaction, salt bridge, molecular dynamic simulation.

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I. INTRODUCTION

Extensive use of chromium in different industrial sectors such manufacturing of dye, leather tanning, mining and others results in the contamination of soil, ground water and agricultural field with release of chromium. Hexavalent chromium Cr(VI) is reported to be harmful to all the living forms including humans, plants and microorganisms and has been classified as major pollutant by United States Environmental Protection Agency (USEPA) (Cheung and Gu, 2007). Hexavalent chromium Cr(VI)is highly soluble and enters the cell through sulphate transporter pathway and through enzymatic and nonenzymatic way they get converted to trivalent chromium Cr(III)which is less toxic, insoluble and less bioavailable. Reactive oxygen species are created during this reaction, which interact with protein and nucleic acid to cause harmful effects on cells (Cheung and Gu, 2007).Detoxifying Cr(VI) by turning it into Cr(III) is therefore crucial for the environment. The most cost-effective and safe approach to reduce Cr(VI) to Cr(III) is the use of microorganisms which has a lot of appeal. The primary mechanism for Cr(VI) reduction is the use of enzymechromate reductases. Due to their crucial role in mediating chromium toxicity and their prospective applications in bioremediation and biocatalysis, chromate reductases have attracted a great deal of attention from researchers throughout the world in recent years (Ackerley et al., 2004). As a result, numerous chromate reductase bacterial species are isolated, characterised, and used to convert Cr(VI) into Cr(III) in a procedure that is relatively more environmentally friendly than the traditional ones. The major bacteriain this regard are *Escherichia coli*, Arthrobacter sp., *Ochrobacterium* sp., *Agrobacteriumradiobacter*, *Alcaligens* sp., *Bacillus* sp. *Rhodobactersphaeroides*, *Pseudomonas ambigua*and *Pseudomonas putida* and others(Conceição et al., 2009, Thatoi et al., 2014). Although there are reports on common mesophilic bacteria such as *E. coli*, *Pseudomonas* sp. as potential producer of chromate reductases, however there are no such reports on the structure and sequence analysis of this enzyme.

In this chapter an attempt has been taken to compare the sequence and structure of chromate reductase enzyme from common mesophilic bacteria*Acetobacter aceti, Escherichia coli,* and *Pseudomonas putida*through insilico analysis andthis is the first report on such observation.

II. MATERIALS AND METHODS

1. **Dataset:** The sequences of chromate reductase from 3 mesophilic bacteria i.e., *Acetobacter aceti, Escherichia coli*, and *Pseudomonas putida* were retrieved from the largest protein database Uniprot (UniProt Consortium, 2015). In the case of protein structures, only *Escherichia coli* had one structure in the RCSB PDB (Kouranov et al., 2006) database. The other protein structures from *Acetobacter aceti* and *Pseudomonas putida* were created through homology modeling.

2. **Homology Modelling:** In homology or comparative modelling, the shape of a protein with a similar amino acid sequence is predicted using experimentally known protein structures. The technique is based on the observation that, in nature, a protein's structural conformation is better preserved than its amino acid sequence, and that; in general, modest to moderate changes in the sequence only little alter the 3D structure of a protein.

Fold assignment, sequence alignment, model creation, and model refining are the four processes that typically make up the homology modelling process. The fold assignment procedure locates proteins with known three-dimensional (template) structures that are connected to polypeptide sequences with unknown structures (Hillisch and Pineda, 2004). Modelling was done through a python-based programme Modeller V10.4 (Webb and Sali, 2016). After the creation of models, all models were validated through several online servers as earlier process (Biswas et al., 2020).

3. **Analysis of Protein Sequences:** All protein sequences were subjected to MSA through Clustal omega to prepare the block of the sequences. Non-block format of protein sequences was taken for amino acid distribution analysis which was done by the Protparam server (Gasteigeret al., 2005; Mitra et al., 2021). Window-dependent properties like, Kyte-Doolittle hydrophobicity, and Grantham polarity were calculated through the Protscale server (Gasteigeret al., 2005).

4. **Analysis of Protein Structures:** All protein structures were minimized through Chimera 1.15rc (Goddard et al., 2005) with an Amber forcefield (Wang et al., 2005). Minimized protein structures were subjected to a PIC server (Tina et al., 2007) to identify the intra-protein interactions. The secondary structure was investigated by the PDBsum server (Laskowski, 2001).

5. **Molecular Dynamics Simulations**: In recent years, molecular dynamics (MD) simulations have had a significant increase in influence on molecular biology and drug development. Validation of docking complex is tested by dynamics to check their stability (Mitra and Das Mohapatra, 2021c). These simulations completely capture the atomic level details and extremely fine temporal resolutions of the behaviour of proteins and other biomolecules. Molecular dynamic simulations were performed using GROMACS (Abraham et al., 2015; Mitra et al., 2022c) and the GROMOS96 43a1 force field. After equilibration, the steepest descent method with 5000 steps was used to reduce the energy on the solvated systems. At 300 K temperatures, the final production run's molecular dynamic simulations lasted for 50 ns. The radius of gyration (Rg), solvent accessible surface area (SASA), root mean square deviation (RMSD), and root mean square fluctuation (RMSF), as well as hydrogen bonding, were determined through molecular dynamic simulations.

III. RESULTS AND DISCUSSIONS

1. **Diversity in Protein Sequences:** There is some evidence that amino acid composition of proteins is readily influenced by their size. The components of the amino acids are connected in a straight line by substituted amide bonds. Diversity in amino acid compositions in the same protein in different organisms makes them more stable and functional from one another (Mitra et al., 2022a; Mitra and Das Mohapatra, 2021a).

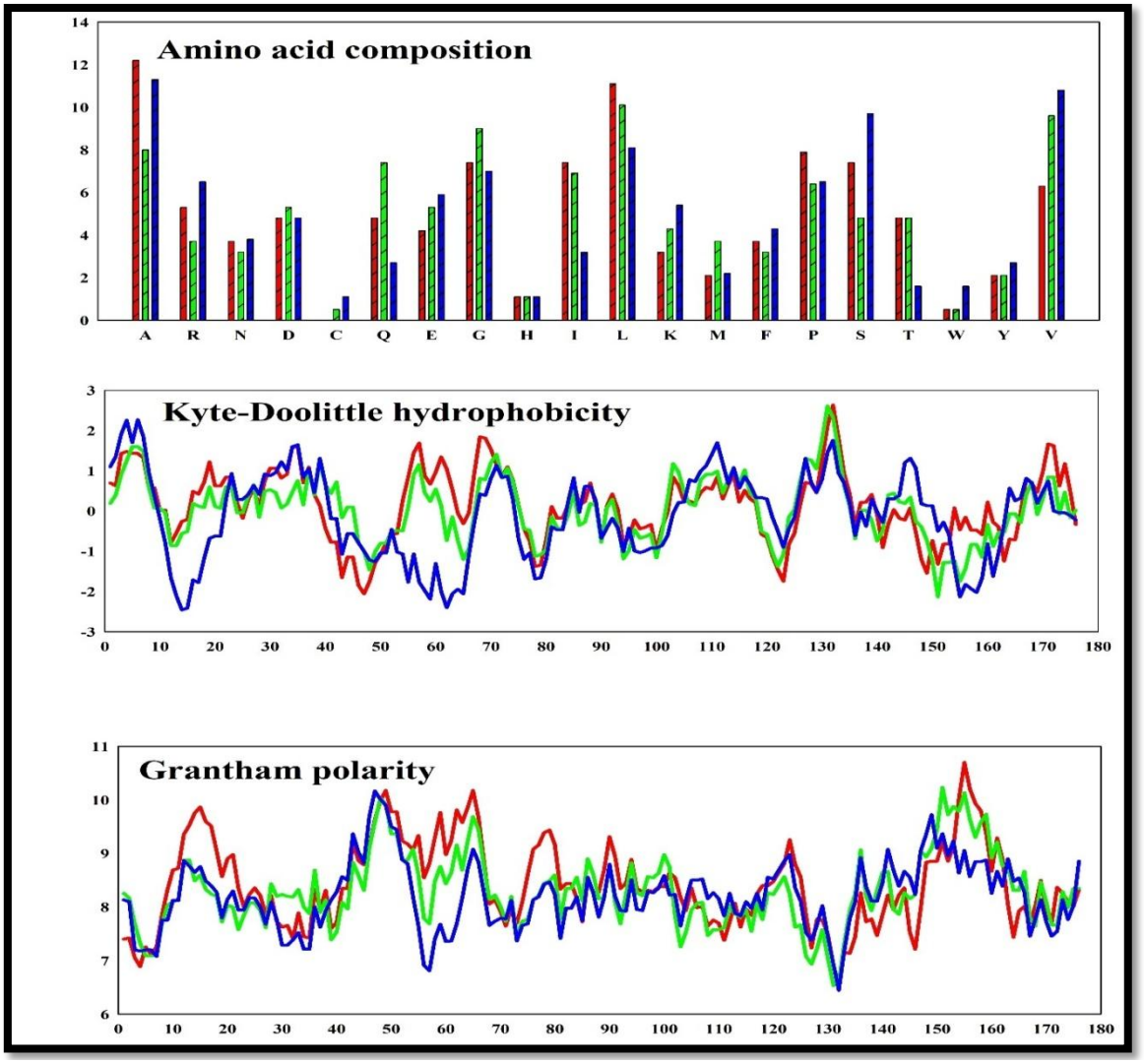

Figure 1: Amino acid compositions, Kyte-Doolittle hydrophobicity, Grantham polarity in chromate reductase of Acetobacter aceti (red), Escherichia coli (green) and Pseudomonas putida (blue).

Charged polar amino acid residues and uncharged polar amino acid residues have the highest abundance in *P. putida* and *A. aceti* rather than *E. coli* (Fig. 1). The increasing ratio of Tyr residues in chromate reductase of *P. putida* increases the protein stability (Panja et al., 2020). Some hydrophobic residues like Ala, Phe, and Val were also high in *P. putida*. *E. coli* showed some specific amino acid abundance like Asp, Gln, Met. Charged residues play a key role in protein stabilization in adverse conditions. Increasing in number of such amino acids is highly observed in extremophiles (Mitra and Das Mohapatra, 2021b). In the case of hydrophobicity, *P. putida* showed the highest hydrophilic nature followed by *A. aceti* and *E. coli*. The hydrophilic nature of protein is often more stable and can easily interact with aqueous medium. Hydrophilic proteins have more use for industrial purposes (Biswas et al., 2022). Although, the polarity shows

a similar result of hydrophobicity. *A. aceti* showed the highest polarity with some noticeable pick points followed by *P. putida*.

2. **Validation of model structures:** In the context of protein structure refinement, which has recently been identified as one of the bottlenecks limiting the quality and utility of protein structure prediction, the development, and improvement of reliable computational methods intended to assess the quality of protein models is pertinent. A variety of scoring functions have been created to analyze protein structures; these scoring functions may be divided into several groups based on the principles and structural aspects taken into account during the evaluation (Mereghetti et al., 2008). From the Ramachandran plot (Fig. 2), the model structure of chromate reductase from *A. aceti* contained 87.2% amino acid residues in its most favored regions whereas, 12.8% residues were present in additional allowed regions. No amino acid residues were present in disallowed regions, while, the model chromate reductase structure of *P. putida* showed 86.6% residues in the most favoured regions and 12.7% residues in additional allowed regions. Only 1 amino acid residue i.e., 0.7% was present in disallowed regions (Table 1). The above results indicate the excellent model structures which were used for further analysis.

Table 1: Details of Ramachandran plot frommodel structures from *A. aceti*and *P. Putida*

Name	Residues in most favoured regions	Residues in additional allowed regions	Residues in generously allowed regions	Residues in disallowed regions
Acetobacter aceti	87.2	12.8	0	0
Pseudomonas putida	86.6	12.7	0	0.7

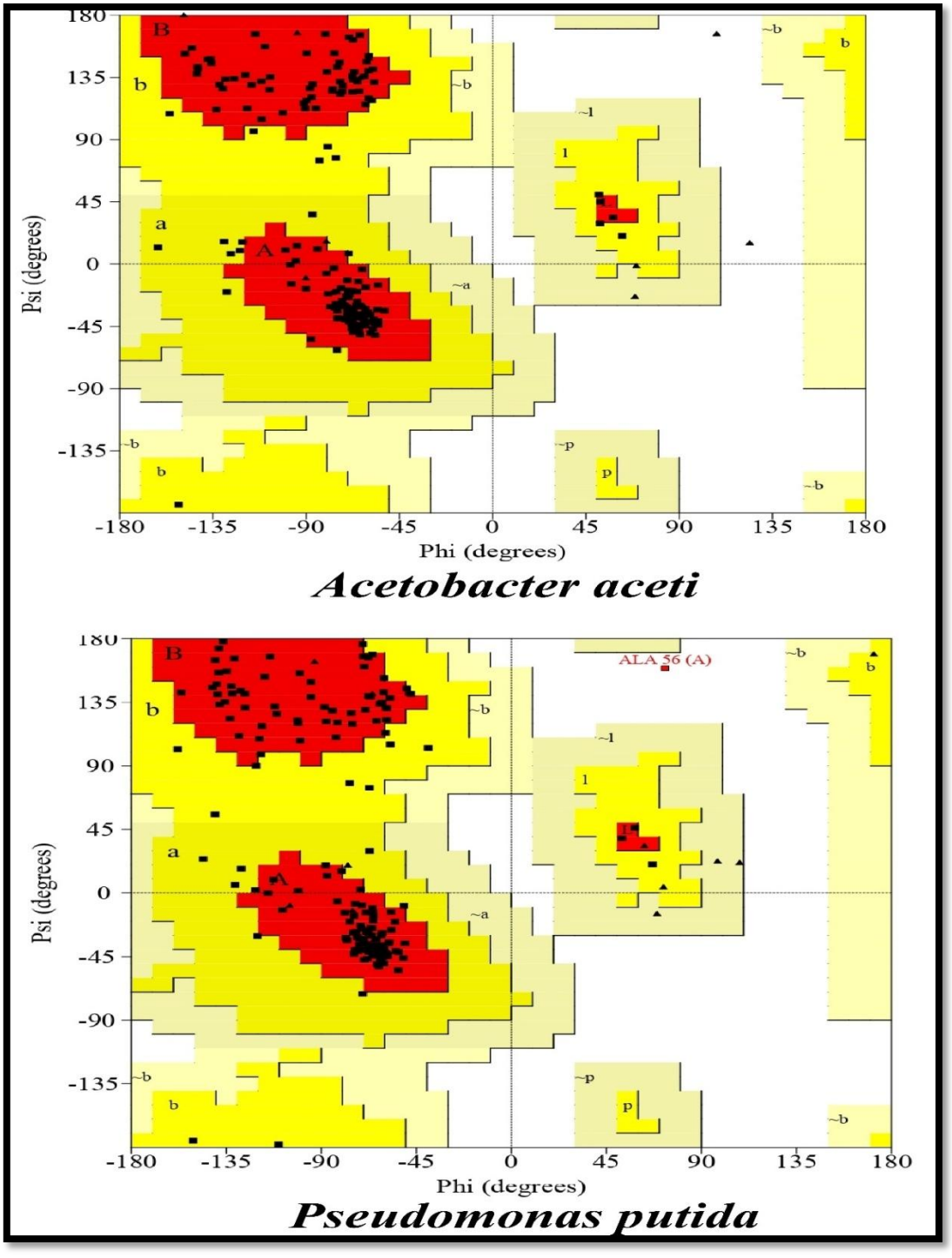

Figure 2: Ramachandran plot of chromate reductase model structures from A. acetiand P. putida

3. **Variations in Secondary Structures:** An argumentative reaction is frequently prompted by the assertion that secondary structure dictates tertiary structure: While some believe it to be manifestly incorrect, others believe it to be true but unimportant. The true-but-trivial group thinks that after α-helices, β-strands, and β-turns are fixed, little conformational latitude is left. In terms of protein folding and structure, secondary structures are crucial (Fleming et al., 2006). Chromate reductase of *A. aceti* consisted of 1 sheet, 3 beta-alpha-beta units, 6 strands, 8 helices, 4 helix-helix interaces, 14 beta turns, 1 gamma turn (Fig. 3). The protein from *E. coli* showed 2 sheet, 3 beta-alpha-beta units, 1 beta-hairpin, 1 beta bulge, 8 strands, 8 helices, 4 helix-helix interaces, 14 beta turns, 1 gamma turn in its secondary structure. It had an additional beta-hairpin and beta bugle structure. Chromate reductase of *P. putida* had a very much similar structure to *A. aceti*. It contained 1 sheet, 3 beta-alpha-beta units, 5 strands, 8 helices, 4 helix-helix interaces, 14 beta turns, 2 gamma turns.

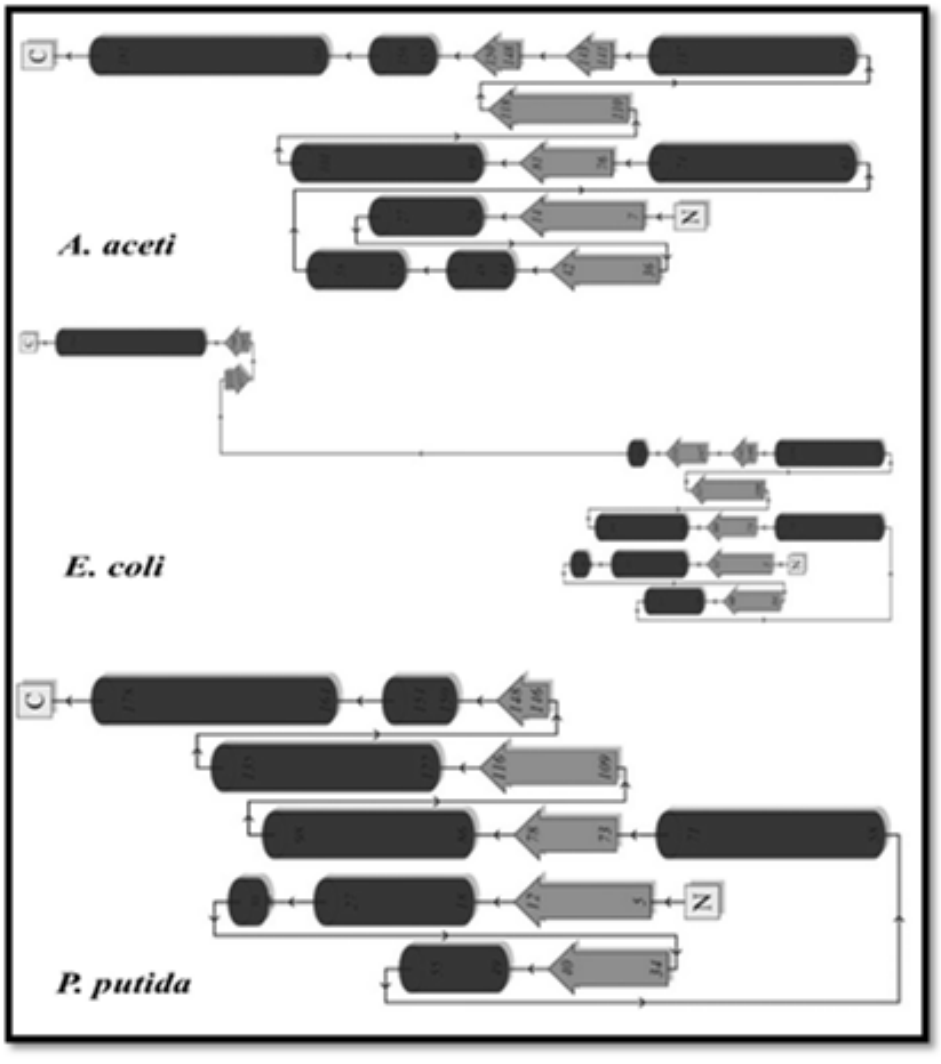

Figure 3: Secondary structure of chromate reductase from*A. aceti, E. coli*and *P. Putida*

4. **Formation of intra-protein interactions:** Non-covalent interactions are significant in the fields of physics, chemistry, and particularly the bio-disciplines. Weak interactions between atoms or molecules known as non-covalent interactions occur when there is no chemical reaction. Non-covalent interactions are frequently referred to as non-bonded interactions since no new chemical bonds are formed or broken as a result of them (Scheraga, 2012). Salt bridges are very important ionic interactions in proteins which increase their stability. Salt bridges are generally two types i.e., isolated and network. However, A new concept of a special type of salt bridge i.e., cyclic salt bridge was introduced in structural biology recently (Mitra and Das Mohapatra, 2021b). Chromate reductase of *A. aceti* showed 5 isolated salt bridges (Table 2). Chromate reductase from E. *coli* had 4 isolated and 1 network salt bridges whereas chromate reductase of *P. putida* had 6 isolated and 1 network salt bridges. The higher formation of salt bridges in the chromate reductase of *P. putida* makes it more stable than the other two studied sources.

Table 2: Isolated and network (coloured pair) salt bridges of chromate reductase from *A. aceti, E. coli* and *P. Putida*

Organisms name	Residue no.	Residue name	Residue no.	Residue name
A. aceti	83	GLU	126	ARG
	93	LYS	97	ASP
	145	LYS	147	GLU
	156	LYS	166	ASP
	171	GLU	175	ARG
E. coli	29	LYS	162	GLU
	82	GLU	125	ARG
	92	LYS	96	ASP
	155	LYS	165	ASP
	68	GLU	71	ARG
	71	ARG	103	ASP
P. putida	17	GLU	22	LYS
	21	ARG	41	GLU
	29	GLU	160	LYS
	53	GLU	98	ARG
	59	GLU	63	ARG
	90	LYS	94	ASP
	66	GLU	69	ARG
	66	GLU	70	ARG

Aromatic-aromatic interactions are very common in bacterial proteins which helps to maintaining their activity in adverse conditions (Mitra and Mohapatra, 2021a). Chromate reductase of *A. aceti* possesses two isolated aromatic-aromatic interactions whereas *E. coli* had 1 isolated and 1 network aromatic-aromatic interaction (Table 3). The

abundance of aromatic-aromatic interactions was increased in the chromate reductase of *P. putida*. So, the chromate reductase of *P. putida* was more stable than the other two chromate reductase.

Table 3: solated and network (coloured pair) of aromatic-aromatic interactions of chromate reductase from *A. aceti, E. coli* and *P. Putida*

Species name	Position	Residue	Position	Residue	D(centroid-centroid)	Dihedral Angle
A. aceti	51	TYR	98	TRP	4.84	78.4
	60	PHE	98	TRP	6.12	76.67
E. coli	179	PHE	182	PHE	6.46	17.53
	49	TYR	97	TRP	4.86	71.66
	59	PHE	97	TRP	5.97	71.22
P. putida	19	TYR	155	PHE	4.96	147.23
	48	TYR	61	TRP	6.26	21.82
	123	PHE	147	TYR	5.35	88.23
	172	PHE	176	PHE	6.8	167
	64	PHE	105	TRP	5.77	104.58
	76	PHE	105	TRP	5.98	138.49

It was discovered that the protein's aromatic-sulphur interactions aided in folding and electron transport. The bulk of protein structures include aromatic-sulphur interactions, yet little is understood about how these interactions operate in ion channels. Compared to only hydrophobic interactions, aromatic-sulphur interactions offer extra stabilization (Yeung et al., 2020; Mitra et al., 2022b). The aromatic-sulphur interactions were absent in the Chromate reductase of *A. aceti* (Table 4). Although, *E. coli* had 1 isolated and 1 network aromatic-sulphur interaction. Chromate of *P. putida* had only 2 isolated aromatic-sulphur interactions.

Table 4: Isolated and network (coloured pair) of aromatic-sulphur interactions of chromate reductase from *A. aceti, E. coli* and *P. Putida*

Species name	Position	Residue	Position	Residue	D(Centroid-Sulphur)	Angle
E. coli	182	PHE	142	MET	4.84	141.64
	19	PHE	118	MET	4.98	73.82
	19	PHE	22	MET	4.49	122.87
P. putida	105	TRP	138	MET	5.3	137.12
	176	PHE	141	MET	5.21	106.3

An essential, all-encompassing force for molecular recognition in biological receptors is the cation-pi interaction. Novel binding sites for cationic ligands, such as acetylcholine, can be built through the sidechains of aromatic amino acids (Mecozzi et al.,

1996). Chromate reductase of *A. aceti* and *E. coli* showed 1 and 2 isolated cation-pi interactions respectively. *P. putida* had a higher abundance of cation-pi interactions. It formed 2 isolated and 1 network cation-pi interactions. Cation-pi interactions can play a crucial role in protein stabilization by contributing significant binding energies (Mitra and Das Mohapatra, 2023). Cation interactions can boost binding energies by 2–5 kcal/mol, according to several studies, making them compatible with hydrogen bonds and ion pairs in drug-receptor and protein-protein connections (Dougherty, 2013).

Table 5: Isolated and network (coloured pair) of cation-pi interactions of chromate reductase from *A. aceti, E. coli* and *P. Putida*

Species name	Position	Residue	Position	Residue	D(cation-Pi)	Angle
A. aceti	86	TYR	126	ARG	3.56	18.73
E. coli	19	PHE	15	ARG	5.26	134.66
	85	TYR	125	ARG	3.56	20.72
P. putida	100	TYR	98	ARG	5.91	64.25
	155	PHE	22	LYS	5.34	55.85
	19	TYR	15	ARG	4.69	166.28
	19	TYR	22	LYS	5.34	92.38

5. **Stability Check through Simulations:** Molecular dynamic simulations provide real-time reports on the stability, flexibility, packing conditions, etc through RMSD, RMSF, Rg, SASA, and hydrogen bonds (Mitra et al., 2022a). Root mean square deviation (RMSD) values are regarded as accurate predictors of variability when used with highly similar proteins, such as different protein conformations. However, because the RMSD value depends on the number of atoms included in the structural alignment, RMSD values obtained for structure pairings of different sizes cannot be directly compared. The difference between the backbones of a protein from its initial structural conformation to its ultimate location is measured using the RMSD. The variations generated during the simulation of the protein can be used to gauge its stability concerning its conformation. The protein structure is more stable when the smaller the variations are (Mitra and Das Mohapatra, 2022a). For a 50 ns simulation, the RMSD value for the C-alpha backbone was determined to assess the stability of both systems.

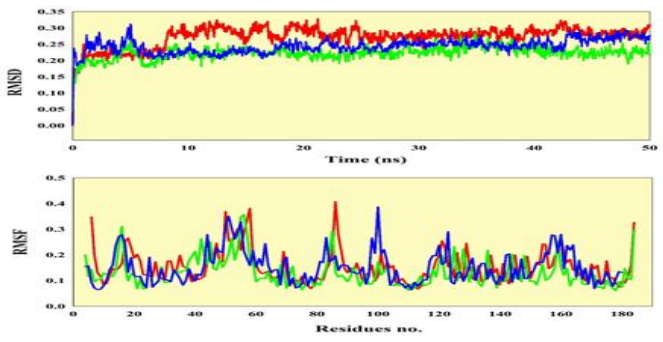

Figure 4: RMSD and RMSF of chromate reductase from*A.aceti* (red), *E. coli* (green) and *P. putida* (blue).

RMSD of *E. coli* showed the lowest trajectory throughout the path (Fig. 4). Initially, all 3-chromate reductases started at the same position, but up to 5ns chromate reductase of *P. putida* showed a slight deviation from others. But it normalized after that, and remain maintain a steady plot to the end of 50ns. *A. aceti* starts deviation at 10ns and follow the same trajectory throughout the run and ended at 0.3A. RMSD of *E. coli* and *P. putida* were very close to each other, showing their similar deviation pattern.

The RSMF can show which parts of the system are the most mobile, whereas the RMSD measures how far a structure deviates from a reference over time. Proteins are often only subjected to the backbone or alpha-carbon atom RMSF analysis since they are more indicative of conformational changes than the more flexible side chains (Mitra et al., 2023). High fluctuation in dynamic simulations was observed in *A. aceti*. The starting and the ending of the simulations run showed the same high fluctuation for chromate reductase of *A. aceti*. *E. coli* showed high fluctuation at residues no. 19, 55, 85, and 184. Chromate reductase of *P. putida* showed fluctuation at residue no. 45 and 100. The overall trajectory of *P. putida* showed quite less fluctuation than others.

When a polypeptide unfolds, its radius of gyration (Rg) varies, and this can serve as a key indicator of how the equilibrium unfolding process is progressing (Johnson et al., 2009). The analysis of Rg revealed the lower value in chromate reductase from *A. aceti* followed by *P. putida.* The lowest Rg indicates the tight packaging of protein. So, the chromate reductase from *P. putida* was more tightly packed than others.

Proteins' solvent-accessible surface area (SASA) has long been regarded as a key variable in research on protein folding and stability. It is described as the surface characterized by a hypothetical solvent sphere's center and the molecule's van der Waals contact surface all around a protein. A protein's amino acid residues can be categorized as buried or exposed based on SASA values. SASAs come in a variety of forms, ranging from those with relative solvent accessibility to those with absolute surface areas (Ausaf et al., 2014). Although, the chromate reductase from *P. putida* showed high SASA at residue no. 100, but its remaining part showed lower SASA than another chromate reductase. Lower SASA revealed the better folding pattern of chromate reductase in *P. putida.*

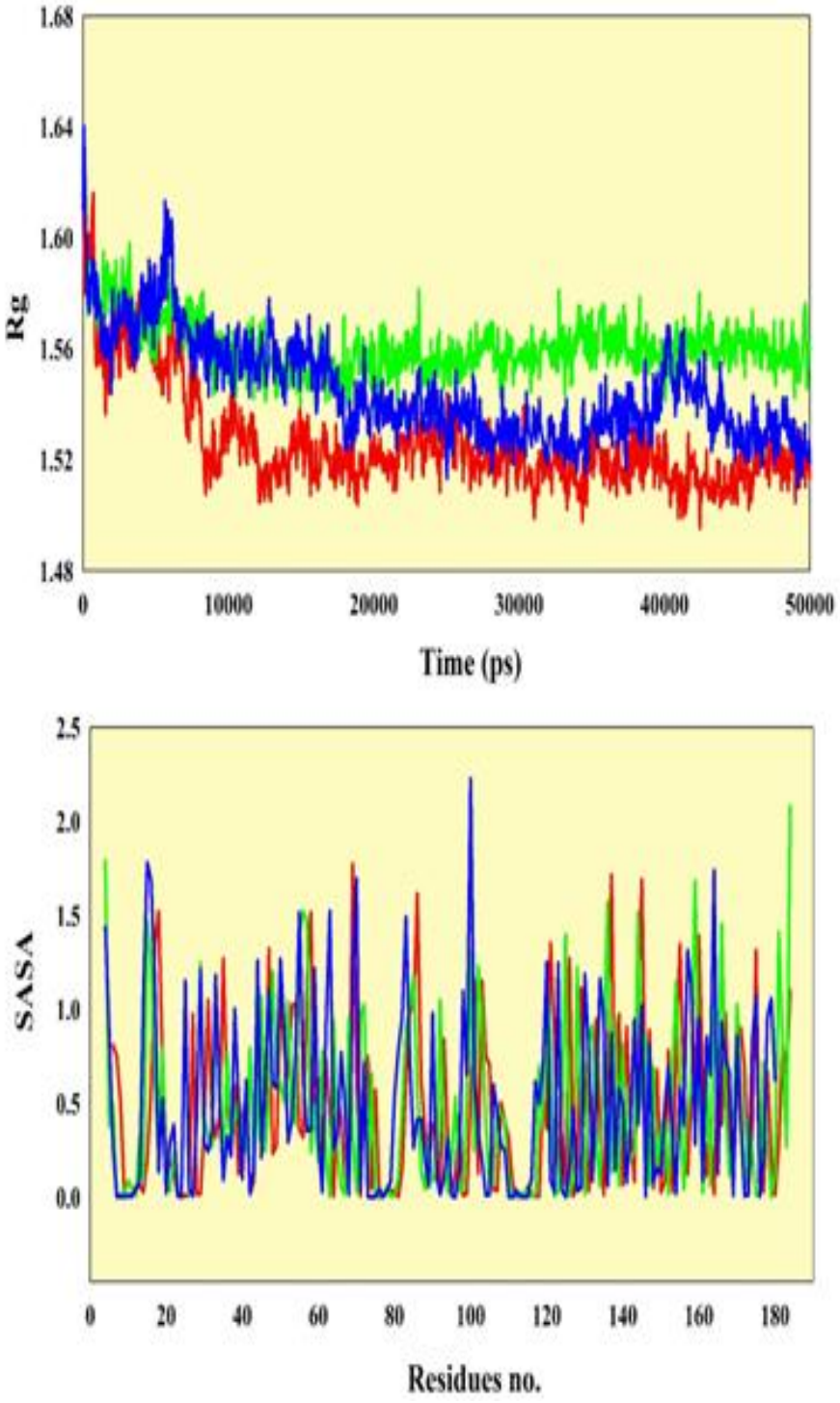

Figure 5: Rg and SASA of chromate reductase from*A.aceti* (red), *E. coli* (green) and *P. putida* (blue)

6. **Hydrogen bond:** In order to accurately simulate protein folding, hydrogen bond interaction must be accurately described. The hydrogen bond is crucial for sustaining the secondary structures of proteins. Hydrogen bonding is viewed as a nonbonded interaction in contemporary classical force fields, were electrostatic interaction reigns supreme.

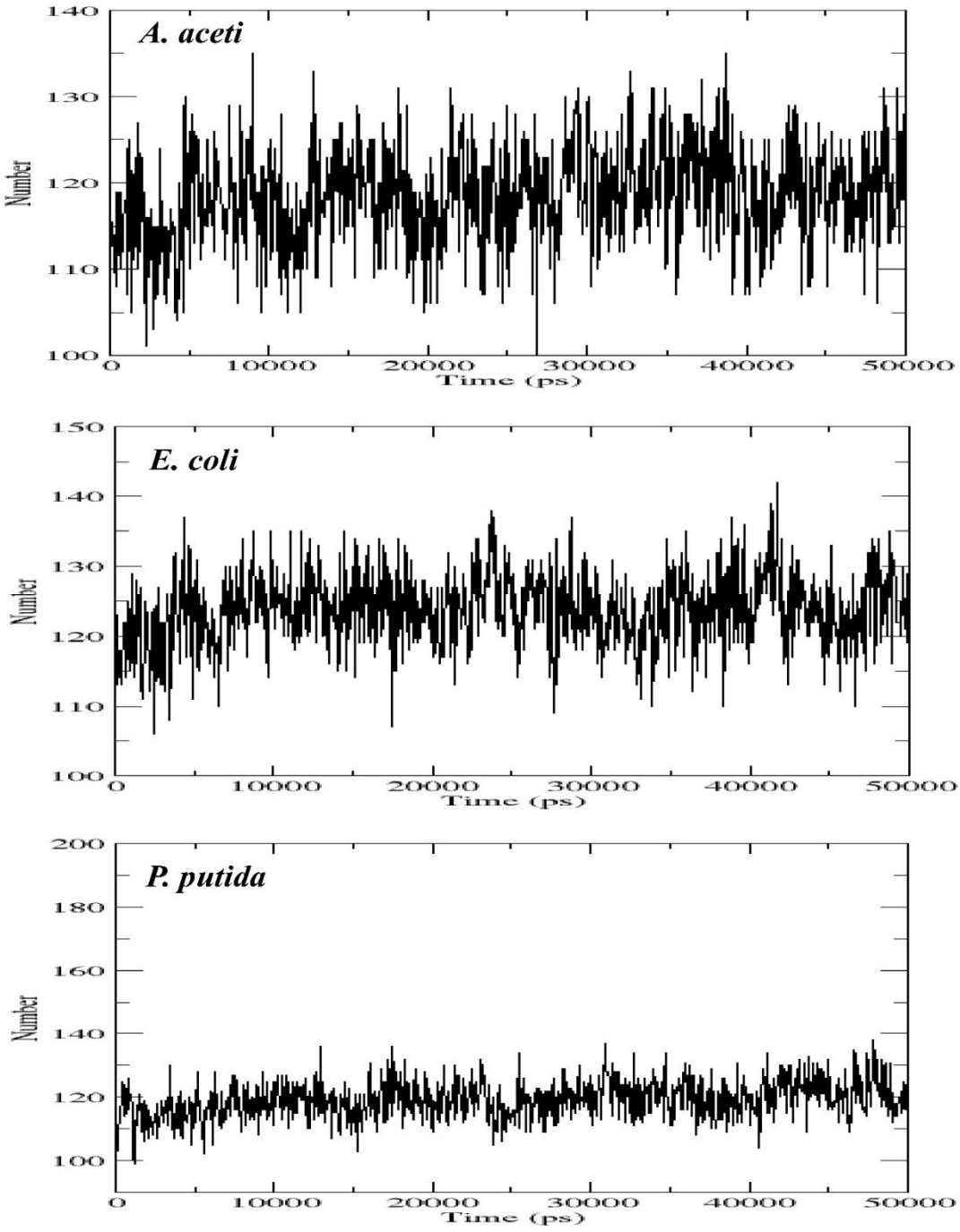

Figure 6: Hydrogen bonds in chromate reductase from*A.aceti, E. coli*and *P. putida*

Higher number of hydrogen bonds was formed in chromate reductase in *A. aceti* followed by *E. coli*. Not only were the numbers, the length of hydrogen bonds in P. putida lower than others. A continuous steady trajectory was maintained there.

IV. CONCLUSION

Hexavalent chromium is a very hazardous, transportable environmental contaminant that is also regarded as a priority. Chromate reductases have lately drawn interest due to their possible application in the bioremediation process. They are known to catalyze the reduction of Cr(VI) to Cr(III) and are present in chromium-resistant bacteria. To resist the pollution, chromate reductase of different mesophilic bacteria was taken for this study to find the best enzyme among them. In silico analysis revealed the amino acid diversity and the effectiveness of polar residues. Through the formation of a high number of intra-protein interactions chromate reductase of *P. putida* appeared as most stable in extreme conditions. Molecular dynamics simulations proved the chromate reductase of *P. putida*to be more stable, more tightly packed with better folding pattern. This study will emphasise in field of protein engineering to make more stable chromate reductase for counter the chromium pollution.

CONFLICT OF INTEREST

None

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None.

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