ORIGINAL ARTICLE

Structural and Functional Characterization of *Bacillus* Azoreductase: A Computational Study for Sustainable Azo Dye Bioremediation

Y. Ananth Kumar[1], M. Swetha[2], C. Dev Krishna Bharathi[3], Nagarajan Kalimuthu[4], V. Ragupathi[5], Mukesh Kumar Dharmalingam Jothinathan[6]

[1]Department of Biotechnology, APA College of Arts and Science, Tirunelveli, Tamil Nadu, India, [2]Department of Community Medicine, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India, [3]Department of Urology, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India, [4]Department of Ophthalmology, Centre for Global Health Research, Saveetha Institute of Medical College and Hospital, Saveetha University, Chennai, Tamil Nadu, India, [5]Department of Biotechnology, St. Joseph College (Arts and Science), Chennai, Tamil Nadu, India, [6]Department of Biochemistry, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India

Abstract

Background: Azo dyes are major environmental pollutants due to their toxicity, persistence, and resistance to conventional degradation methods. Azoreductases from *Bacillus* species play a crucial role in the reductive cleavage of azo bonds, offering an eco-friendly solution for dye bioremediation. Computational approaches provide valuable insights into enzyme structure and function for sustainable applications. **Aim:** To perform structural and functional characterization of azoreductase from *Bacillus velezensis* using *in silico* approaches to evaluate its potential in azo dye bioremediation. **Methodology:** The azoreductase sequence (Accession No. B3VPZ9) was retrieved and subjected to homology modeling using MODELLER, based on *Bacillus subtilis* azobenzene reductase (PDB ID: 1NNI). Structural validation was carried out using PROCHECK and Verify-3D. Molecular docking with selected azo dyes was performed to assess binding interactions and affinity. **Results:** The sequence showed high similarity (98.24%) with the template, enabling a reliable 3D model. Validation confirmed good stereochemical quality, with 98.7% residues in favored regions and a Verify-3D score of 94.61%. A conserved Rossmann fold and catalytic residues were identified. Docking studies revealed strong binding affinities, with Congo red showing the highest binding energy ($\Delta G = -7.99$ kcal/mol), supported by hydrogen bonding and hydrophobic interactions. **Conclusion:** The findings demonstrate the structural stability and functional efficiency of *Bacillus velezensis* azoreductase, highlighting its potential for sustainable azo dye bioremediation and eco-friendly wastewater treatment.

Key words: Azoreductase, *Bacillus velezensis*, comparative modeling, molecular docking, Sustainable Development Goals-12, Sustainable Development Goals-14, Sustainable Development Goals-6

INTRODUCTION

The synthetic dyes are crucial to the textile, dyeing, and printing industry, and the most popular category includes the azo dyes because they are structurally diversified, highly stable, and easy to prepare.[1,2] Although very important in industry, azo dyes have the potential to be environmental and health hazards due to their persistence, level of toxicity, and insensitivity to natural degradation.[3] They are usually present in

Address for correspondence:
Nagarajan Kalimuthu, Department of Ophthalmology, Centre for Global Health Research, Saveetha Institute of Medical College and Hospital, Saveetha University, Chennai, Tamil Nadu, India.
E-mail: nagabp@gmail.com

Received: 15-02-2026
Revised: 23-03-2026
Accepted: 30-03-2026

water bodies and may decrease the intensity of light reaching the water surface, eliminate photosynthesis, and release mutagenic or carcinogenic aromatic amines when they decompose.[4,5]

The physio-chemical treatment methodology (coagulation, adsorption, and advanced oxidation) has been utilized conventionally in the removal of dyes.[6] However, these are also very expensive, energy-consuming and can generate toxic sludge as a by-product.[6,7] On the contrary, microorganism and enzyme-based biological treatment is also a cost-effective and environmentally friendly option.[8,9] Of special interest are the enzymatic systems that are of interest, such as the azoreductases, which are capable of catalyzing the reduction cleavage of the azo bond (–N=N–) in either aerobic or anaerobic conditions, resulting in decolorization and detoxification of azo dyes.[10,11]

The majority of azoreductases are common in bacteria, fungi, and intestinal microbes, and they could be categorized as flavin-dependent or flavin-independent according to their cofactor needs.[12,13] In specific case, bacterial azoreductases have been getting considerable attention due to their stability and versatility to various environmental factors.[14,15] Bacillus genus is also characterized by having a strong enzymatic repertoire to break down a range of synthetic dyes, and they can be considered excellent biosensors in terms of bioremediation at the industrial scale.[16]

This research paper is dedicated to azoreductase of *Bacillus velezensis* (primary accession number: *B3VPZ9*, gene name: azr). This enzyme consists of 175 amino acids of about 19,360.31 Da. The other prior research has stated that *B. velezensis* strains have the potential to degrade azo dyes like Direct Red 28 and, by implication, the encoded azoreductase can be a key factor in this process.[17] In an effort to determine the structural and working properties of such an enzyme, the present study has been undertaken that utilizes bioinformatics, such as homology modeling and molecular docking, to forecast the 3D arrangement of the azoreductase and examine its relationship with the selected azo dyes. This form of *in silico* characterization makes a basis to work out enzyme substrate specificity as well as future experimental validation and enzyme engineering toward bioremediation.

MATERIALS AND METHODS

Retrieval of sequences and template selection

The three-dimensional structure of the azoreductase was built by MODELLER, an extensively used homology modeling software which uses spatial constraints, which predict protein structures using homologous templates already known. An alignment file in its PIR format was first created and matched the target sequence (B3VPZ9_9BACI) with that of the template (1NNI). This orientation guarantees the necessary structuring of the transfer of structural information, especially preserved residues, which are vital in catalytic activity and substrate binding.

MODELLER builds the 3D model with the help of an optimization algorithm that meets spatial constraints based on the template structure, dihedral angles, bond angles, and stereochemical parameters. This strategy takes advantage of the structural motifs of the azoreductase family being conserved throughout evolutionary history, permitting the conservation of the main functional domains. The refinement process consists of iterative refinement of the overall violation of the spatial constraints and this produces a model that preserves the architectural integrity of the enzyme active site and fold. Models of homology type are precious to explain the mechanism of enzymes, to direct experiments in mutagenesis, and to design inhibitors. The produced framework with its conserved characteristics of azoreductases offers a solid model to intelligible predictive support of substrate specificity, catalytic processes, and application opportunities in the bioremediation and enzyme engineering. The 3D structure that was modeled was assessed through the use of the Procheck and Verify 3D software. Procheck created a Ramachandran plot, which represents the permitted and prohibited regions on the backbone dihedral angles of the protein. The quality of the model was evaluated using Verify 3D the compatibility between the amino acid sequence and the 3D environment was analyzed.

Retrieval of ligands from PubChem

Aniline Yellow, Congo Red, Methyl Orange, and Chrysoine Resorcinol ligands were found in the PubChem database, a source of both information on small molecules and their biological activity. The chemical structures of the 2D chemical structures of these compounds were downloaded as SMILES strings, which will be used in later docking and virtual screening of the ligands, which are Aniline Yellow, Congo red, Methyl orange, and Chrysoine resorcinol. PubChem is a database, which contains information on the biological activities of small molecules. These ligands were 2D in nature and the structures were downloaded as Smiles strings.

Generation of 3D coordinates

The SMILES bibliographies of the ligands, namely the Aniline Yellow, the Congo Red, the Methyl Orange, and the Chrysoine Resorcinol, were transformed into three-dimensional chemical structures in CORINA, the most advanced tool of computational chemistry that is famous for the effectiveness of the creation of realistic 3D atomic coordinates based on the molecular formulae. CORINA uses a systematic algorithm which takes into account stereochemistry, bond lengths, bond angles, and torsional degrees of freedom to generate energetically favorable conformations which proved useful in docking studies. The resulting 3D structures were

observed and studied with the help of PyMOL that provides an opportunity to study the geometry of the molecules, the presence of functional groups, and possible sites of interaction in detail. This 3D implementation of 2D systems is a significant milestone in the design of structure-based drugs because it can give the spatial context of ligand-receptor interactions, affinity to bind, and specificity.

Molecular docking

It was carried out to examine the interactions between azoreductase and azo dye ligands with AutoDock 4.1. PDBQT formatted protein and ligand structures were made in a bid to do correct simulations. The search for binding conformations was efficient using Monte Carlo simulated annealing algorithm and genetic algorithms developed in AutoDock. It employed a grid-based technique to quickly calculate binding energies, such as those of van der Waals binding, electrostatic binding, and hydrogen bonds. Docking results were analyzed to give an understanding of binding conformations, energies, and mechanisms of interaction that are useful in understanding the enzyme specificity as well as to improve bioremediation strategies.

RESULTS AND DISCUSSION

Sequence retrieval and homology analysis

The amino acid sequence of azoreductase of *B. velezensis* has been obtained from the Swiss-Prot database (Accession No. B3VPZ9), which provides an excellent annotation accuracy. An analysis using the BLASTp against the Protein Data Bank (PDB) revealed the best structural template to be azobenzene reductase of *Bacillus subtilis* (PDB ID: 1NNI) with a very high sequence identity of 98.24 [Figure 1]. This extraordinary homology has a high prediction of much homology conservation, especially of regions of catalytic interest. Earlier research has determined that sequence identities of more than 30 40% are adequate to have a high homology modeling, and the accuracy rate rises significantly above 60%.[18] Thus, the high similarity here gives a high degree of confidence to the structural fidelity of the modeled enzyme and facilitates a proper inference on active-site architecture as well as substrate recognition properties.

Comparative modeling and structural features

In *B. velezensis* azoreductase, a three-dimensional structure was produced using MODELLER which was used upon the 1NNI as a template. The calculated structure presented a preserved Rossmann fold which consisted of 2-alpha-strand that forms the central dinucleotide-binding unit. This is a fold that is characteristic of flavin-dependent oxidoreductases, and it is mandatory to bind nicotinamide adenine dinucleotide phosphate or flavin mononucleotide and transfer electrons in the process of catalysis.[18] This motif indicates that the enzyme belongs to the flavin-dependent azoreductase superfamily and is congruent with earlier reported structures of dye-reducing enzymes of various bacterial species, showing conservation of the motif among the enzymes.[19] The fact that this fold is preserved indicates its functional role in redox reactions and indicates its appropriateness for bioremediation use.

Structural validation of the modeled protein

Originally, the predicted model was stringently evaluated with the help of PROcheck and Verify 3D. The PROCHECK analysis has indicated that 98.7% of the residues [Figures 2a and b and 3] were located in the most preferred locations since the Ramachandran plot, and the remaining 1.3% were located in additional allowed regions of the plot with none being in the disallowed regions. The stereochemical purity of this distribution is high, and the conformational strain is low.[9] Although the analysis done using 3D revealed a framework same compatibility score of 94.61, which is above the established standard of 80, this shows that the residue surroundings correspond with the natural protein structures.[20] On the whole, both of these validation measures would support the idea that the modeled structure is strong and can be used to interpret it functionally, perform docking studies, and make mechanistic predictions. It is advised that such multi-parameter validation should be used to establish the reliability of the model before proceeding with the ligand-binding studies.[21]

Molecular docking and substrate interaction analysis

AutoDock was used to conduct molecular docking experiments on the interaction between the modeled azoreductase and four representative azo dyes, namely Congo Red, Methyl orange, Aniline Yellow, and Chrysoine resorcinol. The binding energies were negative (thermodynamically favorable interactions) in all the ligands. Congo red had the highest binding affinity of all the above, namely −7.99 kcal/mol, Methyl orange −7.52 kcal/mol, Aniline Yellow −5.16 kcal/mol, and Chrysoine resorcinol −5.02 kcal/mol [Figures 2a and b and 4]. Preferential binding of Congo red implies greater affinity of the substrate, which may result in an increased catalytic activity of this dye.

Docking studies have demonstrated that the following conserved residues: SER36, SER38, ASP31, PHE32, and GLN61 are important in interaction with the substrate. All likely reactions in serine and aspartate are that it mediates hydrogen bonding and stabilization of transition states, which is essential for electron transfer during the reduction of azo bonds. PHE32 aromatic residues can form π--S interactions with aromatic dye structures, whereas the GLN61 polar residues can be used to position the substrates using hydrogen bonds. These residues are consistent with the active-site motives given in other

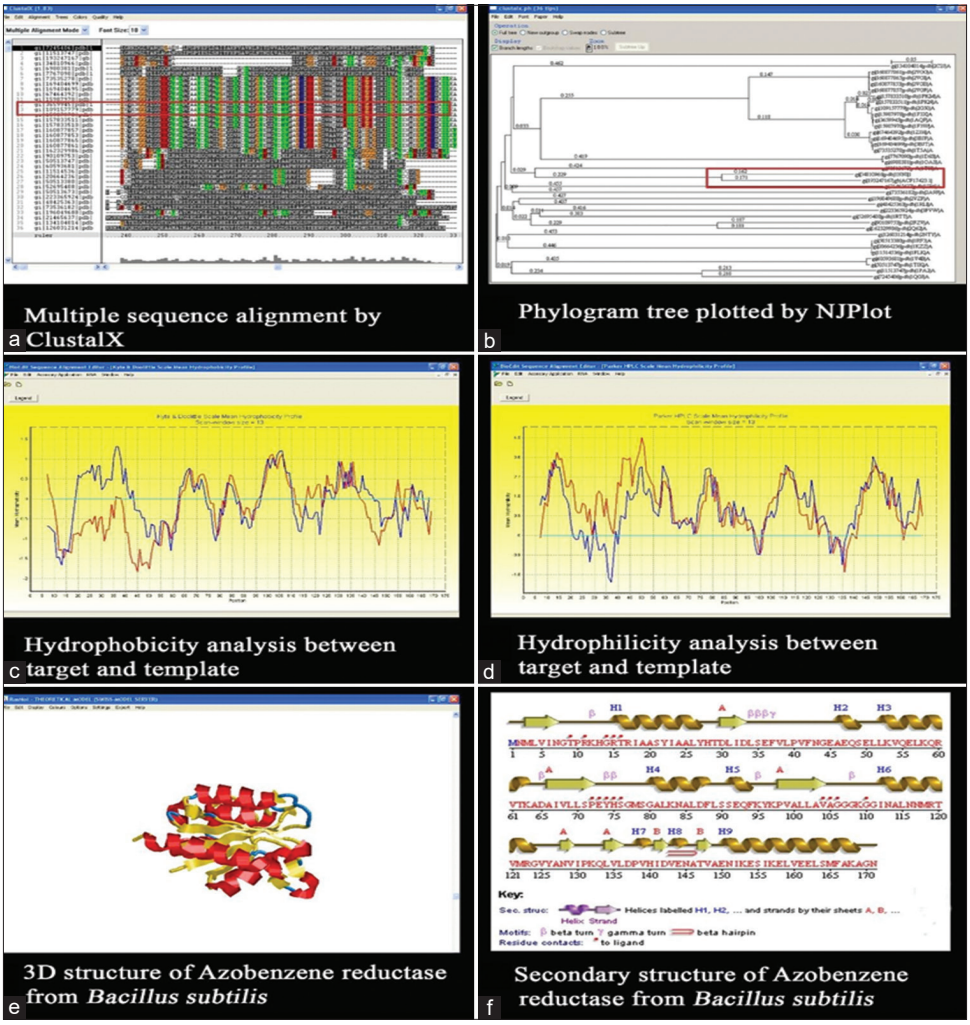

Figure 1: (a-f) Template selection: Template protein has 171 residues and has 67% identity with the target sequence. Template protein has 46% helical (9 helices and 81 residues) and 19% beta sheet (10 strands and 34 residues) and chain type (sandwich) architecture and topologically Rossmann fold

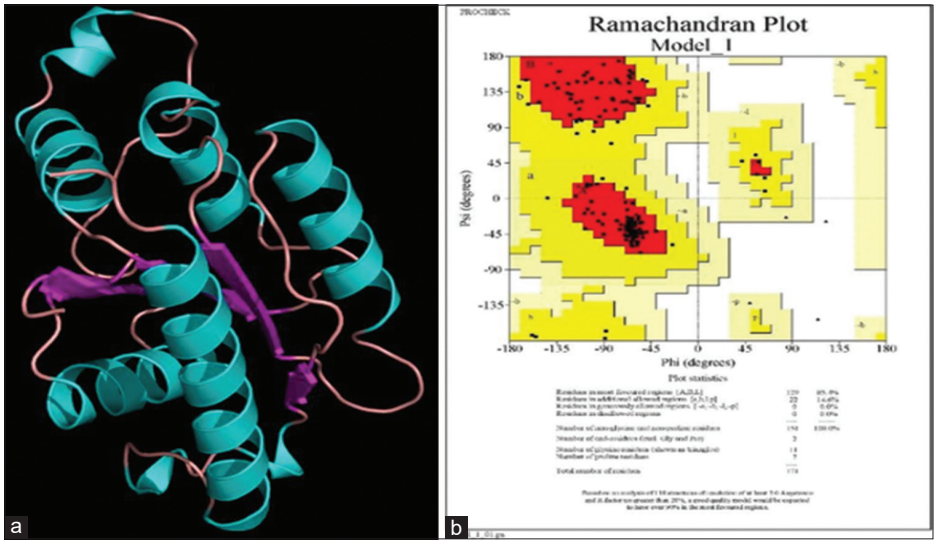

Figure 2: (a) 3D structure of azoreductase from *Bacillus velezensis*; (b) Ramachandran plot (Procheck checks the stereochemical quality of a protein structure which gives and distribution values of Ramachandran plot of non-glycine and non-proline residues and stereochemical quality of overall structure geometry analyzed. Mostly, 100% of the residues were in favored and allowed regions

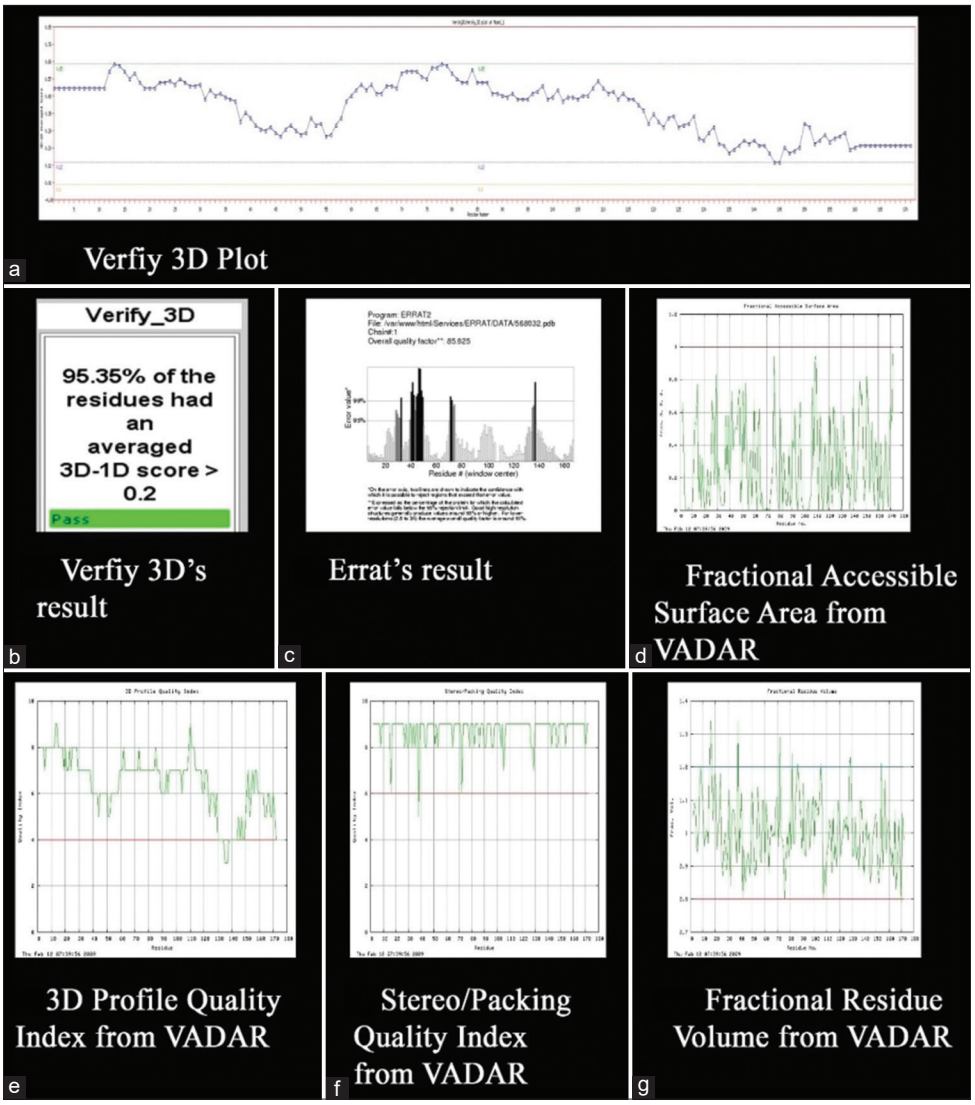

Figure 3: (a-g) Validation of 3D Model of *Bacillus velezensis* azoreductase*. *The Verify-3D graph corresponded to acceptable environment of the model. The high score of 0.65 indicates that the environment profile of the model is good. 85.625% of the overall quality factor obtained by ERRAT which shows that good high resolution of the 3D model of the target protein)

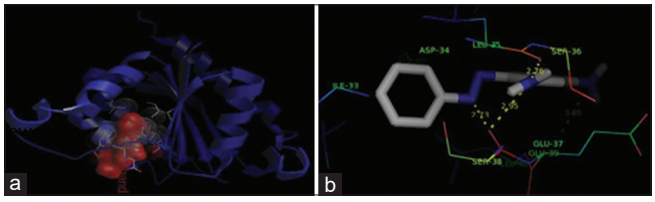

Figure 4: (a) Methyl orange versus Azoreductase. Docking result 1–Python. (b) Docking Result 2–PyMol

bacterial azoreductases, highlighting the fact that the substrate recognition and catalytic process are preserved.[22]

Functional implications and bioremediation potential

The differences in binding affinities between the dyes observed give some understanding of the substrate selectivity and catalytic selectivity of *B. velezensis* azoreductase [Figure 4]. Greater affinities of the enzyme to structurally complex dyes like Congo red suggest flexibility of the enzyme to large-bulky aromatics which is favorable in the treatment of wastewater. It has been demonstrated that Bacillus-derived azoreductases tend to be more stable and possess wider and broader substrate profiles than those of other genera, which augment their interest in industry.[22,23] It is also indicated by the conservation of the Rossmann folds and active-site residues that this enzyme can be rationally engineered to enhance catalytic activity, substrate affinity, or environmental stability based on earlier studies in which the optimization of enzymes was accomplished through mutagenesis.[24,25]

Overall significance

The *in silico* studies presented here suggest that computational studies may be useful in explaining the relationship between enzyme structures and their functions. The conserved

catalytic architecture, validated 3D model, and desirable docking interactions all combined in support of the potential of *B. velezensis* azoreductase as a robust biocatalyst for azo dye degradation. These results not only support the literature that exists on the bacterial azoreductases but also form a good basis for experimental validation and rational enzyme engineering with the view of coming up with an efficient and eco-friendly solution to the environmental bioremediation process.

CONCLUSION

In silico analyses in this paper provide a very in-depth insight into the structure and functional characteristics of azoreductase of *B. velezensis* that supports its use as a useful biocatalyst in decomposing azo dyes. A high-quality 3D model was constructed by homology modeling of bacterial *B. subtilis* azobenzene reductase using the structurally related model which has been confirmed by various stereochemical analyses. The conservation of Rossmann fold and the clear architecture of active-site is a hint of the evolutionary conservation and catalytic significance of the enzyme. The outcome of molecular docking supports the high affinity of the enzyme to azo dyes, especially Congo red, and shows the correlation with the usage of azo dyes in bioremediation practice. The active site residues found in docking are key active-site residues in known globally conserved catalytic motifs in closely related bacterial azoreductases, confirming the predictable interaction details. Together, as a body of computational work, these computational results provide a basis for future functionality-checking and rational engineering work in the laboratories. The research proves that combined bioinformatics strategies can be used to describe enzyme systems and give meaningful information which could be employed in future plans of improving the environmental and industrial significance of azoreductases.

HUMAN AND ANIMAL RIGHTS DECLARATION

All authors hereby declare that there are no ethical concerns related to human or animal rights in this study.

CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

All the authors agreed to publish the data in this journal.

AVAILABILITY OF DATA AND MATERIALS

The datasets used or analyzed in this study are available from the corresponding author upon request.

REFERENCES

1. Bafana A. Identification and characterization of azoreductase enzyme AzoR2 from *Bacillus velezensis* for biodegradation of azo dyes. Inter Biodeterior Biodegradation 2022;167:105351.
2. Baker D, Sali A. Protein structure prediction and structural genomics. Science 2001;294:93-6.
3. Chen H, Hopper SL, Cerniglia CE. Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. Microbiol (Reading) 2005;151:1433-41.
4. Chung KT, Stevens SE Jr. Degradation of azo dyes by environmental microorganisms and helminths. Environ Toxicol Chem 1993;12:2121-32.
5. Colovos C, Yeates TO. Verification of protein structures: Patterns of nonbonded atomic interactions. Protein Sci 1993;2:1511-9.
6. Forgacs E, Cserháti T, Oros G. Removal of synthetic dyes from wastewaters: A review. Environ Inter 2004;30:953-71.
7. Holkar CR, Jadhav AJ, Pinjari DV, Mahamuni NM, Pandit AB. A critical review on textile wastewater treatments: Possible approaches. J Environ Manage 2016;182:351-66.
8. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 2015;10:845-58.
9. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: A program to check the stereochemical quality of protein structures. J Appl Crystallogr 1993;26:283-91.
10. Leelakriangsak M. Molecular approaches for bacterial azoreductases. Songklanakarin J Sci Technol 2013;35:647-57.
11. Mustafa G, Zahid MT, Kurade MB, Patil SM, Shakoori FR, Shafiq Z, *et al*. Molecular characterization of azoreductase and its potential for the decolorization of Remazol Red R and Acid Blue 29. Environ Pollut 2023;335:122253.
12. Ngo AC, Tischler D. Microbial degradation of azo dyes: Approaches and prospects for a hazard-free conversion by microorganisms. Inter J Environ Res Public Health 2022;19:4740.
13. Oliveira JM, Poulsen JS, Foresti E, Nielsen JL. New insights into the mechanism of azo dye biodegradation by *Lactococcus lactis*. J Environ Chem Eng 2024;12:113670.
14. Oyetade JA, Machunda RL, Hilonga A. Photocatalytic degradation of azo dyes in textile wastewater by polyaniline composite catalyst-a review. Sci Afr

2022;17:e01305.
15. Pandey, A, Singh P, Iyengar L. Bacterial decolorization and degradation of azo dyes. Inter Biodeterioration Biodegr 2007;59:7384.
16. Pinheiro LR, Gradíssimo DG, Xavier LP, Santos AV. Degradation of azo dyes: Bacterial potential for bioremediation. Sustainability 2022;14:1510.
17. Rane A, Joshi SJ. Biodecolorization and biodegradation of dyes: A review. Open Biotechnol J 2021;15:97-108.
18. Rao ST, Rossmann MG. Comparison of super-secondary structures in proteins. J Mol Biol 1973;76:241-56.
19. Robinson T, McMullan G, Marchant R, Nigam P. Remediation of dyes in textile effluent: A critical review on current treatment technologies with a proposed alternative. BioresourTechnol 2001;77:247-55.
20. Singh RL, Singh PK, Singh RP. Enzymatic decolorization and degradation of azo dyes: A review. Inter Biodeterioration Biodegr 2020;153:105053.
21. Stolz A. Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 2001;56:69-80.
22. Wang CJ, Hagemeier C, Rahman N, Lowe E, Noble M, Coughtrie M, *et al*. Molecular cloning, characterisation and ligand-bound structure of an azoreductase from *Pseudomonas aeruginosa*. J Mol Biol 2007;373:1213-28.
23. Zafar S, Bukhari DA, Rehman A. Azo dyes degradation by microorganisms - an efficient and sustainable approach. Saudi J Biol Sci 2022;29:103437.
24. Zimmermann T, Kulla HG, Leisinger T. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. Eur J Biochem 1982;129:197-203.
25. Zollinger H. Color Chemistry: Syntheses, Properties, and Applications of Organic Dyes and Pigments. 3rd ed. Cambridge: Wiley-VCH; 2003.

Source of Support: Nil. **Conflicts of Interest:** None declared.