**Veritas** Volume1 |Issue1| July 2026
Edmundian Journal of Multidisciplinary Research (EJMR) ISSN: pp: 18-32

# Deciphering the Binding Mechanism of Azodyes to *Pseudomonas putida* Laccase enzyme: A Computational Biology approach

[1]Sibange Paul, [1]Barihun Thyrniang, [2]Rangraj Koch, [1]Dominic Lyngdoh, [1]Shekinah Challam, [1]Sumit Deb and [1]Samrat Adhikari*

*[1]Advanced Biotechnology Research Laboratory, Department of Biotechnology, St. Edmund's College, Shillong-793003, Meghalaya, India*
*[2]Department of Agricultural Biotechnology, Dundalk Institute of Technology, Dublin University, Ireland*

Received: 17 March 2026| Reviewed: 16 Jun 2026| Revised: 10 July 2026 |Accepted: 12 July 2026

## ABSTRACT

Laccase, a metalloenzyme that contains copper which readily oxidizes a wide variety of organic and inorganic substances and could degrade phenolic and non-phenolic compounds. This property could possibly be exploited to detoxify a varied range of environmental pollutants including commercially important dyes viz., Crystal Violet, Congo Red, Indigo, Direct Blue 9 etc. These dyes are very important commercially, but their disposal becomes an issue as it pollutes the water sources wherein, they are readily thrown. Enzymes from bacteria and cyanobacteria have shown much promising results in degrading the dyes into simpler compounds and their decolorization makes them less harmful so they can be degraded by nature subsequently. In our current study, we have tried to model the structural interaction of Laccase enzyme derived from *Pseudomonas putida* and these industrial dyes, which in turn gives us insights into the potential of degrading these harmful dyes for downstream wastewater treatment. This study can be further taken into consideration to learn more about bacterial laccase structural features and its detoxifying activities to remove hazardous environmental pollutants.

**KEYWORDS:** Laccase, Multiple Sequence Alignment, domains and motifs, homology modelling, molecular docking, *Pseudomonas putida*.

## INTRODUCTION

Rapid industrialization and modernization have led to the extensive release of untreated industrial effluents into the environment, making wastewater pollution one of the most pressing environmental challenges worldwide. Among various industrial pollutants, synthetic dyes discharged from textile, paper, leather and dye manufacturing industries constitute a major source of aquatic contamination due to their persistence, toxicity and resistance to biodegradation (Thakuria et al., 2015). The continuous discharge of dye-containing effluents not only imparts intense coloration to water bodies but also reduces light penetration, disrupts aquatic ecosystems and poses serious health risks to humans and other living organisms. Consequently, there is an urgent need to develop sustainable and environmentally friendly strategies for the treatment of dye-contaminated wastewater. The synthetic dye decomposition techniques such as adsorption on various sorbents, chemical decomposition by oxidation, photodegradation and microbiological decolouration are developed (Forgacs et al., 2004).

Microorganisms have emerged as promising agents for the biodegradation of recalcitrant organic pollutants owing to their remarkable metabolic diversity and adaptability. Soil bacteria hold a high contribution in the catalysis of aerobic treatment of the wastes from chemical plants (Levine, 1991). Anaerobic bacteria such as *Clostridium* species demonstrated treatment of various azo dyes

*Author for Correspondence: Dr Samrat Adhikari
secbiotech@gmail.com

without leading to mutagenicity in the *Salmonella typhimurium* strains TA98 and TAI00 (Raffi et al., 1997). Most of the dyes and the chemicals were observed to be utilized by an alkalophilic microorganisms' consortium parallel to nitrogen consumption by the consortia for growth (Brown & De Vito, 1993). Among them, *Pseudomonas putida*, a Gram-negative bacterium widely distributed in soil and aquatic environments, has attracted considerable attention because of its exceptional ability to metabolize a broad spectrum of aromatic compounds and industrial contaminants. This metabolic versatility is attributed to its diverse enzymatic machinery, enabling the degradation of numerous xenobiotic compounds, including synthetic dyes. Although azoreductases have been extensively investigated for their role in azo dye degradation, increasing awareness has recently been focussed towards bacterial laccases as multifunctional oxidative enzymes capable of degrading structurally diverse pollutants.

Laccases (EC 1.10.3.2) are multicopper oxidoreductase enzymes that catalyse the oxidation of a wide range of phenolic and non-phenolic substrates while simultaneously reducing molecular oxygen to water (Madhavi and Lele, 2009). Their broad substrate specificity, coupled with their ability to catalyse oxidation without requiring additional cofactors, has made laccases highly attractive for numerous biotechnological and environmental applications. Compared with fungal laccases, bacterial laccases exhibit several advantageous characteristics, including greater thermal stability, wider pH tolerance, enhanced resistance to harsh environmental conditions, and improved biochemical stability, making them particularly suitable for industrial wastewater treatment processes. *Pseudomonas* sp. SU-EBT intracellular laccase activity induction describes its role in the decolorization of Congo red (Telke, et al., 2010). The textile, pulp, paper, leather and dye manufacturing industries generate enormous volumes of coloured wastewater containing synthetic dyes with complex aromatic structures such as azo, anthraquinone and triarylmethane chromophores. These structural characteristics render the dyes highly resistant to natural biodegradation and conventional wastewater treatment methods (Chen, 1989; Zollinger, 1991). It is estimated that approximately 10–15% of the dyes used during textile processing are released into industrial effluents, causing severe environmental contamination (Yu, 2004).

Conventional physicochemical treatment methods, including coagulation, adsorption, and chemical oxidation, are often expensive, energy-intensive and may generate secondary pollutants. In contrast, microbial and enzymatic degradation approaches offer environmentally benign, cost-effective and sustainable alternatives for the detoxification and mineralization of these hazardous compounds. For practical industrial applications, detailed characterization of biodegradative enzymes is essential to understand their catalytic mechanisms, substrate specificity, structural stability, and potential for immobilization, which can significantly enhance enzyme reusability and operational efficiency in continuous wastewater treatment systems (Velu et al., 2011).

Advances in computational biology have further enabled comprehensive structural and functional analyses of enzymes through *in silico* approaches, providing valuable insights into enzyme–substrate interactions prior to experimental validation. In this context, the present study aims to establish a comprehensive *in silico* computational framework for the structural characterization of laccase from *Pseudomonas putida*. The study focuses on predicting the three-dimensional structure of the enzyme, evaluating its physicochemical and structural properties, and investigating its molecular interactions with three commercially important synthetic dyes—Congo Red, Indigo, and Crystal Violet—using molecular docking approaches. The findings of this study are expected to enhance our understanding of the catalytic potential of *P. putida* laccase and provide a theoretical basis for the development

of efficient enzyme-based bioremediation strategies for the treatment of dye-contaminated industrial wastewater.

## METHODOLOGY

### Sequence retrieval

The primary amino acid FASTA sequence of the laccase enzyme from *Pseudomonas putida* was retrieved from the NCBI database with the reference accession number WP_016713391.1 (Bernstein et al, 1977). The sequence analysis indicated a length of 246 amino acids for the monomeric protein chain. The three-dimensional chemical structures of the target industrial dyes—namely Congo Red (PubChem CID: 6166), Crystal Violet (PubChem CID: 11057), and Indigo (PubChem CID: 5318597)—were retrieved from the PubChem compound database in SDF format (Wang et al, 2009). The laccase protein sequence was subsequently subjected to BLASTp analysis against the non-redundant protein database, from which 15 closely related homologous sequences were selected based on high query coverage and sequence identity for subsequent alignment (Altschul S.F. et al, 1990).

### Multiple Sequence Analysis

The 15 homologous hits retrieved from the BLASTp results were aligned for multiple sequence analysis using the CLUSTALW algorithm within the MEGA software package (Tamura K et al, 2007; Larkin M.A. et al, 2007). Based on the multiple sequence alignment, a phylogenetic tree was reconstructed using the Neighbour-Joining (NJ) method. To address critical statistical reliability and ensure tree robustness, the phylogenetic tree was evaluated using 1000 bootstrap replicates, and the final sequence conservation, consensus regions, and evolutionary relationships were analysed and visualized using the Jal View workbench (Waterhouse A.M., et al, 2009).

### Domain analysis and Motif searching

Domain analysis was performed using the Conserved Domain Architecture Retrieval Tool (CDART) at NCBI (Geer et al, 2002) to map the functional and putative domain architectures of the enzyme. To achieve higher sequence-level resolution of functional motifs, structural motif searching was completed using the Motif-Scan server against the Pfam database profiles (Naughton B.T. et al 2006), ensuring precise identification of signature multicopper oxidase binding sites.

### Homology Modelling

The three-dimensional structure of the *P. putida* laccase enzyme was predicted using the SWISS-MODEL workspace web server (Waterhouse, A., et al, 2018). The target FASTA sequence was modelled using the high-resolution crystal structure of the multi-copper oxidase from *Escherichia coli* (PDB ID: 1u05) as the primary structural template, which was chosen based on optimal Global Model Quality Estimation (GMQE) and QMEAN scoring parameters generated by the SWISS-MODEL pipeline for the target-template alignment (template PDB ID 1u05; the template-derived sequence identity, sequence coverage, template resolution, GMQE score, and QMEAN score for the selected model are reported in Table S1). While the target monomeric chain contains 246 residues, the functional template (1u05) exists as a homo-oligomer; hence, the target model was generated in its biological multimeric configuration to closely mirror native functional architectures, resulting in a total of 412 residues evaluated in the final structural coordinate file. The structural and stereochemical validity of the generated 3D coordinates was thoroughly evaluated using PROCHECK via the generation of a Ramachandran plot (Laskowski R.A., et al, 1993).

### Molecular Docking studies

To investigate the geometric complementarity and specific non-covalent interactions between the *P. putida* laccase and the industrial dyes, molecular docking studies were conducted using the PATCHDOCK web server (Duhovny et al., 2002; Schneidman et al., 2005). Patch Dock utilizes a rigid-body shape complementarity algorithm to evaluate receptor-ligand fits. Because specific catalytic active-site residues for this novel bacterial laccase variant have not been previously mapped through *in vitro* experimental assays,

an unbiased blind docking approach was executed across the entire protein surface to comprehensively identify potential binding pockets. Default docking parameters were maintained. The resulting top-scoring docked complexes were subsequently processed for structural refinement and atomic contact energy (ACE) minimization using the Fire Dock algorithm, followed by final local model quality assessments using the QMEAN server functionalities (Benkert P. et al., 2009; Benkert P. et al., 2008).

## RESULTS

### Sequence retrieval and BLAST

The multi-copper polyphenol oxidoreductases from closely related *Pseudomonas* and Gamma proteobacteria species demonstrated the highest alignment scores and sequence identities, reaching up to 100% and 98%, respectively. Across all 15 selected homologous hits, the sequence identity remained remarkably high, decreasing to a minimum of 96% for the lowest hit, while consistently maintaining a 100% query coverage. This confirms a highly conserved evolutionary lineage for the laccase enzyme target within the genus *Pseudomonas*.

### Multiple Sequence Alignment

Multiple sequence alignment performed using CLUSTALW via MEGA revealed significant structural conservation (Fig. 1). Visualization through Jal View underscored high-density blocks of conserved domains, strict consensus segments, and high-quality alignment profiles. The reconstructed Neighbour-Joining phylogenetic tree highlighted clear evolutionary distances and branching lineages among the 15 sequences (Fig. 2). The high bootstrap value support confirms that the target sequence is highly homologous to established bacterial multi-copper oxidases.

### Domain analysis and Motif searching

Domain characterization via CDART identified a signature multicopper oxidase architecture, showcasing relevant domain arrangements across related bacterial groups such as Enterobacteriaceae, Teredinibacter, and others (Fig. 3). Motif-Scan analysis successfully mapped a definitive 'Cu-oxidase_4' multi-copper polyphenol oxidoreductase laccase signature (under Pfams's: Cu-oxidase_4) spanning residues 34-243. This motif returned an exceptional raw score of 401.5, a normalized N-score of 128.673, and an E-value of 4.5e-122 (Fig. 4). Additionally, a minor Seven Residue Repeat (SRR) motif (pfam_fs: SRR) was detected between residues 167-176 with a raw score of 1.2 and an E-value of 1.6, confirming the biological annotation of the protein as a true laccase family member.

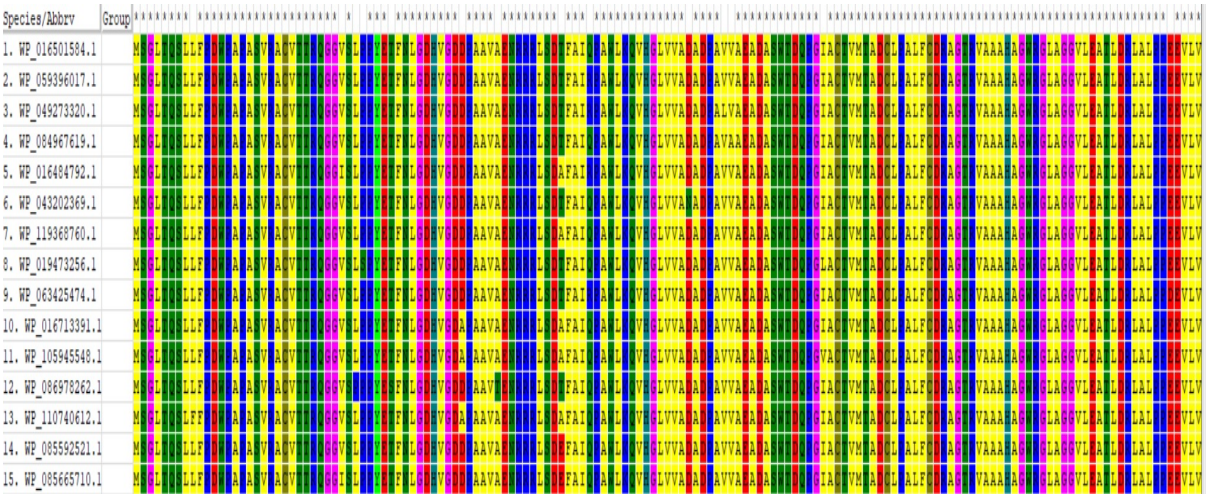


Figure 1: Showing the alignment of 15 sequences that were taken from the BLAST result page. All these sequences taken showed the highest of 100% identity and the lowest of 96% till the last sequence taken. The alignment is done in MEGA using the ClustalW alignment program. The sequences show a very good alignment with the query sequence.

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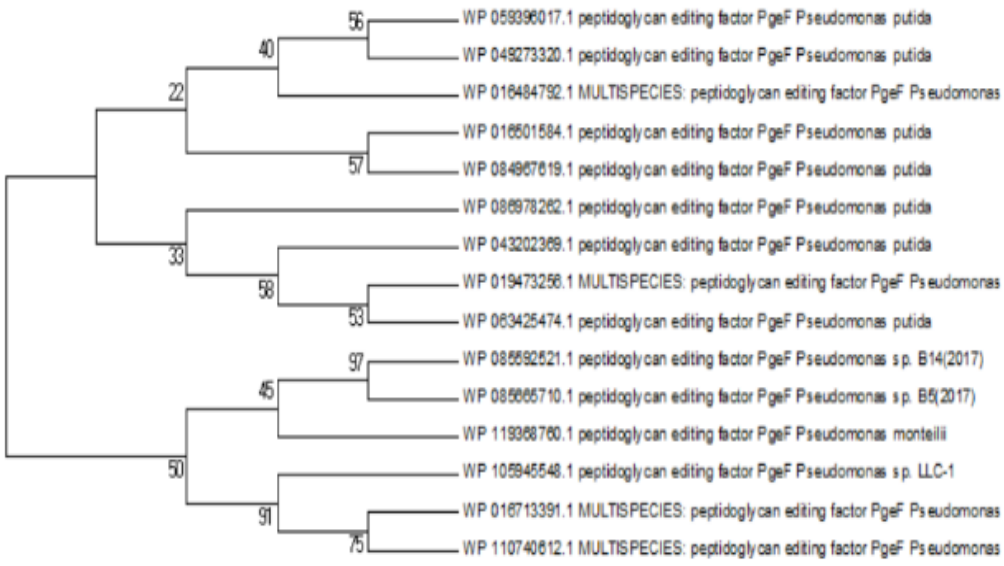


Figure2: Showing the Neighbour Joining Phylogenetic tree with 500 bootstrap replicates generated via MEGA and viewed in Jal View. The tree is also provided with the branch lengths in them. Since Neighbour Joining is considered accurate, the tree was constructed with that method and since only 15 sequences were taken, hence 500 bootstrap steps are enough.

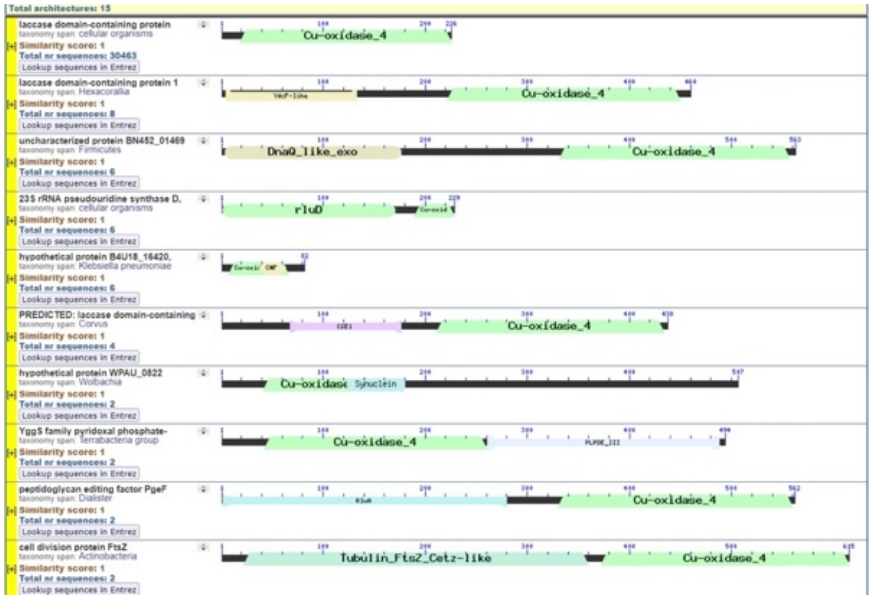


Figure 3: Showing the domains present in the laccase enzyme from *P. putida*. CDART was utilized for generating the domains present in the enzyme. The query sequence was pasted, and the domains were shown as per the figure above

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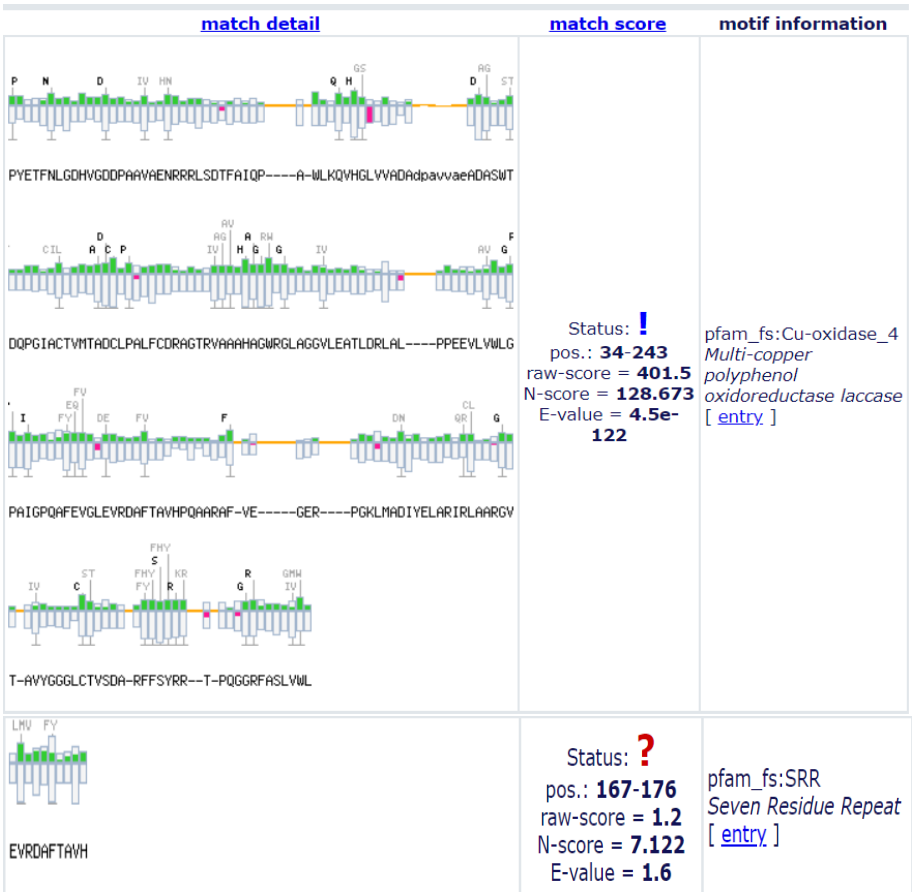


Figure 4 : Showing the motif scan analysis of laccase enzyme from *P. putida*. It also shows the raw score, N-Score and E-value also followed by the motif information

**Homology Modelling**

The 3D structural model of the laccase enzyme was successfully built using the SWISS-MODEL server based on the template multi-copper oxidase structure (PDB ID: 1u05) (Fig. 5).Stereochemical assessment via PROCHECK generated a detailed Ramachandran plot to evaluate backbone conformation stability. The statistics revealed that 80.1% of the residues (330 residues) were positioned inside the most favoured regions, 18.2% (75 residues) inside additional allowed regions, 1.0% (4 residues) inside generously allowed regions, and a minor 0.7% (3 residues) fell within disallowed regions, out of 412 total evaluated non-glycine and non-proline residues. While the presence of only 3 residues in the disallowed regions indicates a stable structural core, the percentage of residues inside the most favoured region (80.1%) is somewhat lower than the standard 90% benchmark typically expected for high-resolution experimental structures. This variation can be attributed to the loop flexibilities inherent to modelling a bacterial multimeric enzyme state. Consequently, this model serves as a reliable structural prototype for initial *in silico* target screening, though subsequent molecular dynamics (MD) simulations would be beneficial to refine local loop geometries and optimize regional backbone stability.

**Molecular Docking studies**

Rigid-body molecular docking via the PATCHDOCK server yielded valuable insights into the prospective binding configurations and surface shape complementarity between the laccase model and the three dyes (Table 1, Fig. 6). Among the evaluated ligands, Congo Red achieved the highest geometric complementarity docking score of 7434 with an

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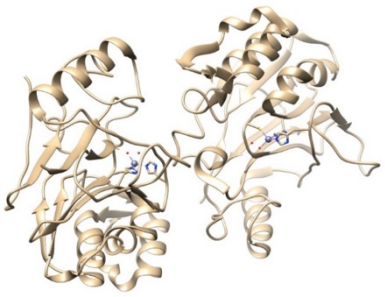
interacting surface area of 949.60 Å² and a favourable atomic contact energy (ACE) of -74.63 kcal/mol. Crystal Violet achieved an intermediate docking score of 5964 (Area: 678.80 Å², ACE: -129.79 kcal/mol), while Indigo displayed the lowest geometric score of 4112 (Area: 450.80 Å², ACE: -91.78 kcal/mol). Residue-level interaction analysis indicated that the Congo Red-laccase complex was uniquely stabilized by specific hydrogen bonding interactions, yielding a hydrogen bond fraction score of 0.2, involving proximal residues highlighted in the 2D interaction maps (Fig. 6b). In contrast, Crystal Violet and Indigo relied exclusively on non-bonded hydrophobic interactions and Van der Waals forces, exhibiting an absence of conventional hydrogen bonds (0.0).Decisively, it must be emphasized that Patch Dock scores reflect structural shape matching and pseudo-atomic contact energies rather than true thermodynamic free energy of binding (ΔG) or active-site chemical turnover rates. Furthermore, since a rigid-body blind docking approach was implemented, it does not simulate induced-fit conformational adjustments. Therefore, the prominent score for Congo Red confirms excellent geometric fit and surface recognition but does not inherently establish a faster rate of catalytic biodegradation. These findings provide a compelling structural rationale for prospective binding affinity, which must be validated using experimental *in vitro* kinetic assays.

**DISCUSSION**

The present study establishes an integrated computational workflow to decipher how *Pseudomonas putida* laccase engages three industrially important dyes. Sequence retrieval and BLASTp analysis confirmed maximal identity with polyphenol oxidoreductases from *Pseudomonas* and Gamma proteobacteria species, and this identification was independently reinforced by multiple sequence alignment, CDART domain mapping, and Pfam motif scanning, which together confirmed the canonical multicopper oxidase architecture and the diagnostic Cu-oxidase_4 signature. This convergence across three independent bioinformatic approaches gives confidence that the modelled protein is a genuine laccase rather than a structurally related but non-catalytic oxidase, and it justified carrying the sequence forward into homology modelling and docking.



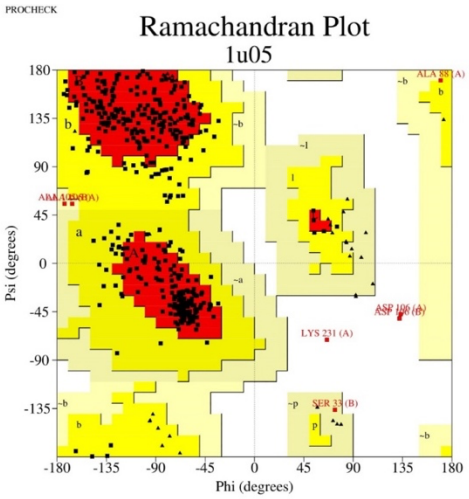



Figure 5: Showing the homology model of laccase enzyme from *P. putida* and its corresponding Ramachandran Plot from Pro Check server. Most favoured regions were found to have 330 residues with 80.1%, additional allowed regions with 75 residues with 18.2%, generously allowed regions with 4 residues with 1.0%, followed by the disallowed regions with 3 amino acid residues with 0.7% and non-Glycine and non-Proline residues with 412 residues

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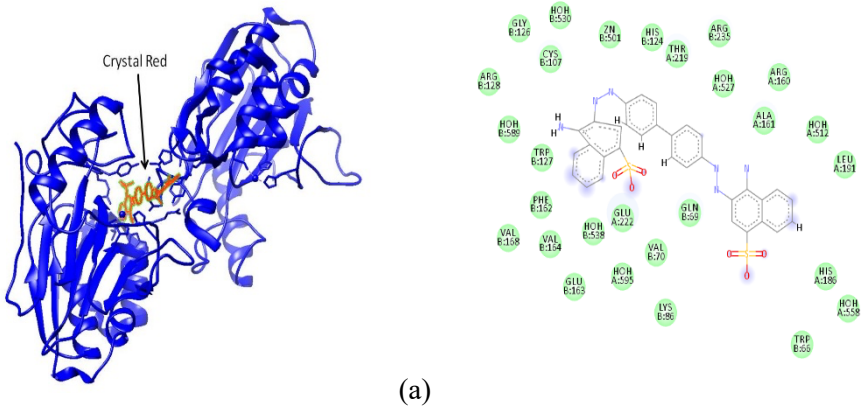
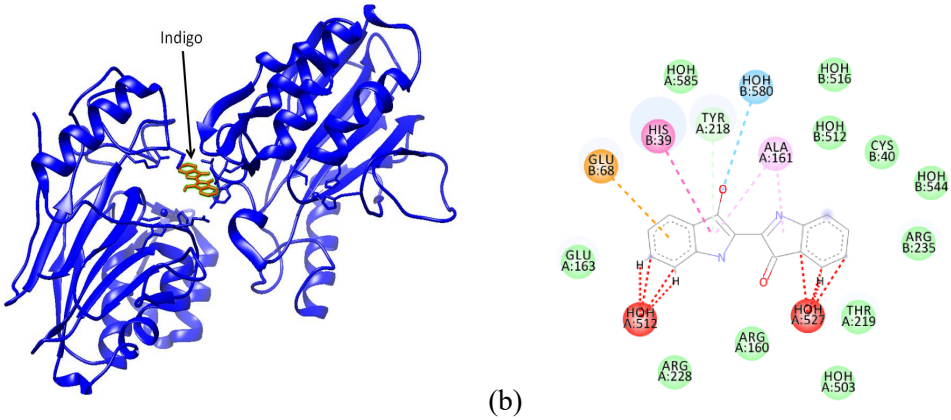
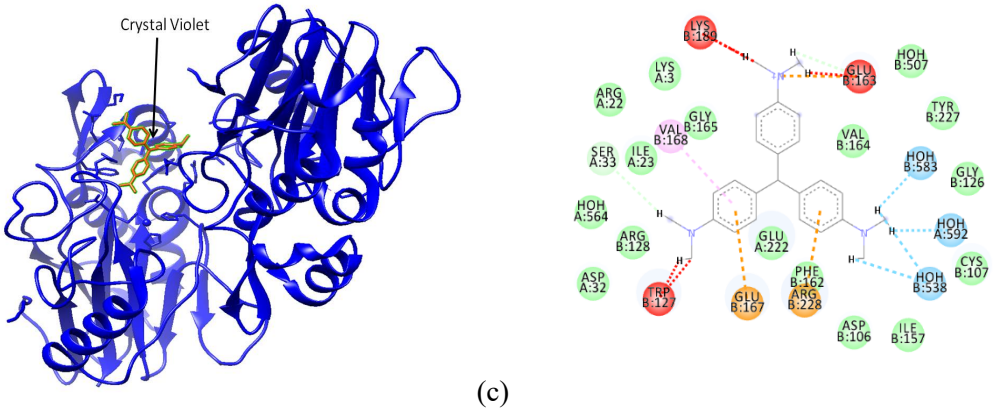



(a)




(b)




(c)

Figure 6: Showing the molecular interactions of (a) 1u05 and crystal red, (b) 1u05 and indigo and (c) 1u05 and crystal violet. The dyes (ligands) are marked in the above figures in orange red colour and the receptor is coloured in blue. The ligands are in ball and stick format. Also, the amino acid residues (with the chain of the enzyme they belong and positions) interacting with them are provided.

Table 1: Showing the molecular docking analysis of laccase as the receptor and the dyes as ligands. It includes the docking score, area covered and ACE (atomic contact energy) and Hydrogen Bonds (HB). The results were further processed via Fire Dock and then the values are provided.

| Receptor | Ligands | Docking Score | Area | ACE | HB |
|---|---|---|---|---|---|
| **(1u05)** | Congo Red | 7434 | 949.60 | -274.63 | 0.2 |
| | Indigo | 4112 | 450.80 | -91.78 | 0.0 |
| | Crystal Violet | 5964 | 678.80 | -129.79 | 0.0 |

The homology model, built on the *Escherichia coli* multicopper oxidase template (PDB 1u05) in its biological multimeric configuration, showed an overall stable fold, with 80.1% of residues in the most favoured Ramachandran regions and only 0.7% in disallowed regions. This value sits below the approximately 90% benchmark generally expected of high-confidence structures, a shortfall that is not unusual for homology models of bacterial multicopper oxidases built without an exact-sequence experimental template; comparable models used directly for blind docking of dyes and other xenobiotics against bacterial and fungal laccases have reported similar loop-region deviations (Buzzo et al., 2023). Consequently, we consider explicit-solvent molecular dynamics refinement of the flexible loop regions – as has been used to stabilize analogous laccase-substrate models before docking (Bhatt et al., 2023), a necessary next step before this structure is used for active-site engineering rather than for the present exploratory screening. Interpreting the docking hierarchy considering dye chemistry, rather than as a simple activity ranking, is instructive. Congo Red is a large, bis-azo, desulphonated diamino biphenyl dye; its extended and comparatively flexible aromatic backbone and anionic sulfonate/amine substituents are consistent with the largest interacting surface area recorded here (949.6 Å²) and the only appreciable hydrogen-bond contribution among the three ligands (fraction 0.2). Sulfonated azo dyes have similarly been reported to form more extensive hydrogen-bonded contacts with surface loops of bacterial and fungal laccases in comparable docking studies (Li et al., 2025). Crystal Violet, a delocalized cationic triarylmethane dye, engaged the modelled surface predominantly through hydrophobic and van der Waals contacts without conventional hydrogen bonding, a pattern also reported for triarylmethane dyes docked against laccases from other sources, which tend to associate through π-stacking with aromatic side chains rather than through polar contacts (Li et al., 2025). Indigo, the smallest and most rigid of the three ligands, occupied the least surface area and returned the lowest geometric score, which more plausibly reflects its limited number of solvent-accessible polar groups than any inherent resistance to enzymatic attack. It bears repeating, that Patch Dock and Fire Dock scores quantify geometric shape complementarity and pseudo-atomic contact energy rather than true binding free energy (ΔG), and they do not identity directly about the single-electron oxidation chemistry that occurs at the laccase type-1 copper centre during catalysis. Because no experimentally confirmed catalytic residues exist for this bacterial variant, docking here was necessarily performed in blind mode across the entire modelled surface; the resulting poses, however favourable, describe candidate surface-recognition events rather than confirmed, catalytically productive orientations relative to the copper cluster. Reviews of docking-based bioremediation studies draw the same distinction, noting that a favourable score can prioritize candidates for downstream testing but cannot, on its own, establish degradation efficiency (Iniguez-Luna et al., 2025). The present ranking – Congo Red greater than

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Crystal Violet greater than Indigo – should therefore be read as a structural hypothesis about relative surface affinity to be tested against real decolorization and degradation data, not as a confirmed biodegradation order. This caveat does not diminish the practical relevance of the findings. Bacterial laccases have repeatedly been shown, once immobilized or otherwise stabilized for continuous operation, to decolorize structurally diverse azo, anthraquinone, and triarylmethane dyes in wet-laboratory systems; immobilization strategies such as alginate encapsulation, nanoparticle conjugation, and vault-nanocage packaging have each been used to improve laccase stability and reusability for dye-wastewater treatment (Velu et al., 2011; Gao et al., 2022; Thathola et al., 2024; Chauhan & Choudhury, 2021). The present structural model and its residue-level contact maps therefore provide a rational starting point for prioritizing Congo Red for early wet-lab or immobilization-based follow-up, while parallel assays would still be required to establish whether the weaker, purely hydrophobic docking profiles of Crystal Violet and Indigo translate into meaningfully lower degradation rates in practice. Taken together, three concrete next steps (i) explicit-solvent molecular dynamics refinement of the homology model to resolve the loop regions responsible for the sub-90% Ramachandran score; (ii) active-site-directed rather than purely blind docking once mutagenesis or structural data localize the catalytic type-1 copper pocket, ideally using flexible-ligand or induced-fit protocols capable of capturing conformational adjustment on binding; and (iii) *in vitro* validation through UV-visible decolorization assays, HPLC/LC-MS degradation-product profiling, and enzyme kinetic measurements, following experimental designs used for other bacterial laccase-dye systems (Dai et al., 2021; Li et al., 2025). Only this combined computational-experimental evidence can convert the present structural ranking into a validated statement about which dye *Pseudomonas putida* laccase degrades most efficiently.

## CONCLUSION

In summary, this is a bioinformatics-driven approach to characterize the Laccase enzyme from *P. putida* and evaluate its potential for dye degradation. Through sequence analysis and homology modelling, the enzyme's structure was validated as stable and functional, specifically showing a high density of conserved domains that are essential for the catalytic activity of these enzymes.

The *in silico* docking results highlight that while the enzyme interacts with multiple dyes, its geometric and hydrogen-bonding complementarity with Congo red is the strongest among the three dyes tested, a structural finding that is suggestive of, but does not by itself confirm, superior degradation potential for this pollutant; this hypothesis would need to be evidenced through molecular dynamics refinement and in vitro kinetic and decolorization assays. These findings support the further investigation of *P. putida* laccase particularly in the sustainable treatment of industrial effluents.

## ACKNOWLEDGEMENTS

The authors also express their heartfelt gratitude to Br Sunil Britto, Principal, St. Edmund's College, Shillong for his support & encouragement throughout the work.

## FUNDING

This study was supported by the Department of Biotechnology (DBT), Govt. of India-sponsored project Advanced Level Biotech Hub, sanctioned to Dr. Samrat Adhikari.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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