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In Silico Comparative Analysis of Azo Dye Interactions with Oxidoreductase, Laccase, and Peroxidase from Acinetobacter junii

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ABSTRACT: *The textile industry releases significant amounts of azo dyes in aquatic bodies, which rigorously contributes to water pollution. Microorganisms effectively transform organic and inorganic pollutants into the lesser toxic form during the process of bioremediation with the aid of enzymes. Modern technology has exhibited remarkable advancement in last years. The incorporation of omics approach in the field of bioremediation is an exciting venture to research. It will significantly reduce the screening time. Therefore, this study employs a computational strategy to compare the azo dye degrading potential of three prominent enzymes from Acinetobacter junii, i.e. oxidoreductase, laccase, and peroxidase. For this purpose, six azo dyes, Acid violet 7, Acid orange 19, Congo red, disperse red 1, Disperse red 13, and Reactive brilliant red, were considered for the analysis of molecular interactions with the three enzymes. After 3D structure modeling through I-TASSER and validated by RAMPAGE, the enzymes were docked with the azo dyes by using auto dock vina, and their binding energies and molecular interactions were evaluated. The comparative analysis of enzymes-dye complexes revealed that both oxidoreductase and laccase formed highly stable complexes with all azo dyes, showing strong binding affinities (>-5kcal/mol). Moreover, NAMD used for the molecular dynamic simulations have revealed the minimum conformational deviations for oxidoreductase and laccase compared to the docked complex of peroxidase. These findings suggest that oxidoreductase and laccase of A. junii are highly promising for efficient remediation of toxic azo dyes. However, these enzymes need further experimental characterization before their use in large-scale setups.*

Keywords: *Acinetobacter junii,* Azo dyes, Bioinformatics, Dye-degradation, Laccase, Molecular Docking

INTRODUCTION

The textile industry plays a pivotal role in augmenting the economic growth of the country, but it is also known for significant environmental concerns, including soil, air and water pollution. Among various types of dyes used in textile industry, synthetic azo dyes impart vibrant and long-lasting colours to products. However, their widespread usage and the release of untreated dye effluents in the lakes, rivers and other aquatic bodies is posing a threat to aquatic life and human health as they can persist in the environment for a longer period (Godswill et al., 2020; Okafor et al., 2021).

Bioremediation of azo dyes is considered as an appealing, environmental-friendly, cost-efficient, and functionally simple approach compared to physicochemical strategies (Majumdar et al., 2022). It involves the screening and utilization of bacteria and fungi, for enzymes such as laccases, peroxidases, and oxidoreductase, which reduce the azo bonds (–N=N–) using flavin containing NADH/NADPH to colorless amines (Singh, 2022). The enzyme laccases (EC 1.10.3.2) belong to copper oxidases family, which plays a significant role in oxidative bioremediation of contaminated environment. On the other hand, enzymes belonging to the oxidoreductase family catalyse the NAD(P)H-dependent azo bond reductive cleavage. These enzymes contribute to the metabolic breakdown of azo dyes within bacterial cells.

However, microbial screening for dye degradation is tedious and a time taking process. Modern system biology offers robust and fast mode of screening the target microorganism for bioremediation. *In silico* approaches including structure modelling, molecular docking analysis, and molecular dynamic simulations help in predicting the 3D structure (Tülek et al., 2021; Rathour et al., 2023) and assessing the binding affinities of azo dyes with bacterial enzymes (Kumar et al., 2020) saving the time and reduing experimental costs in future. Although molecular docking analysis has been extensively used in drug discovery but its applications in determining targets for bioremediation will provide a new impact.

The present study employs a computational strategy to explore the potential of enzymes laccase, peroxidase, and oxidoreductase from *Acinetobacter junii* to degrade the toxic azo dyes by assessing their molecular interactions with disperse red 13, acid violet 7, acid orange 19, disperse red 1, Congo red, and

reactive brilliant red. For this purpose, molecular docking analysis was performed to recognize the suitable dye and enzyme arrangement while MD (Molecular Dynamic) simulations revealed the conformational stabilities of enzyme-dye complexes. This study can be an effective approach in early screening of suitable dye degrading enzymes which can further be used in remediation of textile effluent.

MATERIALS AND METHODS

Azo Dyes and Bacterial Strain

Six textile azo dyes, namely, acid violet 7, disperse red 1, acid orange 19, Congo red, disperse red 13, and reactive brilliant red, were used in present study. *A. junii* was chosen due to its presence in wastewater samples.

Bacterial Enzymes

The database NCBI (https://www.ncbi.nlm.nih.gov/) was employed to recover the amino acid sequence of the A. junii enzymes (laccase, peroxidase, and oxidoreductase).

Physicochemical Characterization of Enzymes

To analyze the physical and chemical properties of three selected enzymes, ProtParam tool (http://web.expasy.org/protparam/) was used. This tool provides insights into several key parameters including molecular weight, stability, aliphatic index (AI), theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), and instability index (II) (ProtParam, 2017).

Structure Modelling

The three-dimensional structures of three selected enzymes were built using online I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) server, followed by the selection of precise protein mockups on the basis of RMSD values, TM score, and C-score. Furthermore, the quality of protein models was evaluated through Ramachandran plots that were designed using RAMPAGE software (Wang et al., 2016). Likewise, the three-dimensional structures of selected azo dyes were fetched in SDF format from the PubChem database (pubchem.ncbi.nlm.nih.gov).

PubChem database presents the repository of small molecules, substances, and compounds along with their molecular information (Kim et al., 2016).

Protein and Ligand Preparations

The protein and ligand preparation are a prerequisite for molecular docking analysis (Madhavi et al., 2013). This involves the conversion of 3D structures to the PDBQT format using AutoDock Vina. In the case of proteins, Discovery studio was used to remove potential clashes and water molecules. Missing residues, gap-filling, and

optimization of hydrogen-bonding networks were also incorporated by Discovery studio (Huang et al., 2006).

Molecular Docking Analysis

The docking of selected enzymes (laccase, peroxidase, and oxidoreductase) with each of the six selected azo dyes was accomplished using Autodock Vina. The most appropriate binding position and binding energies ($kcalmol^{-1}$) for each docked complex was calculated (Li et al., 2019).

Molecular Dynamic Simulations

Finally, stabilities of docked complexes were assessed using MD simulations that were executed via NAMD software and QwikMD interface plugin in VMD (Alonso et al., 2006; Sung, 2011). The simulations were carried out using equilibration phase of 2 ns and production phase of 10 ns at 315 K. The conformational trajectories were analyzed based on the RMSD (Root Mean Square Deviations) and RMSF (Root Mean Square Fluctuations) (Martínez, 2015).

RESULTS

Azo Dye Degrading Enzymes

The amino acid sequences of three enzymes, oxidoreductase, laccase, and peroxidase were retrieved from NCBI using SUU15883.1, SUU18727.1, and ATU46662, accession IDs for these three enzymes, respectively.

Physicochemical Characterization

The isoelectric point of oxidoreductase, laccase, and peroxidase were 8.02, 5.85, and 4.55, respectively. The GRAVY value for all three enzymes was negative. The Instability Index (II) of oxidoreductase, laccase, and peroxidase were 29.54, 23.00, and 38.08, respectively. The aliphatic index of oxidoreductase (93.07), laccase (84.11), and peroxidase (87.39) proclaim them as thermally stable (Table 1). These features have marked the stable nature of the three selected enzymes.

Table 1: The physiochemical parameters of laccase, oxidoreductase and peroxidase enzymes of *Acinetobacter junii* were evaluated through ProtParam tool

Enzymes	A. A	MW	pI	AI	GRAVY	S/IS	II
Laccase	246	27287.15	5.85	84.11	-0.084	S	23.00
Oxidoreductase	551	61014.34	8.02	93.07	-0.113	S	29.54
Peroxidase	306	34337.61	4.55	87.39	-0.228	S	38.08

A. A= No. of amino acid residues; MW= Molecular Weight; pI=Isoelectric point; AI=Aliphatic Index; GRAVY= Grand Average of Hydropathicity; S/IS= Stable/Instable; II= Instability index

Structural Modelling

I-TASSER was used to model the three structures of enzymes followed by the model selection based on RMSD values, C and TM score (Figure 1). The confidence score (C-score) is used to estimate the quality of protein model. The higher value of this score indicates the good quality models. The 3D structures of enzymes oxidoreductase, laccase, and peroxidase with C-score values of -1.91, 1.15, and 1.43 were found to be good quality models. Similarly, the TM score (0.49±0.15, 0.87±0.07, and 0.91±0.06) and RMSD values (12.1±4.4Å, 12.1±4.4Å, and 3.3±2.3Å), of oxidoreductase, laccase, and peroxidase were within the acceptable ranges, further affirming the fidelity of protein structures.

Finally, the Ramachandran plots revealed the 83.298%, 89.815%, and 91.513% residues of oxidoreductase, laccase, and peroxidase in the favorable and acceptable areas of the plot (Figure 1).

Molecular Docking Analysis

The molecular interactions of enzymes laccase, peroxidase, and oxidoreductase with six azo dyes are shown in Figure 2, 3, 4 respectively.

The analysis of binding affinities of these docked complexes shows that all enzymes establish interactions with the selected azo dyes. However, oxidoreductase exhibits the strongest binding affinities with these dyes, followed by laccase and peroxidase (Table 2).

Table 2. The binding affinities of each enzyme and azo dye complex in kcal/mol.

Name of dye	Binding Affinities (kcal/mol)		
	Laccase	**Oxidoreductase**	**Peroxidase**
Acid Violet 7	-6.2	-8.5	-6.3
Acid Orange19	-7.1	-9.0	-6.6
Congo Red	-7.5	-8.9	-6.9
Disperse Red 1	-5.9	-7.9	-4.3
Disperse Red 13	-6.0	-7.3	-4.9
Reactive Brilliant Red	-6.8	-9.1	-6.6

Consequently, the order of best interactions is oxidoreductase > laccase > peroxidase.

Furthermore, the analysis of hydrogen bonds indicates that laccase and oxidoreductase

establish a greater number of hydrogen bonds with the azo dyes, creating more stable complexes (Table 3).

Table 3. The details of amino acid residues from the selected enzymes of Acinetobacter junii forming H-bonds with azo dyes

	Laccase	Oxidoreductase	Peroxidase
Acid Violet 7	Y198	Q188, Y277, D442	S5, R41, T46
Acid Orange19	Q71, T72, R238	A158, D180, Q188	L54, D42
Congo Red	G6	R95, A158, D408	R43
Disperse Red 1	Q71, H127, R238	L312, D442, A452	0
Disperse Red 13	N41, Q73, H127, R238	G155, Q188	L8
Reactive Brilliant Red	H39, Q71, H73, M106, H127, R238	0	R41

Molecular Dynamic Simulations

The docked complexes of laccase, oxidoreductase, and peroxidase with azo dye acid orange 19 were further analyzed by the MD simulations. The conformational deviations of these complexes

were analyzed based on the RMSD and RMSF (Figure 5). The trajectory analysis revealed that the enzyme oxidoreductase and laccase show minimum conformational deviations over the time compared to the peroxidase. These results indicate that the enzyme oxidoreductase and laccase from *Acinetobacter junii* forming the stable complexes with dyes can be used for the degradation of azo dyes.

DISCUSSION

The textile industry plays a pivotal part in the economy of Pakistan, however, the extensive use of azo dyes in this industry has raised significant environmental concerns (Hashemi and Kaykhaii, 2022). These dyes find their way into the water bodies where they block light penetration resulting in the inhibition of photosynthesis, resulting in an increase in the chemical and biological oxygen demands (Meghwal et al., 2020). Despite the availability of different wastewater treatment options, biological treatment using microbial enzymes appears to be the most promising method for clean degradation of azo dyes (Mishra et al., 2020). Numerous microbial enzymes have been reported for their ability to effectively decolorize and degrade azo dyes. Among these, peroxidases, laccases, and oxidoreductase are the most

promising enzymes (Mahmood et al., 2017).

Recently the *Acinetobacter junii* has been identified in the wastewater samples of various regions in Pakistan (Fatima et al., 2015). The natural occurrence of this microorganism in wastewater underscores the potential of its enzymes to degrade the toxic azo dyes. However, the efficacy of this degradation depends upon the binding affinities of its enzymes with the azo dyes (Raj et al., 2014; Medfu et al., 2020). The present study is focused on analysis of molecular interactions and binding affinities of three enzymes: azoreductase, laccase, and peroxidase from *A. junii* with the six azo dyes, acid violet 7, acid orange 19, congo red, disperse red 1, disperse red 13, and reactive brilliant red. The rationale behind the selection of these three enzymes from *A. junii* lies in their well-established potential to degrade organic pollutants. To assess the molecular interactions of these enzymes with azo dyes, the three-dimensional structures of the selected enzymes were built using I-TASSER. As they lack experimentally determined 3D structures, so the structures of respective enzymes predicted by software-based modelling by using an ab initio method (Chopra et al., 2023; Xuejiao et al., 2023). The quality of resulting protein models was rigorously assessed through a variety of parameters, including the Ramachandran plot, C-score, TM score, and RMSD values. In the absence of experimental structures, the model quality estimation is crucial to determine its potential applications for the further analysis (Zhang, 2008; Wang et al., 2016). The outcomes of these quality evaluators have revealed that the protein models exhibit a high level of accuracy and are suitable for subsequent analysis (Kim et al., 2016). Subsequently, the three-dimensional structures of enzymes and azo dyes were prepared for the molecular docking analysis through AutoDock Vina. This preparation step ensures that proteins and ligands are in appropriate formats for studying their molecular interactions.

The molecular docking analysis calculates the binding energies of each enzyme-dye complex in kcal/mol, providing insight into the affinity of each enzyme for the azo dyes. The more negative binding energy suggests the more stable complex where enzymes form stable interactions with the azo dyes and can degrade it effectively (Henrich et al., 2010). The results of molecular docking analysis revealed that enzyme oxidoreductase has the highest binding affinities towards all six selected azo dyes, followed by laccase, and peroxidase as indicated in Table 3. This suggests

that oxidoreductase from *A. junii* is the most suitable enzyme for degrading the chosen azo dyes, followed by laccase and peroxidase. In addition to strong binding affinities, oxidoreductase and laccase also formed more stable complexes with azo dyes forming more H-bonds compared to peroxidase (Tables 2 and 3) (Figures 2, 3 and 4). While the various types of interactions contribute to the protein-ligand interactions, hydrogen bonding is the most stable, contributing stability to the complex (Bitencourt-Ferreira et al., 2019). Consequently, the enzyme making the highest number of hydrogen bonds with azo dyes is expected to form the most stable complexes resulting in effective degradation (Chen et al., 2016).

Molecular docking analysis, due to its static nature, may not provide the comprehensive representations of protein-ligand interactions alone, as it overlooks the dynamic interplay between the protein and ligands. Therefore, the stability of protein-ligand complexes was further assessed through molecular dynamic simulations that provides insight to the into atomic-level conformational patterns of protein-ligand complexes (Du et al., 2016; Fu et al., 2018; Haghshenas et al., 2016). The analysis of conformational deviations of the docked complexes, based on RMSD and RMSF values (Figure 5), revealed that the complexes of oxidoreductase and laccase with the chosen azo dyes exhibited minimal conformational deviations. This finding indicates that these two enzymes are better suited for forming stable complexes with the prevalent and toxic azo dyes present in wastewater, compared to peroxidase.

CONCLUSION

Our study demonstrates that oxidoreductase and laccase from *A. junii* can be considered for bioremediation applications in the treatment of textile and paper mill effluent based on binding affinities and molecular interactions with azo dyes. However, further wet-lab experimentation is imperative to determine the suitability of these enzymes for efficiently addressing the environment required for the elimination of azo dyes using biological tools.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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