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DECOLORIZATION OF REACTIVE DYES USING POLYPHENOL OXIDASE ENZYME: IN-SILICO AND IN VITRO APPROACH

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ABSTRACT

Reactive dyes are environmentally hazardous due to their toxicity, even at low concentrations, and their potential carcinogenic and mutagenic effects. Their high solubility in water makes them difficult to remove using conventional methods. This study aims to use the in-silico and in-vitro approach to explore the potential of polyphenol oxidase in dye remediation. In in-silico studies binding affinities of polyphenol oxidase and azoreductase with five reactive dyes (Reactive Black 5, Reactive Red 1, Reactive Orange 16, Reactive Yellow 17, Reactive blue 19) were compared and the highest binding was validated by molecular dynamic simulation. Further in in-vitro studies, polyphenol oxidase enzyme from peel of *Lagenaria siceraria* was isolated to study the effect of free and immobilized enzyme on reactive dyes decolorization. The in-silico studies revealed that both enzymes demonstrated strong binding affinities with all reactive dyes, with the highest affinity observed for Reactive Blue 19. Molecular dynamic analysis showed the interaction of both the proteins was stable with reactive blue 19 and MM-PBSA calculation indicated that binding affinity of polyphenol oxidase with Reactive blue 19 is -90.901 +/-3.495 (kJ/mol) and binding affinity of azoreductase with Reactive blue 19 is -72.096 +/-3.340 (kJ/mol). This concludes that polyphenol oxidase can be used as a potential enzyme for decolorization of reactive dyes. In-vitro studies, Polyphenol oxidase enzyme was successfully purified with a specific activity of 1374.73 U/mg and purification fold 2.27. Cotton fabric with indirect immobilization showed more recovered activity of 42.4% than direct immobilization i.e. 37%. Decolorization of Novacron Blue C 5X dye was highest in indirect immobilized fabric i.e. 27.6% as compared to direct immobilized (11.66%) and free form of enzyme (14%). This study highlights the potential of polyphenol oxidase in textile dye remediation, providing both in-silico and in-vitro validation of its efficacy.

Keywords: Molecular Docking; Molecular Dynamic Simulation; Azoreductase enzyme; Polyphenol oxidase enzyme; Immobilization; Dye Decolourization

INTRODUCTION

Water pollution is a pervasive environment concern caused by pollutants like dyes, pigments, heavy metals, phenols, microbial, toxic organics etc (Bashir et al., 2020). Among these, dye production is rapidly increasing globally, with an estimated production of $7 * 10^7$ tons annually. Currently, 10,000 dyes are commercially available like acidic, basic, azo, diazo and reactive dyes. These are used by various industries like food, paper, plastic, textile, leather etc. Textile industry is a major consumer of dyes and it is estimated to rise due to increasing fashion trend and population. Approximately 10-25 % of the dyes ends up in water bodies

due to inefficient dyeing or spillage, contributing to water pollution (Alsukaibi, 2022). Its discharge into the environment cause soil pollution, and negatively impact the aquatic organisms, agriculture, and human health. When dyes enter the soil through irrigation water, they alter its physiochemical nature leading to erosion, productivity loss and reduction in growth of plant (Lellis et al., 2019). In aquatic ecosystem it hinders the photosynthesis, thus changing the biological parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD). This disruption restricts plant growth and allows bioaccumulation in the food chain and eventually lead to humans. In humans, exposure to dyes is associated with occupational hazards, skin irritation, kidney damage, cancer and other health issues (Alsukaibi, 2022; Ikram et al., 2022). It is crucial to treat these pollutants before they are discharged into the environment.

Conventional methods used to treat dyes in water include adsorption, bioflocculation, coagulation, ion exchange, flotation, membrane filtration, ultrasonic mineralization, electrolysis, solvent extraction, electrochemical oxidation, photocatalytic techniques, Fenton reagent reaction and biological method (Kumar et al., 2019). These methods often fall short in effectively removing dyes due to resistance to biodegradation, complex chemical structures, production of more toxic by-products, generates large amount of solid waste which increases the disposal cost, requires more time, energy, space and emits hazardous gases. For instance, alum which is a chemical coagulant produces large amount of sludge and decreases the pH of the system which increases cost (Alsukaibi, 2022; Kumar et al., 2019; Ledakowicz and Paździor, 2021). Membrane separation has a major drawback of high investment, high maintenance, produce secondary waste stream, and membrane biofouling (Ren et al., 2020). Ozone used in the ozonation process has low solubility in water which limits its efficiency, leading to increased operational costs. Additionally, the generation of disinfection by-products (DBPs) remains a significant challenge in the practical application of ozonation (Wang and Chen, 2020).

Enzymatic degradation of dyes has been increased in the recent years. Azoreductase, laccase, peroxidase, tyrosinase, polyphenol oxidase are important industrial enzymes for dye degradation and belongs to the class oxidoreductase. Azoreductase is the NADH or $FADH_2$ dependent enzyme that cleaves the azo bonds using Ping Pong Bi-Bi mechanism and results in the formation of arylamines which are further degraded into CO_2 and H_2O in the presence of oxygen. Laccase is a copper containing enzyme that is used extensively for dye degradation due to their oxidizing effects in various dyes. Similar to laccase, tyrosinase oxidise the aromatic compounds without any need for cofactor (Uppala and Muthukumaran, 2021). Polyphenol oxidases are copper containing enzyme that is commonly used for the oxidation of phenolic compounds and in some studies dyes (Tarasov et al., 2023). In the present study, we aim to combine in-silico and in-vitro technique to study the decolorization of dyes using polyphenol oxidase enzyme. For comparison, Azoreductase was used as a control for in-silico studies. Molecular docking for dye degradation helps us to better understand the interaction between the ligand (dye) and the enzyme, predicting enzyme-substrate reaction through binding affinities and orientation, evaluating the effectiveness of dye degradation, prediction of enzyme inhibitors or activators (Tao et al., 2020). Molecular docking of azoreductase and polyphenol oxidase was done with five reactive dyes (Reactive Black 5, Reactive Red 1, Reactive Orange 16, Reactive Yellow 17, Reactive blue 19). Molecular dynamic simulation is a powerful method that aid bioremediation strategies by predicting the structural changes, stability, flexibility in a real-time setup (Oyewusi et al., 2024). Invitro analysis was done by isolating, purifying and characterizing polyphenol oxidase enzyme from the peel of *Lagenaria siceraria*. The isolated enzyme, both in its free and immobilized forms was subsequently utilized for dye decolorization experiments.

MATERIALS AND METHODS

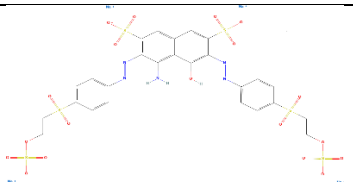
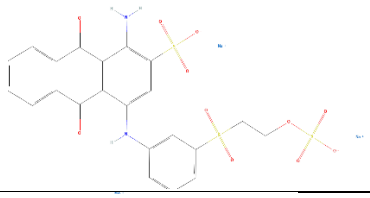
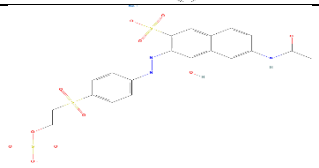
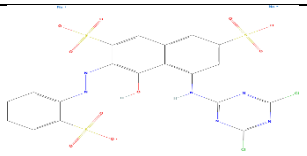
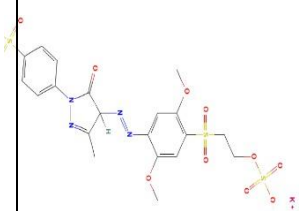
Accession of target protein

The crystal structure of both azoreductase (PDBID-3W77) and catechol oxidase/polyphenol oxidase enzyme (PDBID- 1BUG) was downloaded from Protein data bank (PDB).

Dyes used in the study

The 3D structure of reactive dyes (Reactive Black 5, Reactive Blue 19, Reactive Orange 16, Reactive Red 1, Reactive Yellow 17) was downloaded from PubChem database in SDF file format and converted to pdb file format by using smile convertor tool. The structure, molecular weight, molecular formula with their PubChem ID is been given in Table 1. For in-vitro analysis these dyes were purchased from textile industry with commercial names: Novacron Red orange H3R2, Novacron Yellow C 5G, Novacron Orange EC 3R, Novacron Blue C 5X, Novacron Deep Red ECD.

Table 1: Properties of Reactive Dyes

Dye	Pubchem ID	Molecular weight	Molecular Formula	2D Structure
Reactive Black 5	135442967	991.8	$C_{26}H_{21}N_5Na_4O_{19}S_6$	
Reactive Blue 19	17409	626.5	$C_{22}H_{16}N_2Na_2O_{11}S_3$	
Reactive Orange 16	30082	617.5	$C_{20}H_{17}N_3Na_2O_{11}S_3$	
Reactive Red 1	87291	717.4	$C_{19}H_9Cl_2N_6Na_3O_{10}S_3$	
Reactive Yellow 17	30104	682.8	$C_{20}H_{20}K_2N_4O_{12}S_3$	

Identification of binding site

The software used for the allosteric site and binding site determination is AllositePro and Castp, respectively. AllositePro software (https://mdl.shsmu.edu.cn/AST/) generates a 3D representation of the protein structure with pockets shown in ball and stick and their properties like pocket volume, total solvent accessible surface are (SASA), polar solvent accessible surface area (SASA), Pocket drugability score. The file generated by AllositePro was further used for visualization in Pymol for the selection of allosteric site (Song et al., 2017). CASTp (Computed Atlas of Surface Topography of proteins) is a freely available software that allows user to explore identified pockets and cavities. It is a powerful tool for analyzing surface topography of protein and identifying potential binding site (Binkowski et al., 2003).

Molecular docking

Protein preparation of crystal structure of azoreductase (3w77) and polyphenol oxidase enzyme (1bug) was downloaded from the RCSB Protein Data Bank in the pdb format. Target protein was prepared by a software called Biovia Discovery Studio by removing co-crystallized ligands and addition of Hydrogen atoms. Addition of charges was done in Autodock vina and the structure was saved in PDBQT format. Ligand preparation: Reactive dyes (Reactive Red 1, Reactive Blue 19, Reactive Yellow 17, Reactive orange 16, Reactive Black 5) were downloaded from the PubChem database in the SDF format and converted to PDB format through online smile converter software. Pre-processing of the ligands like removing heteroatoms, delete water, add polar hydrogen groups, addition of charges was done in AutoDock Vina and the structure were saved in PDBQT format. The grid setting was determined based on the binding site residues retrieved from the Castp software. The grid box of azoreductase and polyphenol oxidase enzyme was set at binding site coordinates 35.557, 40.216, 66.167 and 26.253, 106.757, 32.594 for x,y,z, respectively for all docking simulations. Binding affinity was calculated using AutoDock Vina, protein-ligand 2D and 3D interactions were studied and visualized by using Discovery Studio (Dassault Systems BIOVIA, 2021) and UCSF Chimera software (Kakkar and Wadhwa, 2023). Further molecular dynamic simulation was conducted to validate its stability and calculate free binding energy

Molecular dynamic simulation

Molecular dynamics (MD) simulation was performed between azoreductase and polyphenol oxidase with the dye having good binding affinity to check the stability of the complex in the dynamic system using Groningen Machine for Chemical Simulations (GROMACS). This method is widely accepted to check the conformational stability, motion and trajectory and of the complex in a dynamic environment. It also provides knowledge about the real-time dynamics of protein-ligand interaction, any structural changes on binding of ligand, solvation of the molecule. MD simulation of the compound with highest binding affinity was performed using following conditions: pH 7.4, 0.9% NaCl concentration, 310ºK temperature, 1 atm pressure, and 100 ns running time (Hermanto et al., 2022). For determination of structural stability and rigidity of the complex, Root-Mean-Square Deviation (RMSD), Root-Mean-Square Fluctuation (RMSF), ad Radius of Gyration were analysed. Furthermore, the free binding energy between azoreductase and polyphenol oxidase enzyme and dye was calculated using Molecular Mechanics/Poisson-Boltzmann Surface Area (MMPBSA) in combination with MD, utilizing the 'g_mmpbsa' script of GROMACS (Adamu et al., 2023; Oyewusi et al., 2024).

Extraction of polyphenol oxidase (PPO) enzyme

Peel of *Lagenaria siceraria* (Bottle ground) was homogenized in 0.1M phosphate buffer with 1% polyvinylpyrrolidone (PVP) at pH 7.0. The homogenate was filtered through muslin cloth and centrifuged at 10,000 rpm for 30 mins at 4 ºC. From the resulting supernatant the PPO was precipitated using acetone precipitation method by gradually adding 1.5 volumes of cold acetone to 1 volume of crude extract for 2 hours, under continuous stirring, followed by centrifugation for 30 minutes at 10,000 rpm. The precipitate was left overnight at 4 ºC, re-dissolved in phosphate buffer, and centrifuged at 5,000 rpm for 10 minutes. The supernatant obtained is the purified enzyme used for further analysis (Sajjad et al., 2023). Protein estimation of crude extract and purified protein was done by using Bradford dye-binding method using a standard curve of bovine serum albumin (Bradford, 1976).

Gel diffusion assay

Gel diffusion assay was performed by adding 1.5% Agarose in 0.1M phosphate buffer and heated in the microwave oven to dissolve. Catechol (50mM) was added to the buffered solution once the mixture reached 50 ºC. The solution was poured in the petri plate under sterile condition and let it solidify. After that 6 wells were formed in the petri plate and sealed with agarose. Crude enzyme and purified enzyme were loaded (50µl, 100µl, 200µl) in the wells that were formed in the petri plate. It was incubated for 24 hours and the appearance of black ring and zone indicated the PPO activity (Singh and Wadhwa, 2017).

Immobilization of PPO enzyme on cotton fabric

The cotton fabric was pre-treated with cellulase enzyme extracted from *Colocasia esculenta* using the method outlined in our previous study (Kakkar and Wadhwa, 2022). Further, the surface of cotton fabric was modified by 3% acetic acid. The treated and surface-modified cotton fabric was then submerged in a purified enzyme solution for 2 hours at 4ºC on a magnetic stirrer. After immobilization process, the cotton fabric was washed three times with 0.1M phosphate buffer at pH 6 and the remaining supernatant was stored for enzyme activity assessment. The immobilized cotton fabric is stored at -20ºC for further use (Cristóvão et al., 2011).

Measurement of enzyme activity of free and immobilized PPO

The spectrophotometric method was employed to determine PPO activity of Crude extract, purified enzyme, cotton fabric with direct enzyme immobilization, surface modified cotton fabric with acetic acid and enzyme, supernatants of enzyme immobilization process by a method described by (Yemenicioğlu et al., 1999). To assay the free PPO enzyme, 0.1 ml of enzyme, 0.9 ml of phosphate buffer (0.1 M, pH 7), and 1 ml of 50 mM catechol solution were mixed and incubated at 35ºC for 15 minutes. For the immobilized PPO enzyme activity, 25 mg of cotton fabric with immobilized enzyme was used with the same reaction mixture. Under standard assay conditions, the PPO activity was quantified as one unit of enzyme causing an increment of 0.001 unit of absorbance per minute. Immobilization yield (%) is calculated as the difference between the enzyme activity of supernatant before and after immobilization, divided by enzyme activity of supernatant before immobilization, multiply by 100 (Cristóvão et al., 2011).

Effect of pH, substrate and temperature on PPO activity

The effects of pH (ranging from 4 to 8), substrate concentrations (5 mM, 15 mM, 25 mM, 50 mM, 75 mM, 100 mM, and 125 mM), and temperatures (25°C, 35°C, 45°C, and 55°C) on PPO activity were studied.

Dye decolorization

Five Reactive Dyes (commercial names: Novacron Red orange H3R2, Novacron Yellow C 5G, Novacron Orange EC 3R, Novacron Blue C 5X, Novacron Deep Red ECD) were purchased from textile company. Maximum wavelength of each dye and mixture of 5 dyes was observed at varying wavelength. Each dye was incubated with free PPO enzyme (1:1 ratio), and immobilized cotton fabric weighing 25mg (surface modifiedand unmodified i.e. indirect and direct immobilization, respectively) at pH 6 for 24 hours at 35°C. Dye decolorization by PPO was monitored at the specific wavelength and calculated using equation given below (Chittal et al., 2019; Cristóvão et al., 2011).

$$\%Decoloization = \frac{Initial\ Absorbance - Final\ Absorbance}{Initial\ Absorbance} * 100$$

RESULTS AND DISCUSSION

Identification of regulatory Site/allosteric site and binding site

Enzymes have pockets or small cavities on the surface or interior of a protein that can bind to small molecules or other proteins. These pockets contain free amino acid residues which help them to interact with the ligand through hydrogen bond, electrostatic interaction and van der Waals forces. The shape and chemical properties of the pocket determine the selectivity and affinity of ligand binding, making it a critical determinant of protein function (Stank et al., 2016; Zheng et al., 2013). Protein pockets can be categorized into different types, such as active sites, binding pockets, and allosteric sites, depending on their location and functional role. Active sites are pockets on the surface or interior of the protein where catalysis occurs, while binding pockets are regions where ligands bind to regulate protein activity. The identification and characterization of protein pockets are crucial for understanding protein function and designing small-molecule drugs that can specifically target them (Mishra et al., 2019). Therefore, for understanding physiochemical properties of protein-ligand interaction it is important to analyse the binding pockets present in the protein structure. AllositePro is used for the identification of pockets present in both the proteins. It provides knowledge about pocket volume, total solvent accessible surface area (SASA), polar solvent accessible surface area (SASA), Pocket drugability score as presented in Table 2 (Song et al., 2017). Castp software provides details about the binding pockets as well as amino acid residues present in those pockets (Tian et al., 2018). After comparing the binding site residues with the pocket residues provided by Allosite, we concluded that Pocket 1 of Azoreductase and Pocket 0 of polyphenol oxidase enzyme are the allosteric sites while Pocket 0 of Azoreductase and Pocket 1 of polyphenol oxidase enzyme are the Binding sites. The binding site and allosteric site has been depicted well in Figure 1.

Table 2: Properties of Pockets or Cavities Present In Both the Enzymes

Povkets	Pocket volume	Total SASA	Feature Score	Perturbation Score	Allosite Score
Azoreductase enzyme					
Pocket 0	2562.849	1030.756	0.779	0.105	0.645
Pocket 1	1565.071	621.71	0.816	0.104	0.673
Pocket 2	1482.516	703.843	0.797	0.233	0.684
Pocket 3	699.769	306.885	0.795	0.101	0.656
Pocket 4	917.422	302.365	0.630	0.140	0.532
Polyphenol Oxidase Enzyme					
Pocket 0	234.45	145..4	0.641	0.117	0.536
Pocket 1	764.041	371.944	0.526	1.000	0.621
Pocket 2	307.406	124.491	0.792	0.094	0.652
Pocket 3	376.787	201.735	0.380	1.000	0.504

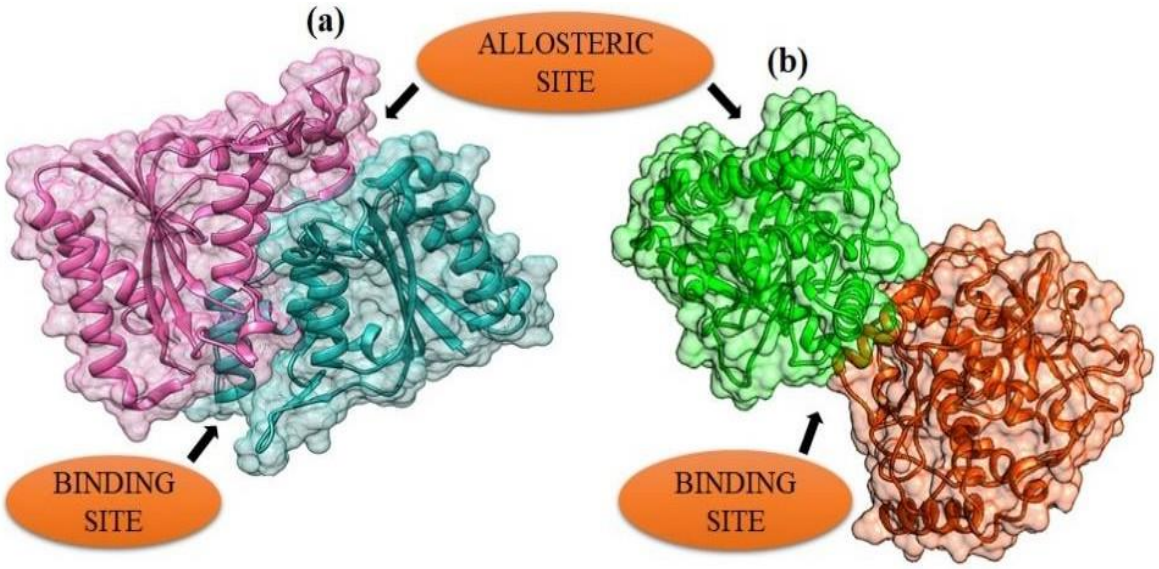


Figure 1. Structure of Enzyme (a) Azoreductase (PDBID- 3W77) (b) Catechol Oxidase (PDBID- 1BUG) with Allosteric and Binding site

Molecular docking

Molecular docking allows us to predict the interaction between substrate and the no. of amino acids that are involved in the catalytic reaction at active site. This gives us better understanding of the protein-ligand interaction, thereby predicting the potential of enzyme for bioremediation. It is a low-cost method to predict the reaction mechanism with high accuracy. These predictions can be used as a source for engineering new enzymes for bioremediation of specific pollutants (Liu et al., 2018). Molecular docking was conducted to examine the interactions between the azoreductase enzyme and the polyphenol oxidase enzyme with reactive dyes. Azoreductase, a well-known dye-degrading enzyme, is frequently utilized in such studies. By comparing the interactions of both enzymes, the potential of polyphenol oxidase to degrade dyes was assessed. Molecular docking studies shows that azoreductase has good binding affinity with reactive dyes: Reactive black 5 (-8.3), Reactive blue 19 (-8.5), Reactive Orange 16 (–7.9), Reactive Red 1 (-8.3), Reactive Yellow 17 (-7.6). In comparison, Polyphenol oxidase has shown good binding affinity with reactive dyes Reactive black 5 (-6.6), Reactive blue 19 (-7), Reactive Orange 16 (-6.9), Reactive Red 1 (-6.8), Reactive Yellow 17 (-6.7). Both the protein showed highest binding affinity with Reactive Blue 19 dye. The docking results were considered favorable because the binding affinities of docked protein were ≤ 0 kcal/mol. Protein amino acid residues interact with the substrate through van der Waals interaction, hydrogen bonding, Pi- Alkyl, Pi-cation, Pi-anion bonds. Table 3 represents the binding affinity, no. of hydrogen bonds, hydrogen bond distance, and the amino acids residues involved in the reaction. The ligand-protein interaction of PPO with different substrate is shown in Figure 2 and Figure 3. Our results are comparable with the other studies on silico bioremediation. In-silico decolorization of commercial dyes was studies using azoreductase (AzrBmH21, AzrBmH22/3, and AzrBmH24/5) from *Bacillus megaterium* H2, whose binding affinity with acid orange 7, cresol red, methylene blue, and malachite green was found to be in a range of -5.4 to -9.4 kcal/mol (Oyewusi et al., 2024).

Table 3: Binding Affinities of 5 Reactive Dyes with Azoreductase and Polyphenol Oxidase (PPO) Enzyme

Dyes	Azo reductase				Polyphenol oxidase			
	Binding Affinity	Hydrogen bond	Hydrogen bond Distance	Interacting Residues	Binding Affinity	Hydrogen bond	Hydrogen bond Distance	Interacting Residues
Reactive black 5	-8.3	4	11.88	PHEA:61, GLYA:64, LEUB:102, ASN A:116, ALAA:118, META:166, PHEA:169	-6.6	5	14.36	SERA:33, ASPA:76, ASPA:77, PROA:78, ARGA:79, GLYA:313, GLNA:314, ALAA:315, ALAB:61, GLUB:68 SERB:162, SERB:163
Reactive blue 19	-8.5	5	12.92	LEUB:102, TYRA:124, PROA:129, PHEA:169, META:166, ASNA:116, THRA:60	-7	6	15.9	PROA:30, SERA:33, ASNA:34, ASPA:77, PROA:78, TYRA:309, GLYA:313, GLNA:314,
Reactive Orange 16	-7.9	5	14.58	ASNA:116, SERA:165, META:166, PHEA:169, TRPB:100, ASNB:101, LEUB:102,	-6.9	8	21.54	PROA:30, ASNA:34, PROA:74, ASPA:76, TYRA:309, GLNA:314 ALAA:315, ALAB:61, ALAB:64, LYSB:65, GLUB:68, SERB:163
Reactive Red 1	-8.3	4	11.29	VALB:18, TRPB:100, ASNB:101 GLYB:145, TYRB:148, VALA:52, ILEA:57	-6.8	4	11.14	PROA:30, PROA:78, ASPA:76, LYSB:57, ALAB:61, ALAB:64, SERB:162, ASPB:159
Reactive Yellow 17	-7.6	6	18.63	ARGB:12, ALAB:17, VALB:18, TRPB:100, ASNB:184, ILEA:57,	-6.7	5	14.25	PROA:30, VALA:31, SERA:33, PROA:74, ASPA:76, PROA:78,

				THRA:60, GLYA:64, ALAA:118				TYRA:309, LYSB:57, ALAB:61, GLUB:68

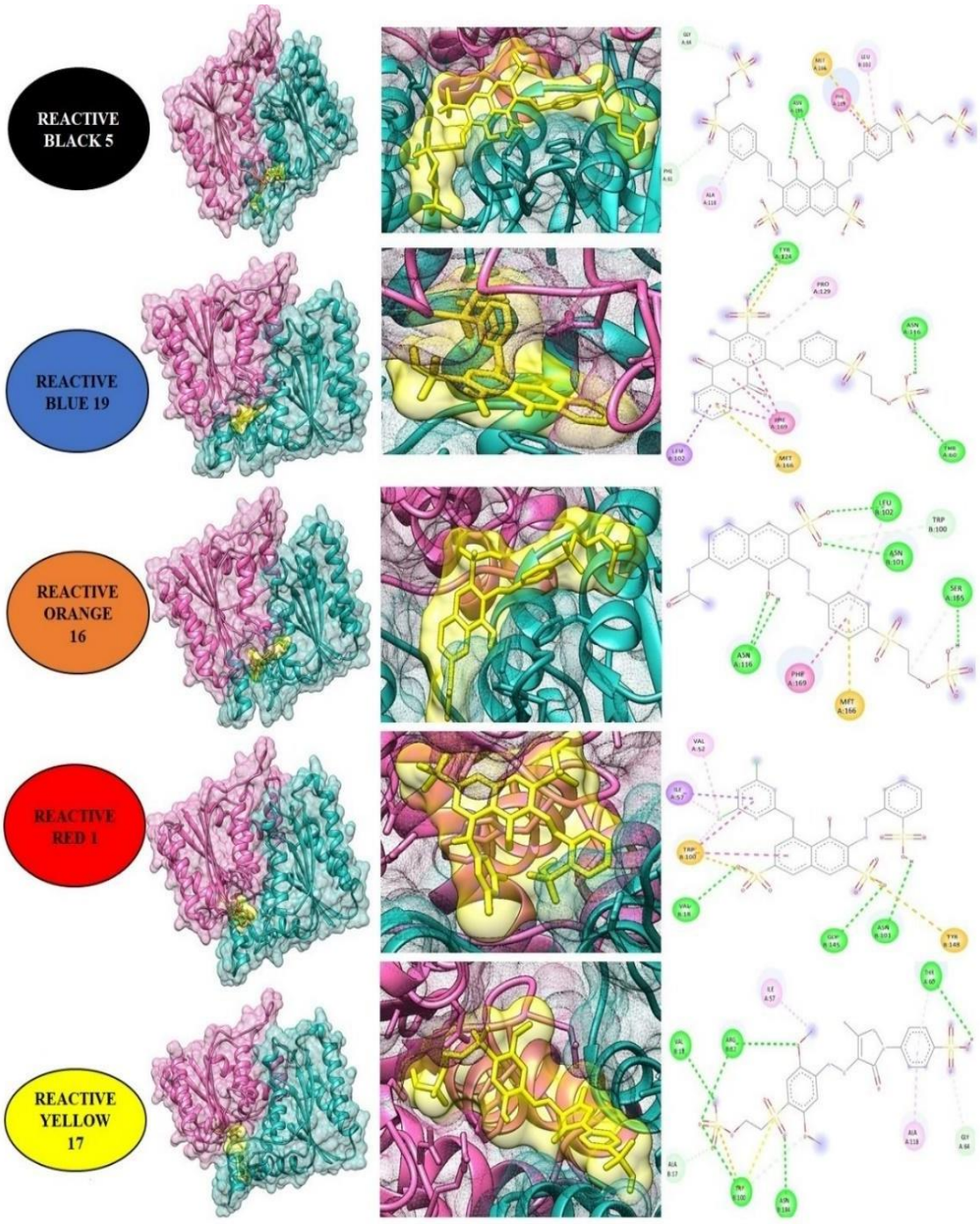


Figure. 2: 2D and 3D representation of Molecular docking of Azoreductase

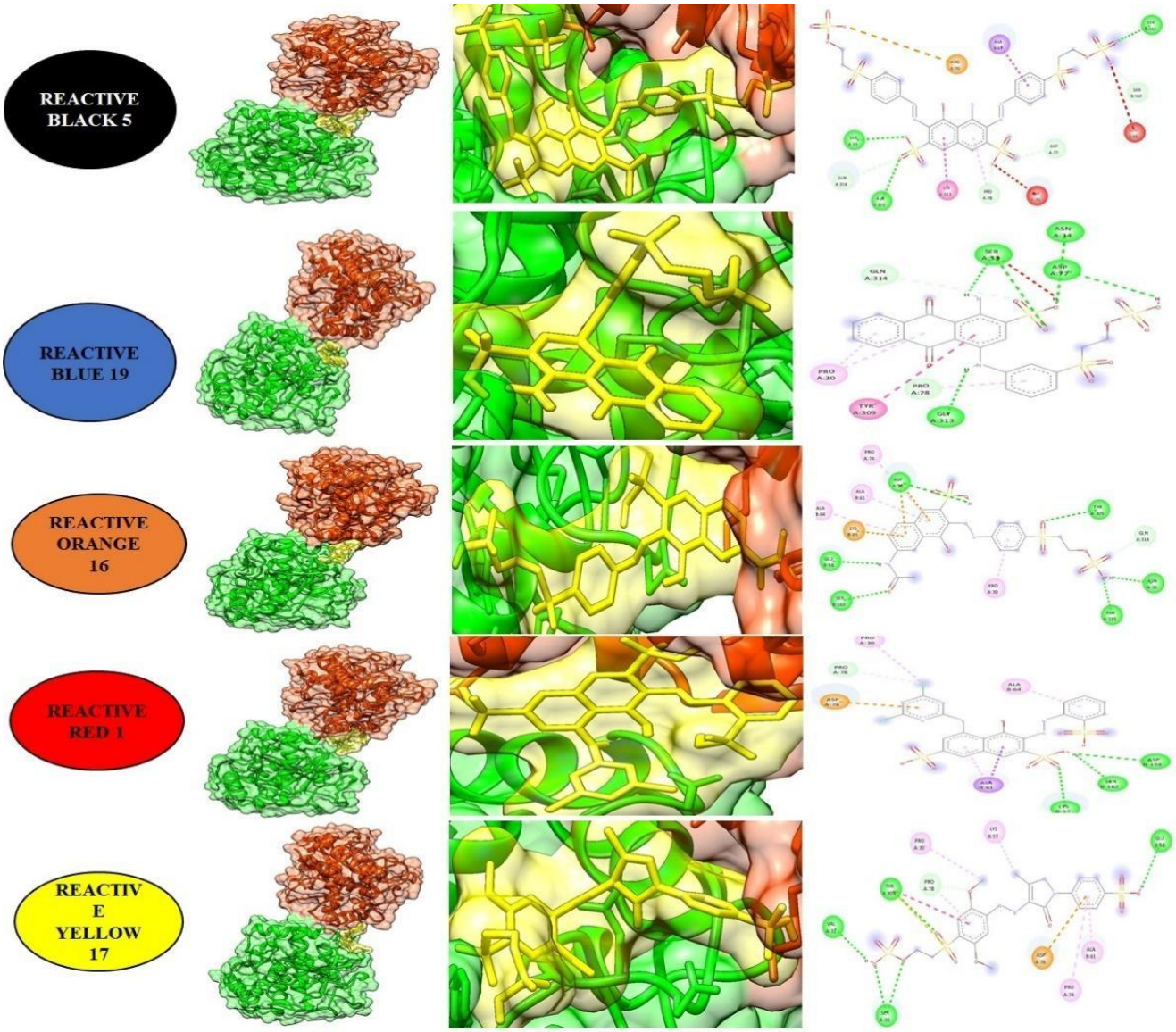


Figure. 3: 2D and 3D representation of Molecular docking of Polyphenol oxidase

Molecular dynamic simulation

Molecular dynamics (MD) is among the most frequently employed and efficient approaches to studying protein ligand interactions, protein structures, solvation of molecules, or real-time dynamics and structural features of proteins (Adamu et al., 2023; Oyewusi et al., 2024). Reactive Blue 19 has shown good binding affinity with both proteins i.e. azoreductase and polyphenol oxidase. MD Simulation study was performed for Reactive Blue 19 Dye with both proteins. To assess system stability, root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration, and MM-PBSA calculations was monitored throughout the production run. The RMSD for Cα atoms of azoreductase protein showed that Protein backbone was less than 0.5nm and remained stabilized throughout the 100ns simulation and no noise was observed in the end. The RMSD profile of Polyphenol Oxidase showed that the protein backbone increased to approximately 0.8 nm from 20 ns to 40 ns, then further increased to 1.2 nm at 40 ns. It subsequently dropped to around 1 nm at 45 ns and stabilized for the rest of 100ns simulation, with no noise observed at the end. The RMSD value of the protein backbone indicated that the presence of Reactive dye 19 did not induce significant alterations in the structural backbone of both the proteins, as shown in figure 4a.

The radius of gyration (Rg), which is used to describe the conformational dynamics, compactness of the protein during the simulation process. A larger Rg value typically indicates a more extended or less compact structure, while a smaller Rg value suggests a more compact or tightly folded structure (Adamu et al., 2023). As shown in Figure 4b, the protein was stable and did not lose its structure throughout the simulation. RMSF stands for Root Mean Square Fluctuation studies was done to analyse any fluctuations in the individual atom or group of atoms during the simulation. The residues that are involved in the binding has shown low fluctuation as shown in figure 4c & 4d. Further estimation of free binding energy using MM-PBSA calculation indicated that binding affinity of polyphenol oxidase with Reactive blue 19 is -90.901 +/- 3.495 (kJ/mol) and binding affinity of azoreductase with Reactive blue 19 is -72.096 +/- 3.340 (kJ/mol) as shown in Table 4. Various components, such as solvent-accessible surface area (SASA), polar solvation energies, intermolecular van der Waals forces, and electrostatic interactions, were combined to determine the MM-PBSA values. Given its strong binding affinity, polyphenol oxidase offers a sustainable approach for dye decolorization.

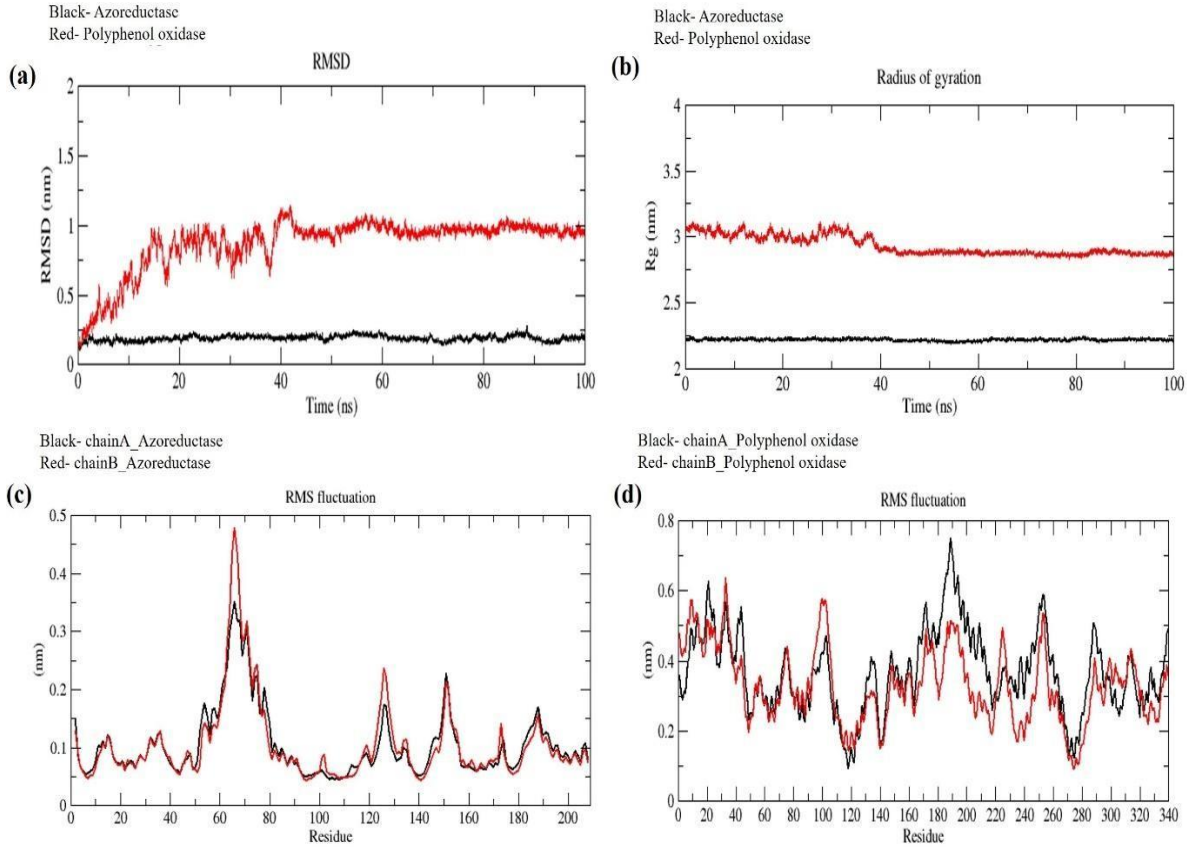


Figure 4: Molecular Dynamics Trajectory Plots a) Root Mean Square Deviation (RMSD), b) Radius of gyration (Rg), c) Root Mean Square Fluctuation Chain A (RMSF), d) Root Mean Square Fluctuation Chain B (RMSF) in a 100ns simulation.

Table 4: Computed MM-PBSA Binding Energy of Azoreductase and PPO with Reactive Blue 19

	Van der Waal energy (kJ/mol)	Electrostatic energy (kJ/mol)	Polar solvation energy (kJ/mol)	SASA energy (kJ/mol)	Binding energy (kJ/mol)
Azoreductase	-160.702 +/- 2.809	-136.620 +/- 7.491	243.877 +/- 8.647	-18.408 +/- 0.224	-72.096 +/- 3.340
Polyphenol oxidase	-129.309 +/- 1.956	-168.665 +/- 5.920	218.578 +/- 6.639	-11.525 +/- 0.252	-90.901 +/- 3.495

Enzyme activity of extracted Polyphenol oxidase

Enzyme activity of Polyphenol oxidase enzyme purified from peel of *Lagenaria siceraria* was studied. Protein concentration was determined using the Bradford assay, results shown in Table 5. The enzyme activity was assessed by monitoring the initial rate of quinone formation, which was detected by an upsurge in absorbance at 420nm. Specific activity of the partially purified enzyme was increased to 1374.73 U/mg with a purification fold of 2.27.

Table 5: Purification of PPO from Peel of *Lagenaria siceraria*

Purification Step	Total Protein (mg)	Total Activity	Specific activity (U/mg)	Percentage Yield	Purification factor
Crude	58.17	35196.92	605.07	100	1
Acetone Precipitated	15.22	20923.39	1374.73	59.44	2.27

Gel diffusion assay

Gel diffusion assay was done to confirm the presence of polyphenol oxidase enzyme. Crude enzyme (50 µl,100 µl and 200µl) and partially purified enzyme (50 µl,100 µl and 200µl) were added to wells in a petri dish containing catechol as the substrate and kept for 12 h at 35ºC. The formation of zones and black rings around the wells indicated the presence of PPO activity as shown in Figure 5.

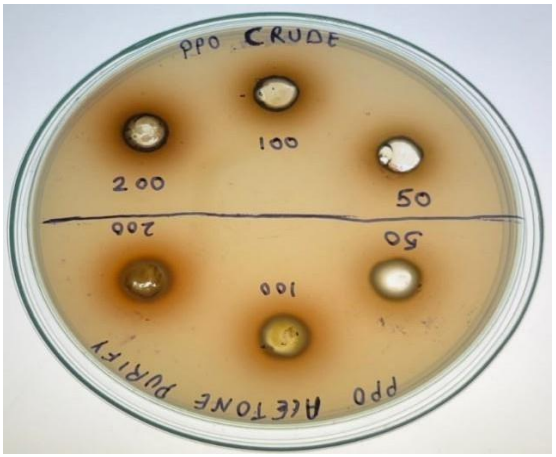


Figure 5: Identification of PPO activity on Catechol Agarose Plate Assay

Effect of pH, substrate and temperature on PPO activity

The effects of pH (ranging from 4 to 8), substrate concentrations (5 mM, 15 mM, 25 mM, 50 mM, 75 mM, 100 mM, and 125 mM), and temperatures (25°C, 35°C, 45°C, and 55°C) on PPO activity were studied. The enzyme activity of PPO gradually increased to pH 6 and then decreased considerably giving a bell-shaped curve. The optimum temperature was found to be 35ºC and the activity decreased as temperature increased. The enzyme activity gradually increased as the concentration increased till 50Mm, after than it dropped and remain constant till 125Mmas shown in Figure 6.

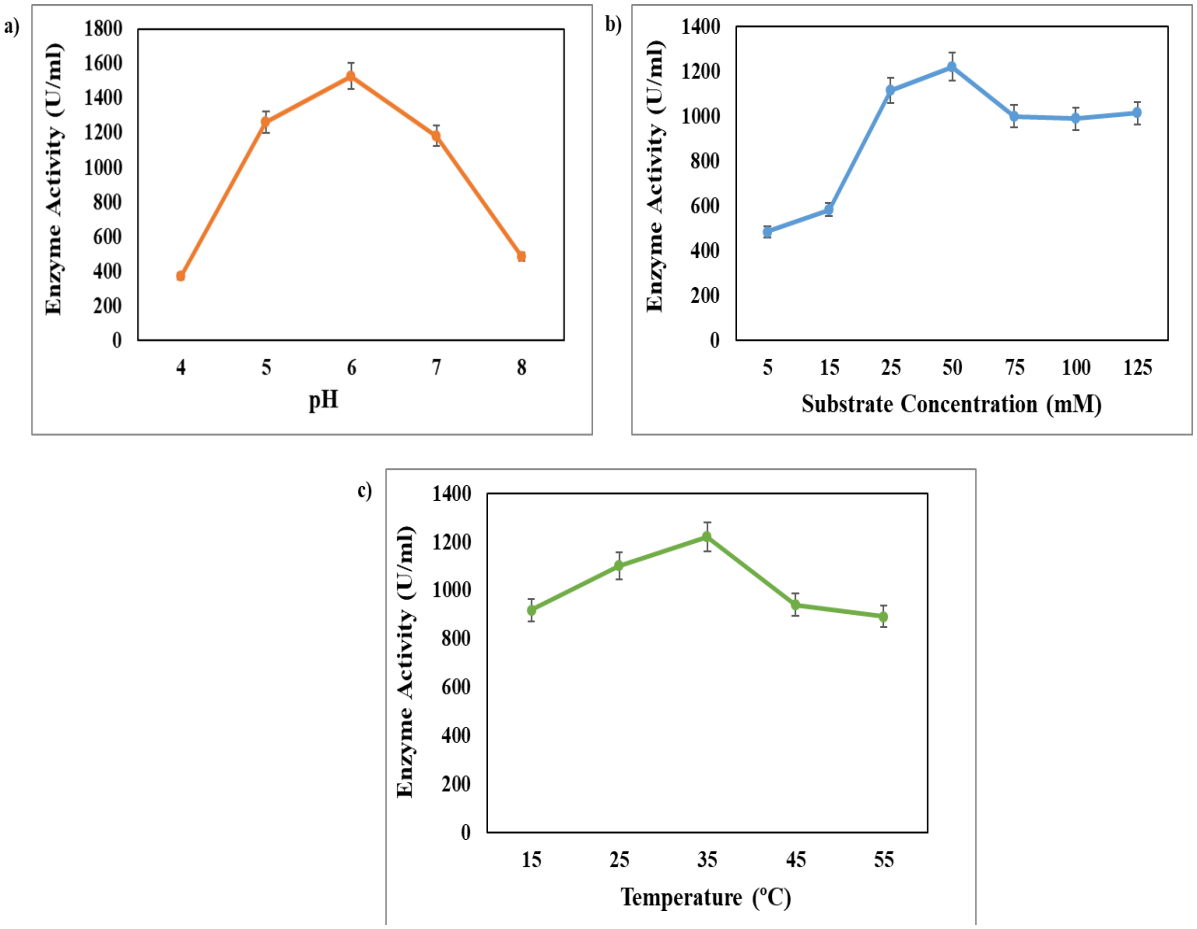


Figure 6: Effect of pH, Substrate Concentration and Temperature on PPO Activity

Decolorization of dye using free enzyme

Absorption spectrum of five reactive dyes was measured by taking the absorbance of each dye at varying wavelengths (325nm to 700 nm). Maximum wavelength of reactive textile dyes was noted to be Novacron Red orange H3R2: (500nm), Novacron Yellow C 5G (425nm), Novacron Orange EC 3R (425 nm), Novacron Blue C 5X (600nm), Novacron Deep Red ECD (525nm) as shown in Table 6. Maximum decolourization was observed in Novacron Blue C 5X Dye of 14 % followed by Novacron Yellow (12%), Novacron Deep Red ECD (9.2%), Novacron Red orange H3R2 (1.16%), Novacron Orange EC 3R (0.57) as shown in Table 7. The Percentage decolorization was increased in all dyes with increase in time except in Novacron Orange EC it decreased as shown in Figure 7. Visualization of the decolorized dye can be seen in Figure 8. The free enzyme was getting denatured after 24 h.

Table 6: Dyes and Their Maximum Wavelength

Dyes	Commercial names	Maximum wavelength (nm)
Reactive Red 1	Novacron Red orange H3R2	500
Reactive Yellow 17	Novacron Yellow C 5G	425
Reactive Orange 16	Novacron Orange EC 3R	425
Reactive Blue 19	Novacron Blue C 5X	600
Reactive Red	Novacron Deep Red ECD	525

Table 7: Percentage Decolorization of 5 Reactive Textile Dyes in 4 Hours

Dyes	Percentage Decolorization (%)
Novacron Blue C 5X	14
Novacron Yellow C 5G-	12
Novacron Deep Red ECD	9.2
Novacron Red orange H3R2	1.16
Novacron Orange EC 3R	0.57

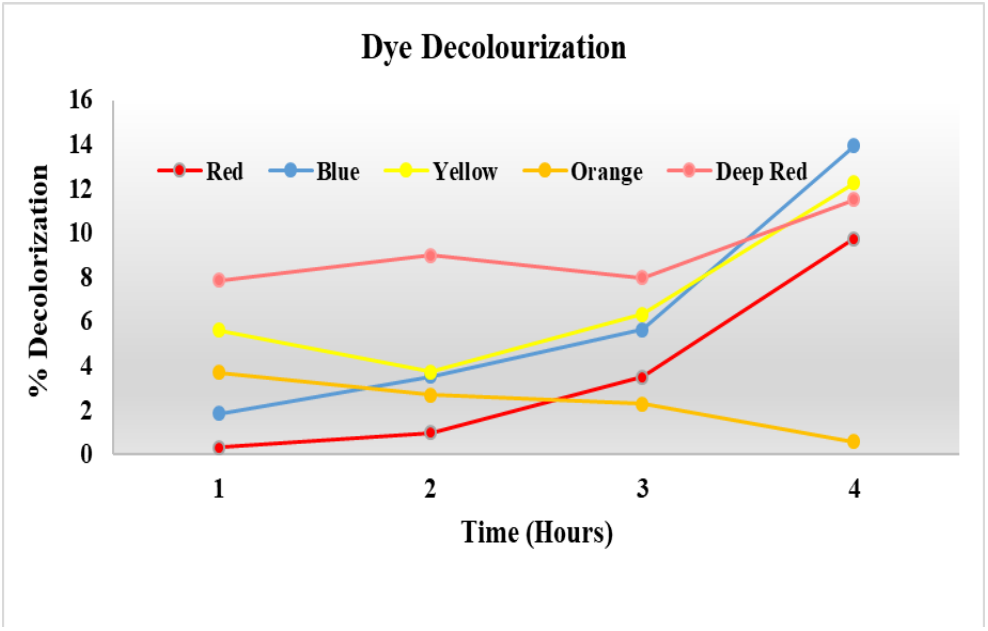


Figure 7: Percentage Decolorization of 5 Textile Dyes with Time

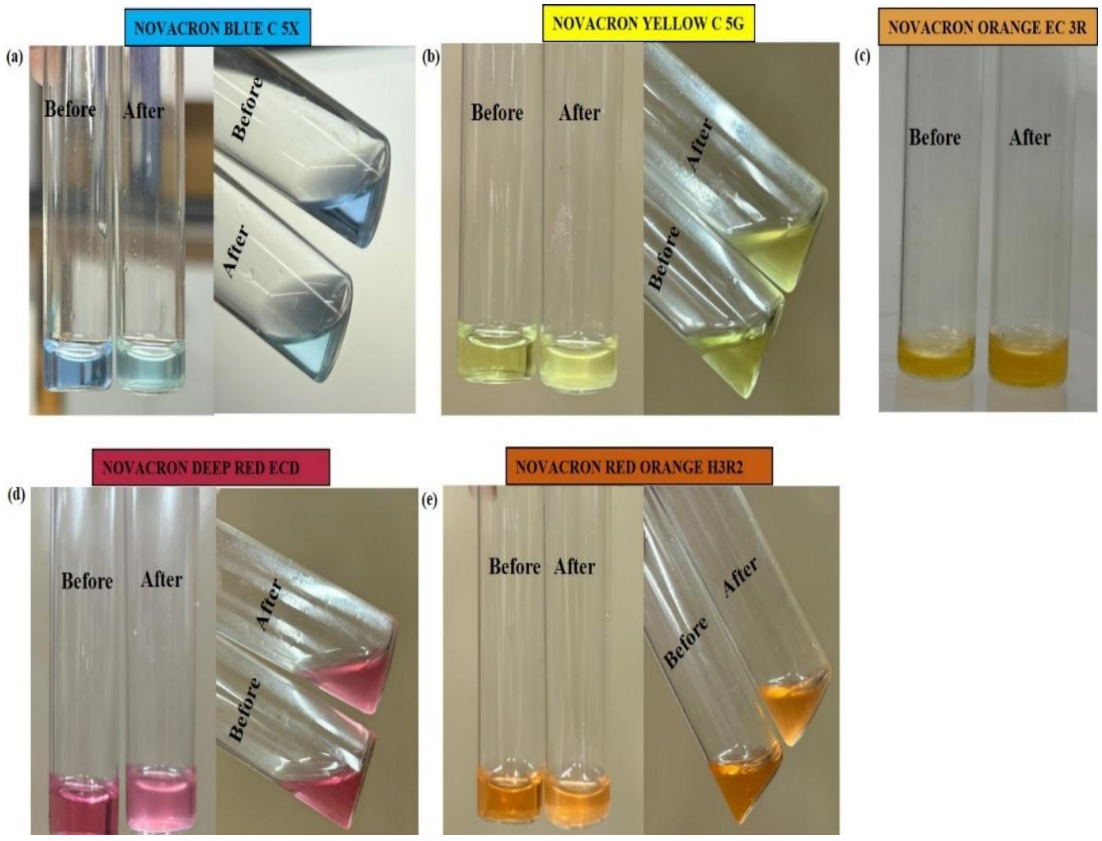


Figure 8: Decolorization of reactive textile dyes in 4 h

Enzyme activity of enzyme immobilized on cotton fabric

Immobilization of enzyme onto a solid support will increase the stability of enzyme and protect them from various inhibitors while maintaining the original enzyme structure. It also makes the reuse of enzyme easier up to several cycles. Immobilized enzyme can eliminate more phenols and dyes due to combined effect of enzymatic reaction and adsorption of pollutants (Al-Sakkaf et al., 2023). Enzyme activity of crude and acetone precipitated enzyme sample was 1034 U/ml and 744 U/ml respectively. This enzyme was further immobilized using adsorption method on two types of cotton fabric: cotton fabric that had been enzymatically treated and cotton fabric that had been both enzymatically treated and surface-modified with acetic acid, representing direct and indirect immobilization approaches. Enzyme activity of cotton fabric with direct and indirect immobilization is 277 U/ml and 316 U/ml, respectively. Enzyme activity of supernatant 1 i.e. leftover solution after the direct immobilization process is 684 U/ml, while in supernatant 2 i.e. leftover solution after indirect immobilization is 614 U/ml. Recovered activity (%) represents the percentage of enzyme activity that remains after immobilization compared to the initial free enzyme activity. Cotton fabric with indirect immobilization showed more recovered activity of 42.4% than direct immobilization i.e. 37%. Immobilization yield (%) represents the percentage of enzyme that was successfully immobilized onto the fabric. It is calculated on the basis of enzyme activity of supernatant before and after immobilization as shown in Table 8. Indirect immobilization retains more enzyme activity.

Table 8: Enzyme Activity of Immobilized Fabric

	Enzyme Activity (U/ml)	Specific activity (U/mg)	Recovered Activity (%)	Immobilization Yield (%)
Crude	1034	467		
Acetone precipitated	744	470.8		
Cotton Fabric (direct immobilized)	277		37	-
Cotton Fabric (indirect immobilized)	316		42.4	-
Supernatant 1 (direct immobilization leftover)	684	487	-	8%
Supernatant 2 (indirect immobilization leftover)	614	401	-	17.4%

Decolorization of dyes using immobilized enzyme

Immobilized enzyme was used for decolorization of dyes. The decolorization percentages are generally lower across all dyes and time points in direct immobilization. For instance, Novacron Blue C 5X shows only 7.88% decolorization after 24 h, and Novacron Yellow C 5G only achieves 1.8% decolorization.Some dyes, such as Novacron Red Orange H3R2 and Novacron Orange EC 3R, show almost no decolorization after 4 hours and very low decolorization after 24 h. In contrast, Indirect immobilization (Cotton fabric modified with acetic acid and PPO enzyme) method shows higher decolorization percentages. For example, Novacron Blue C 5X achieves 11.66% decolorization in 4 hours and 27.6% after 24 h, significantly higher than with direct immobilization. Novacron Yellow C 5G shows 8.62% decolorization after 4 h and 11.98% after 24 h, again much higher than the direct method, shown in Table 9. Visualization of decolorized Novacron Blue C 5X is shown in Figure 9.

Table 9: Decolorization of Dyes by Immobilized Enzymes

Dyes	**Percentage Decolorization (%)**			
	Direct Immobilization (Cotton fabric + PPO enzyme)		**Indirect Immobilization (Cotton fabric modified with acetic acid +PPO enzyme)**	
	4 hours	**24 hours**	**4 hours**	**24 hours**
Novacron Blue C 5X	3.94	7.88	11.66	27.6
Novacron Yellow C 5G-	0.4	1.8	8.62	11.98
Novacron Deep Red ECD	3.2	5.28	2.94	7.36
Novacron Red orange H3R2	-	1.24	1.56	3.22
Novacron Orange EC 3R	-	1	0.345	2.1

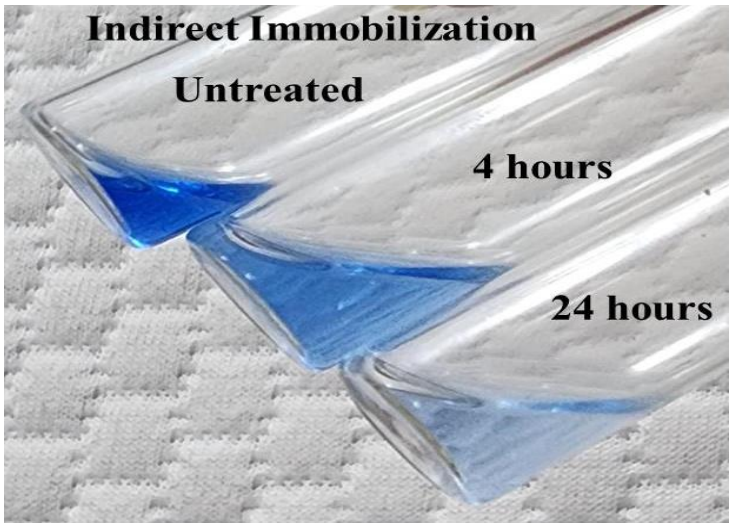


Figure 9: Decolorization of Reactive Textile Dye Novacron Blue C 5X by Immobilized PPO Enzyme

CONCLUSION

Molecular docking studies revealed that the enzymes azoreductase and polyphenol oxidase exhibit strong binding affinity with reactive dyes. Notably, the highest affinities were observed with Reactive Blue 19, showing binding energies of -8.5 and -7, respectively. MD simulation studies showed that the both protein complexes with Reactive Blue 19 is structurally stable and ligand were tightly bound to the protein. MM- PBSA Calculation shows Binding affinity of polyphenol oxidase with Reactive blue 19 is -90.901 +/-3.495 and Binding affinity of azoreductase with Reactive blue 19 is -72.096 +/-3.340. Polyphenol oxidase can be used for decoloration of dye and it's a sustainable approach. Specific activity of the partially purified enzyme from peel *Lagenaria siceraria*of is 1374.73 U/mg. Black rings appeared in the gel diffusion assay confirmed the polyphenol oxidase activity. The optimum parameter required for enzyme activity were found to be pH 6, substrate concentration of 50mM, and temperature 35ºC. Maximum decolorization was observed in Novacron Blue C 5X Dye of 14 % followed by Novacron Yellow (12%), Novacron Deep Red ECD (9.2%). After immobilization of enzyme on cotton fabric, recovered PPO enzyme activity after direct immobilization was 37% and for indirect immobilization was 42.4%. Immobilization yield on fabric with direct immobilization was 8% and for indirect immobilization was 17.4. Dye decoloration was better in indirect immobilization as it has covalent bonding, 27.6% of Novacron Blue C 5X dye got decolorised after the treatment while it showed only 7.88% decolorization in direct and 14% decolorization in free enzyme treatment. So, peels can be a good source of polyphenol oxidase enzyme and used for treatment of dyes and the efficiency of enzyme can be improved by immobilization method.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest and no funding was received to assist with the preparation of this manuscript.

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