

# **Degradation of sulphonated mono and di-azo dye as the sole carbon source in *Serratia marcescens*: Insights from combined wet and dry lab analysis**

**Zarrin Basharat<sup>1\*</sup>, Azra Yasmin<sup>2\*</sup>**

<sup>1</sup>Alpha Genomics (Private) Limited, 45710-Islamabad, Pakistan.

<sup>2</sup>Microbiology & Biotechnology Research Lab, Department of Biotechnology, Fatima  
Jinnah Women University, Rawalpindi 46000, Pakistan.

\*Corresponding author

Email: Z.B. [Zarrin.iiui@gmail.com](mailto:Zarrin.iiui@gmail.com); A.Y. [azrayasmin@fjwu.edu.pk](mailto:azrayasmin@fjwu.edu.pk)

## 18 Abstract

19 The high production volume of azo dyes for manufacturing and treating various  
 20 consumer products leads to deleterious environmental consequences. Bacterial  
 21 agents present in the environment can degrade these dyes. We, hereby, report the  
 22 isolation, decolourization and degradation of a mono (Methyl orange) and di-azoic  
 23 (Congo red) compound of this class of dyes by a versatile bacterium *Serratia*  
 24 *marcescens*. Our isolate showed the capability of sulphonated azo dye  
 25 utilization/degradation i.e. Methyl orange and Congo red usage, with no inhibitory  
 26 effects on its growth in minimal medium. The calorimetric analysis showed 80.83%  
 27 decolourization of Methyl orange and 92.7% decolourization of Congo red after 7  
 28 days of incubation in a shaking incubator at pH: 7 and temperature: 37 °C. An  
 29 azoreductase enzyme of ~25 KDa was detected after SDS-PAGE analysis.  
 30 Quantitative and qualitative testing of the degradation phenomenon was followed  
 31 by *in silico* analysis. Structural modeling followed by molecular docking in  
 32 Molecular Operating Environment revealed numerous residues involved in binding  
 33 and assisting degradation. Changes in the apo, holo, and dye-bound enzyme energy  
 34 profiles were also observed. This is the first study reporting the capability of *Serratia*  
 35 *marcescens* to use azo dyes/sulphonated azo dyes as the sole carbon source and the  
 36 detailed computational analysis of the degradation phenomenon. We hope that these  
 37 findings will be of use to environmental scientists, aid in better dye-degrading  
 38 mutant creation to help craft future remediation strategies for sulphonated azo dyes.

39 **Keywords:** Azoreductase, *Serratia marcescens*, Methyl orange, Congo red, Docking,  
 40 Simulation.

41

42

43

44

45

46

47

## 48 1. Introduction

49 The lucky discovery of azo dyes by Sir William Henry Perkin in 1853, opened a new  
 50 avenue for synthetic coloring [1-2]. Based on the presence of single, double, and  
 51 triple-N=N- bond, azo dyes are termed mono-azo dyes, di-azo dyes, and tri-azo  
 52 dyes [3]. Besides, some azo colorants own auxochrome functional groups like  
 53 sulphonic acid, carboxylic acid, hydroxyl groups, and amino groups. These  
 54 auxochromes do not produce color but only alter solubility, intensity, and the  
 55 wavelength of the absorbed light [4]. Phenolic compounds chiefly form azoic dye  
 56 constituents [5]. Azo dyes are extensively used in numerous industries for dyeing  
 57 textiles, leather, plastics, etc [6]. These dyes and their residues have become a  
 58 potential threat to human and ecological health in complex detrimental ways owing  
 59 to dye recalcitrance to degradation [7]. The estimated release of azoic colorant  
 60 during the dyeing procedure into the water is approximated to be 10–50% [8].  
 61 Extensive usage of these dyes has led to serious pollution problems.

62 Accumulation and ingestion of azo dyes by aquatic organisms in water bodies leads  
 63 to deleterious consequences like teratogenicity [9], biomagnification, etc [10].  
 64 Mutagenic, cytotoxic, and genotoxic effects of an azo dye investigated by Tsuboy  
 65 and his colleagues proved that azo dyes kindle DNA fragmentation, micronuclei  
 66 formation and augment the human hepatoma (HepG2) cell apoptotic index [11]. The  
 67 toxicity of several of these dyes has led to their ban by the European Union [12],  
 68 California (The safe drinking water and toxic enforcement act of 1986 i.e. California  
 69 proposition 65), and other nations [13] but their production and use persists in many  
 70 parts of the world due to their low manufacturing cost and several other desirable  
 71 characteristics.

72 Microbial or enzymatic decolourization of azo dyes is a cost-effective and eco-  
 73 friendly alternative to chemical treatment method [14]. The distinctive metabolic  
 74 aptitudes of certain microbes are momentous for bioremediation purposes as these  
 75 can be utilized for recalcitrant and hazardous environmental pollution cleanup.  
 76 Oxygen utilizing bacterial strains may utilize azo dyes as a sole carbon source,  
 77 whereas azoreductase does the job in other [15]. Azoreductase (EC 1.7.1.6) is vital to  
 78 the degradation of the azoic bond using the ping-pong mechanism and exists in  
 79 several microorganisms [16]. Here, we report dye decolourization and an  
 80 azoreductase (AzoR) encoded by *Serratia marcescens*, a bacterium isolated from the  
 81 effluent of chemical industries that can utilize azo dye as a sole carbon source. This is  
 82 a gram-negative, facultative anaerobe and known for its ability to degrade various  
 83 organic compounds. However, to the best of the authors knowledge, analysis of its  
 84 AzoR enzyme for azo dye (Congo red and Methyl orange) decolourization had not  
 85 yet been attempted. By focusing on this specific bacterial strain, we contribute to the  
 86 understanding of its potential in azo dye degradation and expand the knowledge  
 87 base in this field. We also mined the sequence of this enzyme from the NCBI  
 88 database and modeled it. This was followed by molecular docking and dynamics

simulation analysis for the explanation of the particular biological activity. A combined wet and dry lab experimentation can aid in unearthing the role of catalytic residues and compounds in substrate specificity and activity, which in turn can be used for designing improved mutants and recombinants for enhanced dye degradation. Hence, this work explores the potential of *Serratia marcescens* for azo dye degradation and provides a detailed understanding of the degradation mechanism through enzyme characterization and computational analysis.

## 2. Material and methods

A heavy metal resistant, plant associated *Serratia marcescens* isolated from chemical industrial effluent (Accession no: KJ729142.1), was obtained from the microbial culture collection of our lab. It was initially identified through 16s rRNA sequencing.

### 2.1. Screening of bacteria for dye degradation/utilization capability

Luria-Bertani medium and Bushnell Hass medium were used for primary [17] and secondary screening [18] of dye degradation capability of *Serratia marcescens*. Congo red (CR) (C.I. 22120) and Methyl orange (MO) (C.I. 13025) were purchased from Sigma Aldrich. All reagents used were of analytical grade. The stock solution of MO (0.5 mg/100ml water) and CR (1 mg/90 ml water+10 ml ethanol) was prepared by dissolving the solid chemicals in autoclaved distilled water. The percent decolourization was calculated by the formula [19-20]:

Percent decolourization = (Optical density of control – Optical density of sample) ×100 % / (Optical density of control)

### 2.2. Fourier transform infrared spectroscopy analysis

A 3:1 ratio of Potassium bromide vs pellet of the bacterial degraded product was taken and samples then were ground using mortar and pestle to fine particles and pellets were prepared by establishing a pressure of 100 kg/cm<sup>2</sup> (1200 psi). Infrared spectra were obtained by scanning the prepared pellets with a spectrometer (Schimadzu). The spectrum of air was recorded as background and subtracted automatically by using appropriate software. FTIR spectra of degraded dye samples were recorded in the 4000-400 cm<sup>-1</sup> region [21] at room temperature and the spectrum obtained was overlaid with the dye control and interpreted.

### 2.3. Enzyme extract preparation

The cell-free extract was prepared for activity assay following Aftab *et al.* [22] with necessary modifications. Bacterial cultures were grown in the 100 µg azo dye supplemented with a minimal salt medium for 96 hours at 37 °C. Cells were harvested by centrifugation for 15 minutes at 9000 rpm. The pellets were then

washed twice with 20 mM potassium phosphate buffer (pH:7), frozen at -20 °C, thawed, and then suspended in 10 ml of the same phosphate buffer. Cells were disrupted by the beat-beater method [23]. Universal bottles containing the buffered pellet were supplied with few glass beads and 30 seconds of agitation on vortex was followed by 30-second placement on ice. The process was repeated four times. The homogenate was then centrifuged at 8000 rpm at 4 °C for 20 minutes and the supernatant was used as a crude extract. This was purified by the ammonium sulfate precipitation method followed by dialysis. Solid ammonium sulfate was added to the crude enzyme solution at a concentration of 60%. After dissolving salt in crude enzyme, the solution was placed in cold overnight and then precipitates were collected by centrifugation at 10,000 rpm for 15 minutes. Precipitates were dissolved in the 20 mM phosphate buffer of pH 7 and kept at 4 °C.

A Quantitative Azor assay was then carried out [22] in glass cuvettes with 1ml reaction volume. 400 µL of 20 mM phosphate buffer (pH 7.0) mixed with 200 µL protein sample and 200 µL dye (25 µmol/ml), 200 µL of co-enzyme Nicotinamide adenine dinucleotide hydride (NADH) (1 mM) was monitored spectrophotometrically at 464 nm (for MO) and 498 nm (for CR).

#### 2.4.SDS PAGE analysis

SDS page was carried out following the method of Laemmli [24]. 1 ml culture was collected (purified and crude) and cells were prepared for analysis by adding a final 1 X concentration of SDS-PAGE loading buffer, boiling the sample for 15 min, and loading 20 µl onto a 12.5% SDS-PAGE gel [25]. The gel was stained by Coomassie brilliant blue R-250. The broad range protein ladder was used as a marker for the elucidating weight of AzoR.

#### 2.5.Structure modeling and dynamics simulation

For computational analysis of the AzoR sequence, the 25 kDa AzoR was obtained from NCBI. This was done by the basic local alignment of 16s rRNA of our strain with fully sequenced genomes of *Serratia marcescens* (encoding AzoR). The one with the least E-value and highest similarity (Accession no: ETX40909.1) was procured for dry lab analysis. The structure was determined [26] by homology modeling using iterative threading assembly simulations in the I-TASSER [27-28]. The structure was then simulated for 10 ns [29] using CABS-Flex server [30]. This was done to visualize the flexibility and stability of the protein chain.

#### 2.6.Molecular docking analysis

The substrate molecules Congo red and Methyl orange were downloaded from the PubChem database [31] of NCBI. Open Babel [32] was used for importing SDF files for format conversion and optimization. Profix and TINKER program was run to fix structural defects such as missing atoms of AzoR. The optimized receptor

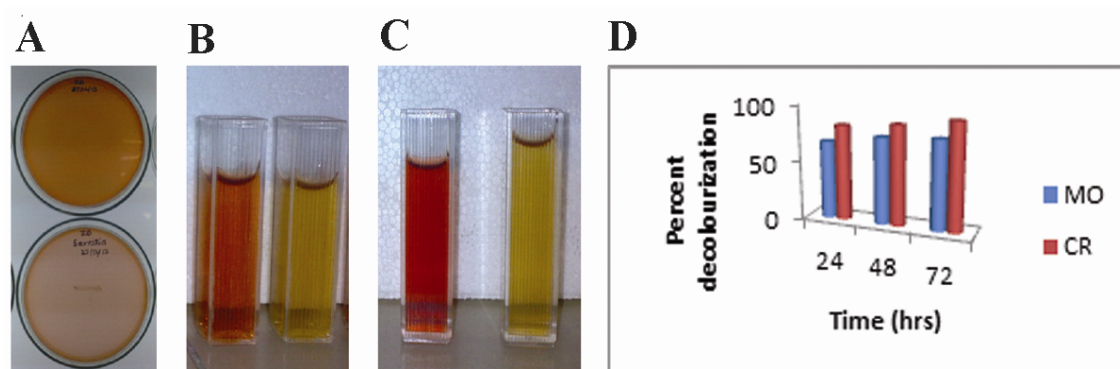
(protonated, energy minimized) and ligand molecules were used for the docking study. Flavin mononucleotide (FMN), NADH, MO, and CR substrate molecules were positioned in the binding pocket by scanning the whole protein surface and flexibly docking the ligands with AzoR in Molecular Operating Environment software. The Triangle Matcher placement method was used for docking. London and affinity dG were used for scoring and rescoring of predicted poses [33]. The top ten structures were retained and the one with the lowest S value was chosen for analysis. Interactions were then visualized. Various energies (angle bend, van der Waals interaction, out-of-plane, bond stretch, dihedral rotation, etc) of ligand-bound and unbound state of AzoR were also studied. The sum of these energies i.e. total potential energy of the system was also recorded.

### 3. Results and Discussion

Genotoxic azo-benzene mediated dye pollution is a threat to both human health and the environment. We are in an urge for fast screening of bacterial varieties with an aptitude for pollutant degradation and hence, find new strains with better degradation capabilities. We explored the potential of *Serratia marcescens*, isolated from chemical industrial effluent, and exploited it for the degradation of sulphonated azo dyes MO and CR.

#### 3.1. Growth in MO and CR supplemented media

The plate and quantitative dye reduction assay were carried out for the strain, showing a high rate of decolourization (Fig. 1). The bacterium was tested for dye tolerance up to 250 µg/ml and showed growth at all tested concentrations. Previously, *Serratia marcescens* has been reported to degrade azo dyes (other than sulphonated dyes) but at a lower scale [34]. The degradation activities were attributed to the manganese peroxidase and laccase enzyme. In a study by Mahmood *et al.* [35], another specie of the genus *Serratia* i.e. *Serratia proteamaculans* was reported to completely degrade a sulphonated azo dye (Reactive black 5) in 12 hrs. A molybdenum reducing *Serratia marcescens* could decolorize less than 20% of MO and CR in 48 hrs [36]. Our strain showed way higher decolourization of MO and CR in 48 hrs (Fig. 1).



**Fig. 1.** (A) Growth and decolourization of the 50 µg CR dye supplemented BH media after 7 days. The upper Petri plate shows the control. (B) 24 hour decolorized MO, (C) CR in LB medium with controls. (D) Percent decolourization for *Serratia marcescens* in LB dye supplemented media.

### 3.2. Use of MO and CR as the sole carbon source

*Serratia marcescens* could utilize the azo dyes as the sole carbon and energy source. The decolourization percentage was calculated for the strain in liquid BH medium after 96 hours of incubation in the shaking incubator. The decolourization in BH medium for MO after 7 days placement in shaking incubator was 80.83% and for CR 92.7%. Decolourization was more for diazo sulphonated dye as compared to the mono azo dye. It might be due to the structure-activity relation of the dye and the decolourizing enzyme pocket. The growth optical density (O.D.) increased with time and decolourization was dependent on it.

Bacteria capable of aerobic decolourization and mineralization of dyes, especially sulphonated azo dyes, have proven difficult to isolate and they need to be specially adapted for this purpose [37-38]. Formerly, a bacterial strain *Hydrogenophaga palleronii* S1 capable of growing on azo dye as the sole carbon and energy source was obtained by continuous adaptation with dye. The strain could grow cleaved 4-carboxy-4'-sulfoazobenzene reductively under aerobic conditions [39]. Dehydrogenases, menaquinones, and cytochromes were found to be essential electron transfer components for azo reduction during the growth of *Shewanella decolorationis* S12 using the azo compound as the sole electron acceptor [40]. Bheemeraddi *et al.* [41] demonstrated that rapid dye decolourization takes place in the presence of glucose and yeast extract as compared to starch, lactose, sucrose, and other nitrogen sources like peptone, beef extract, potassium nitrate, and sodium nitrate. Organic nitrogen sources might be responsible for NADH regeneration acting as an electron donor in azo bond reduction.

This type of decolourization is rare to find in bioremediation studies but recently, Manogaran *et al.* [42] reported decolourization of a sulphonated bis azo dye,

Reactive red 120 by a consortium of bacteria (including *Serratia marcescens*). The consortium required prolonged acclimation for better efficiency or the decolourization was low. Nevertheless, *Serratia marcescens* coupled with *Pseudomonas aeruginosa* and *Enterobacter* sp. could mineralize the dye completely. Several co-substrates or trace elements could enhance decolourization but the addition of yeast showed the opposite effect. It is contemplated that the synergistic effect of the bacterium in consortium might have a beneficial impact on each other to attain the decolourization. Our isolate of the *Serratia marcescens* showed the capability of utilizing azo dyes as a sole source of electron acceptors and with minimal nutritional requirements but it is contemplated that carbon and nitrogen source is imperative to augment the pace of bacterial biodegradation activity. This is the first report of the capability of the axenic culture of *Serratia marcescens* to use azo dyes (sulphonated azo dyes) as the sole carbon source.

### 3.3. FTIR analysis

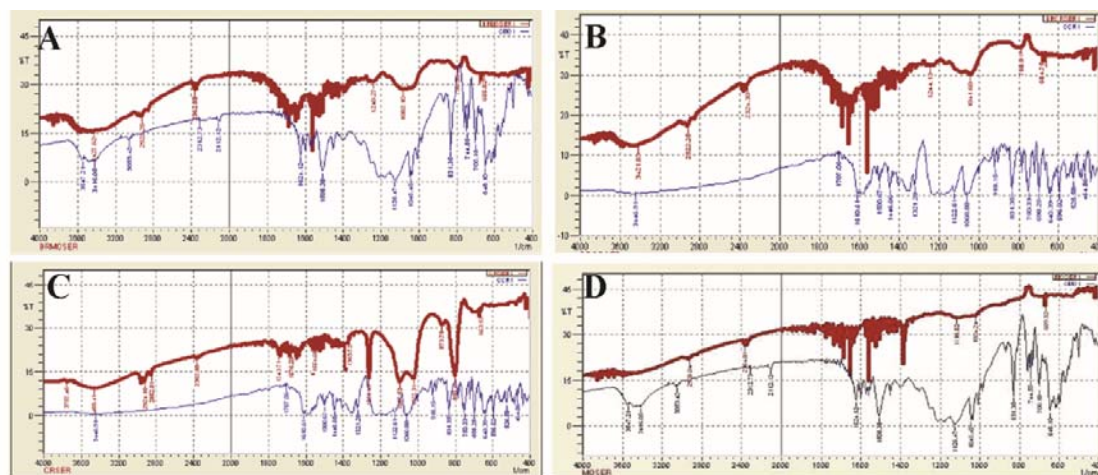
FTIR analysis of MO control showed specific peaks at 3100 cm<sup>-1</sup> ( asymmetric C-H stretching vibration), 3060 cm<sup>-1</sup> (=C-H stretching vibration), 2112 cm<sup>-1</sup> ( C-C and CN stretching vibrations), 1700 cm<sup>-1</sup> (C=C stretching vibration), 1624 cm<sup>-1</sup> (C=C stretching vibration), 1610 cm<sup>-1</sup> (C=C stretching vibration), 1585 cm<sup>-1</sup> (C=C stretching vibration), 1500 cm<sup>-1</sup> (C-H symmetric deformation vibration), 1385 cm<sup>-1</sup> (symmetric C-H deformation vibration), 1186 cm<sup>-1</sup> (C-H skeletal vibration), 1050 cm<sup>-1</sup> (C-H<sub>3</sub> rocking vibration), 1010 cm<sup>-1</sup> (C-C skeleton vibration), 990 cm<sup>-1</sup> (C-C skeleton vibration), 845 cm<sup>-1</sup> (C-H vibration), 800 cm<sup>-1</sup> (aromatic C-H out-of-plane deformation vibrations), 720 cm<sup>-1</sup> (CH<sub>3</sub> rocking vibration), 655 cm<sup>-1</sup> (C-H wagging vibration), 642 cm<sup>-1</sup> (Ring deformation), 595 cm<sup>-1</sup> (C-C skeletal vibrations , 510 cm<sup>-1</sup> (C-C skeletal vibration), 495 cm<sup>-1</sup> (skeletal vibration), 410 cm<sup>-1</sup> (skeletal vibration), 445 cm<sup>-1</sup> (skeletal vibration).

*Serratia marcescens* degraded MO product showed peaks at around 2900 cm<sup>-1</sup> (C-H stretching vibration), 3100 cm<sup>-1</sup> (N-H stretching vibration-associated to =C), 2400 cm<sup>-1</sup> (asymmetric N-H stretching vibration), 1700 cm<sup>-1</sup> (C=C stretching vibration), 1600 cm<sup>-1</sup> (C-H vibration), 1506 cm<sup>-1</sup> (N-H deformation vibrations), 1200 cm<sup>-1</sup> (C-H skeletal vibration), 1050 cm<sup>-1</sup> (C-H symmetrical deformation vibration), 831 cm<sup>-1</sup> (C-H out-of-plane deformation vibration), 770 cm<sup>-1</sup> (N-H out-of-plane bending vibrations).

FTIR analysis of CR control showed specific peaks in the fingerprint region 1650 cm<sup>-1</sup> (for amide functional group), 1300 cm<sup>-1</sup> N=N symmetric stretching vibration of azide group, 1310 cm<sup>-1</sup> (ring structure vibration), around 1400 (naphthalene ring), 1505-1550 cm<sup>-1</sup> (C=C vibration in the naphthalene ring), 1530 cm<sup>-1</sup> (N=N stretching vibration) , 1080 cm<sup>-1</sup> (C-N stretching vibration of amine), around 990 cm<sup>-1</sup> (S=O stretching vibration), 1110 cm<sup>-1</sup> (Ring vibration) including C-S and symmetric SO<sub>3</sub> stretching vibration, 800 cm<sup>-1</sup> (C-C skeletal vibration), 660 cm<sup>-1</sup> (C-S stretching and vibration). For *Serratia marcescens* degraded MO (Fig. 2), peaks became visible at around 2328 cm<sup>-1</sup> (N-H stretching vibration), 2922 cm<sup>-1</sup> (C-H stretching vibration), 2900 cm<sup>-1</sup> (C-H stretching vibration), 2380 cm<sup>-1</sup> (N-H stretching vibration), 1707 cm<sup>-1</sup> (C=C stretch),



263 1690  $\text{cm}^{-1}$  (C=C stretch), 1750  $\text{cm}^{-1}$  (C=O stretch), 1590  $\text{cm}^{-1}$  (N-H bending vibrations),  
 264 1300  $\text{cm}^{-1}$  (N=N deformation vibration for azo group), 1090  $\text{cm}^{-1}$  (C-H symmetrical  
 265 deformation vibration), 1200  $\text{cm}^{-1}$  (C-H skeletal vibration) and 790  $\text{cm}^{-1}$  (C-C skeletal  
 266 and C-H out-of-plane deformation vibration for aromatic group).



267  
 268 **Fig. 2.** FTIR spectra of degraded dye products for (A) FTIR of degraded MO in LB-  
 269 medium, (B) FTIR of degraded CR in LB-medium (C) FTIR of degraded MO in BH-  
 270 medium (D) FTIR of degraded CR in BH-medium.

271  
 272 In IR spectra of MO and CR degradation reported by Quan *et al.* [43], non-uniform  
 273 vibrations of 1584  $\text{cm}^{-1}$  (for -N=N- of azo dye), 1450 and 1510  $\text{cm}^{-1}$  (benzene skeleton  
 274 vibration), 1180  $\text{cm}^{-1}$  (benzene ring in-plane bending vibration), 698 and 536  $\text{cm}^{-1}$   
 275 (outward flexural vibration of benzene ring) were detected. Degradation of the dyes  
 276 caused a lot of peaks to either weaken or disappear, indicating the occurrence of a  
 277 decolourization reaction. Krithika *et al.* [44] report that the FTIR spectra of controls  
 278 showed several peaks in the 3200-3500  $\text{cm}^{-1}$  region (N-H and O-H stretch). After the  
 279 breakdown of dyes, absorption decreased for this region. New bands were formed in  
 280 the carbonyl region (1401.07–1072.83  $\text{cm}^{-1}$ ), attributed to amine groups. Our strain  
 281 also showed amine formation (N-H deformation, bending, or stretching vibrations)  
 282 in both spectra recorded for degraded MO and CR. 1506  $\text{cm}^{-1}$ .

### 283 3.4. AzoR characterization

284 Enzymatic bioremediation is a potentially rapid method involved in the metabolic  
 285 and co-metabolic transformation, detoxification, or degradation of toxic dye [45].  
 286 The preliminary strategy of the azo dye biodegradation is decolourization, where -  
 287 N=N- is cleaved in a reductive manner. This step is catalyzed by the enzyme AzoR  
 288 [46]. A 25 KDa AzoR enzyme band was observed in our isolate of *Serratia marcescens*,  
 289 after SDS PAGE analysis. Eslami *et al.* [47] previously reported a 22 Kda AzoR from  
 290 a halophilic bacterium whereas a 29 KDa AzoR was isolated by Nisar *et al.* [48] from

a *Staphylococcus* sp. Dong *et al.* [49] reported many copies of NAD(P)H-dependent AzoR in *Serratia* sp. S2. They were found to have a role in chromium remediation. Three copies of AzoR enzyme were found to be encoded by a *Serratia* sp. when the whole genome was sequenced by Basharat *et al.* [50].

This proves that *Serratia marcescens* encodes AzoR enzyme that can be used for remediation in case of excessive dye quantity hindering the activity of target molecule or in case of additive induced growth inhibition. Another characteristic of AzoRs is that they produce aromatic amines via reductive cleavage of -N=N-. Our strain also showed a tendency towards amine formation, which was seen in FTIR analysis, so this further strengthens the observation that the decolourization was via AzoR. The feasibility of enzymatic treatment has been demonstrated at laboratory scale in prior studies and we propose that AzoR may be obtained in large amounts from *Serratia marcescens* grown in favorable conditions.

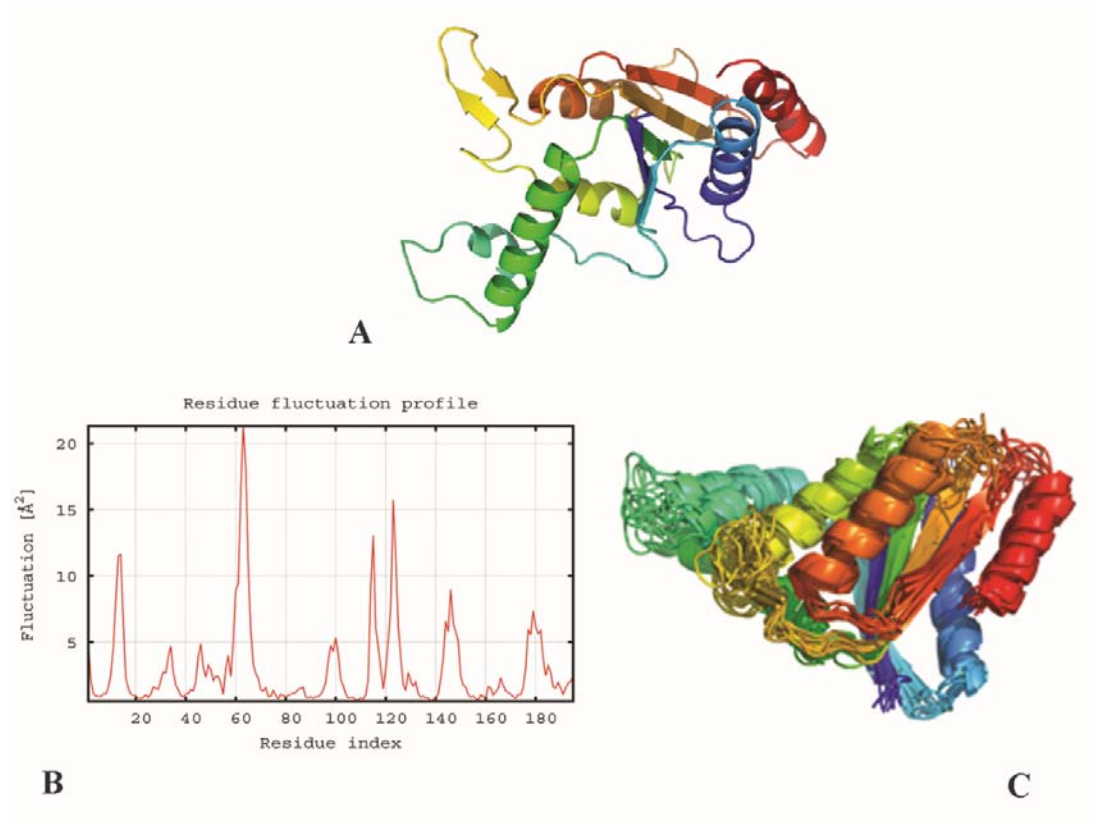
### 3.5. Impact of reducing factors on degradation

Azo dye degradation can be achieved by AzoR because it cleaves the azo bond [46] by specific oxidation or reduction at the expense of a reducing agent. It ensnares azo dyes and acts as an effective dye degrading tool by using the ping pong mechanism; i-e ambushing or locking in the toxic azo dyes in the enzyme, with AzoR being the main entity and using supporting factors (reducing agents) like NAD/NADH/FAD or FADH. Dyes are reduced via a ping-pong bi-bi process which entails two cycles of NAD(P)H-based FMN to FMNH<sub>2</sub> reduction [51] ). During the first phase, the azoic substrate reduces to hydrazine whereas, in the second round, hydrazine is reduced to two amines [52]. AzoR based dye-reduction phenomenon can be divided into two types based on the processing pathway of the enzyme i-e membrane-bound and cytoplasmic AzoR based dye-reduction. Cytoplasmic AzoR enzymes can reduce non-sulphonated azo dyes as they can diffuse through the bacterial cell membrane. For membrane-bound AzoR decolourization, they can use a redox mediator to shuttle the electrons across the membrane barrier in a non-aerobic environment [15]. Azoic dyes comprising sulphonate groups and higher molecular weights are not likely to be transferred across the cell membranes. As a result, the reducing action of the dye is independent of the intracellular dye uptake [53-54]. Reduction in the extracellular environment is based on electron transport and is achieved by the establishment of a link between intracellular electron transport systems of a bacterial cell and azobenzene dye molecules of high molecular weight [15]. It was suggested by Russ *et al.* [55] that bacterial membranes are nearly impermeable to co-factors having flavin, hence, restricting the reduction of sulphonated azo dyes due to equivalent transfer by cytoplasmic flavins. An alternate mechanism for sulphonated azoic-dye reduction in cells with intact membranes occurs due to the genesis of reduced flavins by the cytoplasmic flavin-dependent AzoR [54]. The outer membranous electron transfer apparatus of the bacterial cell wall either makes contact directly with the azo dye substrate or interacts circuitously to the cell surface

redox mediator. Redox mediator compounds with small molecular weight can act as electron carriers among the outer membranous NADH-dependent AzoR and azo dye. Bacteria may synthesize redox mediators during substrate processing or these are added superficially [56]. Various researches have proved that the addition of redox mediators accelerates the dye degradation process [56-58]. Our quantitative AzoR assay showed an external addition of NADH/NADH was not necessary for dye degradation, which means it was encoded by the bacterium itself.

### 3.6.3D structure analysis

Implementation of successful enzymatic bioremediation approaches relies profoundly on intrinsic molecular analysis of the microbial enzymatic structure. Protein structural modeling and docking is now a mature technique and a powerful tool for the understanding and description of protein forms and functions, although careful consideration must be utilized when choosing a template. The protein sequence of *Serratia marcescens* was analyzed computationally to obtain insights about substrate specificity, reaction mechanism, electron transfer, dye positioning, and interaction with amino acid residues of AzoR. Our structure showed more fluctuation at the loop regions as compared to  $\beta$ -sheets and  $\alpha$ -helices (Fig. 3). FMN prosthetic group was most probably bound to the enzyme or AzoR was flavin independent, as there was no requirement for the exogenous addition of flavins. This was in correspondence with the performed AzoR assay where no flavin moiety was added externally but decolourization was observed. The motif GXGXXG was absent from our specie. Although *Serratia marcescens* in this study lacked GXGXXG motif but a general  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  structure was present where FMN might bind (Fig. 3). In *Bacillus* sp. AzoR in monomeric form, FMN has been found to be bound in the loop pocket [15, 59]. These insights also lead to the idea that AzoR in *Serratia* sp. might be flavin free. Due to the lack of experimental structures of AzoR specie till today, we are unable to say conclusively that this might be the case and more studies in this area could help reach valid reasoning. Nevertheless, we did interaction analysis with FMN *in silico* and found several residues to bind with the moiety (section 3.7).

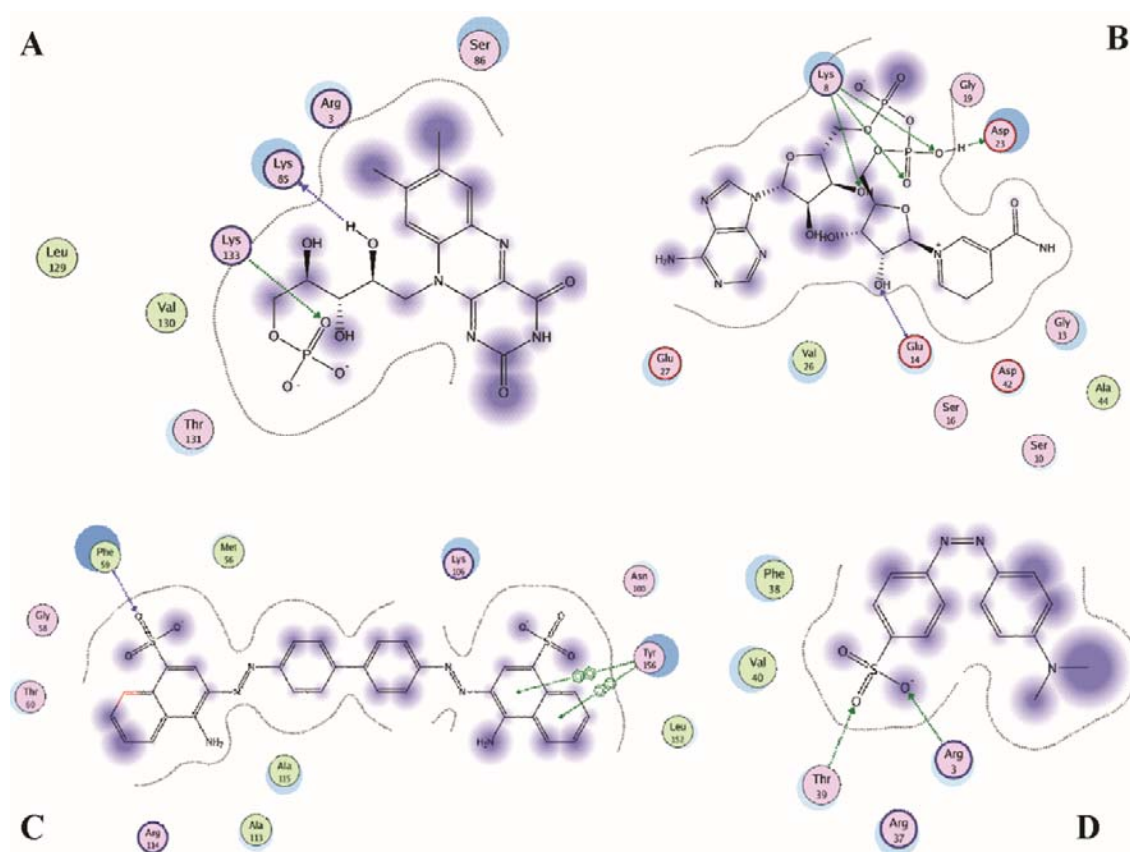


**Fig. 3.** (A) 3D modeled structure of *Serratia marcescens* AzoR. (B) Structural flexibility profile of simulated AzoR, with fluctuations for individual protein residues shown in the red line. The output is based on the all-atom model via trajectory clustering. (C) Refinement of the model and superpositioning (shown in 3D) is centred on the maximum likelihood superposition method of THESEUS (Theobald and Wuttke, 2006).

As far as Rossman binding fold is concerned, Sarkar *et al.* [15] has previously reported five beta sheets and a high percentage of alpha helices in AzoR from *Bacillus* sp. similar to AzoR of our *Serratia* specie. This explains that Rossman binding fold (six parallel beta strands linked to two pairs of  $\alpha$ -helices in the topological order  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$ ) is not universal.

### 3.7. Hydrogen and hydrophobic interactions in dye degradation

A protein is capable of taking complex shapes and its interactions with other proteins or ligands allow it to form versatile conformations. Modeling and abstraction have played a noteworthy role in perceiving protein-ligand associations using computational modus operandi. Molecular docking of the enzyme with various substrates revealed residue and type of interactions present (Fig. 4).



**Fig. 4.** 2D representation of (A) FMN-AzoR complex (B) NADH-AzoR complex (C) CR-AzoR complex (D) MO-AzoR complex. ● depicts polar residue, ● acidic, ● basic, ● hydrophobic, → side chain acceptor, → side chain donor, → backbone donor, ■ ligand exposure, ↔ arene-arene and black dotted line as proximity contour.

A hydride is typically transferred from NADH to FMN to accomplish the first stage of the reduction reaction. Three hydrogen bonds (two with Lys8 and one with Glu14) and eight hydrophobic interactions (with residue Gly13, Ser16, Gly19, Asp23, Val26, Glu27, Asp42, Ala44) were observed between NADH and AzoR. Cao *et al.* [60] reported slightly different interactions for NADH-AzoR binding in *Shewanella onediensis* MR-1. Ser16 and Ala residue formed hydrogen bonds instead of hydrophobic interactions, as observed in our study. Apart from this study, we could not find reduced NAD interacting residues with azoreductase reported in literature till today.

For FMN-AzoR interaction, a hydrogen bond was observed between the nitrogen of Lys133 of AzoR and the oxygen atom of FMN. Lys85, Val130, Thr131, and Arg33 form hydrophobic interaction with oxygen atoms of FMN while Ser86 formed a

399 hydrophobic interaction with the carbon atom of FMN. The pattern of FMN binding  
400 was compared to *Bacillus vazeleanus* [59], and common interacting residues were Arg  
401 and Thr (although hydrophobic interactions instead of hydrogen bonding were  
402 observed). Hydrogen bond interactions of the *Chromobacterium violaceum* [61],  
403 *Bacillus* sp. [62], and *Klebsiella* sp. (PDB ID: 6DXP) were different and entailed only  
404 one residue (i.e. Arg) common to that of FMN-AzoR interacting residues of our  
405 bacterium.

406 MO formed two hydrogen bonds (with residue Thr39 and Arg3) and three  
407 hydrophobic interactions (with residue Arg37, Phe38, Val40). Six of these residues  
408 (Arg, Thr, Phe, Met, Gly, Asn) were common to interactions of AzoR-MO in  
409 *Oedogonium subpalgiostomum* AP1 [63]. None of our MO binding residues were  
410 similar to that of *Pseudomonas putida* [64]. CR formed one hydrogen bond and ten  
411 hydrophobic interactions (with residue Met 56, Gly58, Phe59, Thr60, Asn100, Lys106,  
412 Ala113, Arg114, Ala115, Leu152, Tyr156). Two of these residues were common when  
413 compared to CR-AzoR interaction of *Pseudomonas putida* [64].

414 As azo dyes require polar electron-donating ring substituents (e.g. -OH, -NH<sub>2</sub>, -  
415 NHCH<sub>3</sub>, or -N(CH<sub>3</sub>)<sub>2</sub>) so the reactivity of azo dye substrates is determined by their  
416 electron densities and redox potentials [65-66]. Punj and John [67] demonstrated that  
417 H<sup>+</sup> bond formation can reduce the electron density around the residue making it  
418 more amenable to reductive cleavage. This might explain more degradation of MO  
419 as compared to CR by *Serratia marcescens* AzoR because two hydrogen bonds were  
420 observed in MO-AzoR interaction while only one hydrogen bond was observed in  
421 the case of CR-AzoR interaction. The energy profile of the ligand-bound and  
422 unbound states of AzoR also varied (Table 1).

423 Table 1. Energy profile of AzoR and its ligand-bound complexes. A potential energy  
424 model, equivalently, a forcefield (Amber99 in this case), assigns a potential energy  
425 value to a molecular configuration. E<sub>str</sub> = bond stretch energies, E<sub>ang</sub> = angle bend  
426 energies, E<sub>stb</sub> = stretch-bend cross term energies, E<sub>tor</sub> = dihedral rotation energies, E<sub>oop</sub>  
427 = out-of-plane energies, E<sub>vdw</sub> = van der Waals interaction energies. The potential  
428 energy is the sum of interaction energies, depicted as E<sub>total</sub> in the table.

Protein	$E_{str}$ (Kcal/mol)	$E_{ang}$ (Kcal/mol)	$E_{stb}$ (Kcal/mol)	$E_{tor}$ (Kcal/mol)	$E_{oop}$ (Kcal/mol)	$E_{vdw}$ (Kcal/mol)	$E_{total}$ (Kcal/mol)
AzoR	538.041	1154.411	-28.989	119.986	1150.718	1691.273	4625.440
AzoR-FMN complex	546.187	1190.071	-28.772	1170.768	120.136	1755.589	4753.978
AzoR-NADH complex	583.708	1228.999	-40.010	1187.274	120.578	1789.975	4870.525
AzoR-MO complex	585.553	1194.855	-36.698	1150.051	120.055	1737.544	4751.360
AzoR-CR complex	660.881	1234.639	-42.178	1164.386	123.789	1920.452	5061.968

429

430 These results further advance our knowledge on the molecular aspects of the  
431 interaction of sulphonated azo dyes to AzoR enzyme in *Serratia marcescens*, depicting  
432 the integral role of reductases in environmental cleanup. Thus, this work can also  
433 serve as a baseline for the mechanistic understanding of factors affecting enzyme  
434 reactivity towards a class of substrates rather than just one single substrate. Future  
435 work can be targeted towards the assessment of quantitative structure-activity  
436 relationships, incorporating maximum reaction rate and substrate specificity study  
437 for bioremediation-need driven practical designing of waste-treatment catalysts.

#### 438 4. Conclusion

439 This study aimed to present a lucid picture of the relation and communication  
440 between FMN, NADH, dyes, and substrate molecule. We were able to identify  
441 residues crucial for interaction between AzoR and substrates. Comparison revealed  
442 that these were not totally conserved in comparison to the available studies.  
443 However, if they are conserved within the genus *Serratia* remains to be elucidated.  
444 The acquired information can aid in designing AzoR enzyme mutants for better  
445 accommodation of dye pollutants and thus, be of benefit to the community working  
446 on enzymatic bioremediation. Additional proposed work is an optimization of AzoR  
447 mediated dye degradation studies using response surface models, site-directed  
448 mutagenesis for better dye affinity to the enzyme, and reconstruction of azo-dye  
449 degradation/metabolic pathways in these organisms. This might be helpful to curb  
450 free radical, hydrophobic character, electrophilic species, and arylamine derivative  
451 formation leading to interaction with electron-rich sites in DNA, curtailing mutation,  
452 and DNA adduct formation responsible for cancer. Also, the development of  
453 fluorophores/biosensors for the detection of AzoR activity in bacterial cultures as  
454 well as in environmental samples for monitoring and analysis is proposed.

#### 455 Declarations

#### 456 Funding

457 This research did not receive any specific grant from funding agencies in the public,  
458 commercial, or not-for-profit sectors.

#### 459 **Conflict of Interest**

460 The authors have nothing to disclose in terms of conflict of interest.

#### 461 **Ethics approval**

462 Not applicable.

#### 463 **Consent to publish**

464 All authors consent to be responsible for the published material and have read and  
465 approved the content.

#### 466 **Availability of data and material**

467 This work was done using public data from NCBI. All data and related material is  
468 present in the manuscript and accession numbers mentioned at appropriate places.  
469 No new data was generated that requires submission in a repository and no plant or  
470 animal material was used that requires ethical approval.

#### 471 **Author contributions**

472 A.Y. conceived and designed the project, provided resources, supervised and edited  
473 the paper. Z.B. designed the project, performed the experiments and wrote the  
474 paper.

475

476

477

478

479

480

481



482

483

484

485

486

## 487 **References**

- 488 1. Robinson R (1957) Sir William Henry Perkin: Pioneer of chemical industry. *J*  
489 *Chem Edu* 34(2):54. <https://doi.org/10.1021/ed034p54>
- 490 2. Holme I (2006) Sir William Henry Perkin: a review of his life, work and  
491 legacy. *Coloration Tech* 122(5):235-251. [https://doi.org/10.1111/j.1478-](https://doi.org/10.1111/j.1478-4408.2006.00041.)  
492 [4408.2006.00041.](https://doi.org/10.1111/j.1478-4408.2006.00041.)
- 493 3. Hussain SM, Hussain T, Faryad M, Ali Q, Ali S, Rizwan M, Hussain AI, Ray  
494 MB, Chatha SA (2021) Emerging aspects of photo-catalysts (TiO<sub>2</sub> and ZnO)  
495 doped zeolites and advanced oxidation processes for degradation of Azo  
496 dyes: A Review. *Curr Anal Chem* 17(1):82-97.  
497 <https://doi.org/10.2174/1573411016999200711143225>
- 498 4. Gurses A, Açıkyıldız M, Güneş K, Gürses MS (2016) In: Dyes and pigments:  
499 their structure and properties. *Dyes and Pigments*. Springer, Cham.  
500 <https://doi.org/10.1007/978-3-319-33892-7>
- 501 5. Preethi S, Anumary A, Ashokkumar M, Thanikaivelan P (2013) Probing  
502 horseradish peroxidase catalyzed degradation of azo dye from tannery  
503 wastewater. *SpringerPlus* 2(1):1-8. <https://doi.org/10.1186/2193-1801-2-341>
- 504 6. Pan Y, Wang Y, Zhou A, Wang A, Wu Z, Lv L, Li X, Zhang K, Zhu T (2017)  
505 Removal of azo dye in an up-flow membrane-less bioelectrochemical system  
506 integrated with bio-contact oxidation reactor. *Chem Eng J* 326:454-461.  
507 <https://doi.org/10.1016/j.cej.2017.05.146>
- 508 7. Selvaraj V, Karthika TS, Mansiya C, Alagar M (2020) An over review on  
509 recently developed techniques, mechanisms and intermediate involved in the  
510 advanced azo dye degradation for industrial applications. *J Mol Struct*  
511 1224:129195. <https://doi.org/10.1016/j.molstruc.2020.129195>
- 512 8. Lellis B, Fávaro-Polonio CZ, Pamphile JA, Polonio JC (2019) Effects of textile  
513 dyes on health and the environment and bioremediation potential of living  
514 organisms. *Biotechnol Res Innovat* 3(2):275-290.  
515 <https://doi.org/10.1016/j.biori.2019.09.001>

9. Haque MM, Haque MA, Mosharaf MK, Marcus PK (2021) Decolorization, degradation and detoxification of carcinogenic sulfonated azo dye methyl orange by newly developed biofilm consortia. *Saudi J Biol Sci* 28(1):793-804. <https://doi.org/10.1016/j.sjbs.2020.11.012>
10. Paba GM, Ávila BR, Baena Baldiris D (2021) Application of environmental bacteria as potential methods of azo dye degradation systems. *Global J Env Sci Manag* 7(1):131-154. <https://doi.org/10.22034/GJESM.2021.01.10>
11. Tsuboy MS, Angeli JPF, Mantovani MS, Knasmuller S, Umbuzeiro GA, Ribeiro LR (2007) Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI Disperse Blue 291 in the human hepatic cell line HepG2. *Toxicol in Vitro* 2(8): 1650-1655. <https://doi.org/10.1016/j.tiv.2007.06.020>
12. Rajmohan KS, Ramya C, Seepana MM (2019) Recent Advancements and Perspectives on Biological Degradation of Azo Dye. In *Biochemical and Environmental Bioprocessing*, Ed. Jerold, M. and Sivasubramanian, V., 17-36. CRC Press.
13. Nimkar U (2018) Sustainable chemistry: A solution to the textile industry in a developing world. *Curr Op Green Sust Chem* 9:13-17. <https://doi.org/10.1016/j.cogsc.2017.11.002>
14. Agrawal S, Tipre D, Patel B, Dave S (2017) Bacterial decolourization, degradation and detoxification of Azo dyes: An eco-friendly approach. In *Microbial Applications*, ed. Kalia V. and Kumar P., pp. 91-124. Springer, Cham.
15. Sarkar S, Banerjee A, Halder U, Biswas R, Bandopadhyay R (2017) Degradation of synthetic azo dyes of textile industry: a sustainable approach using microbial enzymes. *Water Conserv Sci Eng* 2(4):121-131. <https://doi.org/10.1016/j.cogsc.2017.11.002>
16. Dong H, Guo T, Zhang W, Ying H, Wang P, Wang Y, Chen Y (2019) Biochemical characterization of a novel azoreductase from *Streptomyces* sp.: Application in eco-friendly decolorization of azo dye wastewater. *Int J Biol Macromol* 140:1037-1046. <https://doi.org/10.1016/j.ijbiomac.2019.08.196>
17. Kumar K, Devi SS, Krishnamurthi K, Dutta D, Chakrabarti T (2007) Decolorization and detoxification of Direct blue-15 by a bacterial consortium. *Biores Technol* 98: 3168-3171. <https://doi.org/10.1016/j.biortech.2006.10.037>
18. PalaniVelan R, Rajkumar S, Ayyasamy PM (2012) Exploration of promising dye decolourising strains obtained from Erode and Tirrupur textile wastes. *Int J Env Sci* 2(4):2470-2481. <https://doi.org/10.6088/ijes.00202030128>
19. Kuberan T, Anburaj J, Sundaravadivelan C, Kumar P (2011) Biodegradation of Azo dye by *Listeria* sp. *Int J Env Sci* 1(7):1760-1770.
20. Mehde AA (2019) Development of magnetic cross-linked peroxidase aggregates on starch as enhancement template and their application for decolorization. *Int J Biol Macromol* 131:721-733. <https://doi.org/10.1016/j.ijbiomac.2019.03.062>

21. Cossolin AS, Reis HCOD, Castro KCD, Santos BAPD, Marques MZ, Parizotto CA, Vasconcelos LGD, Morais EBD (2019) Decolorization of textile azo dye Reactive Red 239 by the novel strain *Shewanella xiamenensis* G5-03 isolated from contaminated soil. *Revista Ambient Água* 14(6). <https://doi.org/10.4136/ambi-agua.2446>
22. Aftab U, Khan MR, Mahfooz M, Ali M, Aslam SH, Rehman A (2011) Decolorization and degradation of textile azo dyes by *Corynebacterium* sp. isolated from industrial effluent. *Pak. J. Zoology*, 43(1), 1-8.
23. Hettwer D, Wang H (1989) Protein release from *Escherichia coli* cells permeabilized with guanidine-HCl and Triton X100. *Biotechnol Bioeng* 33:886-895. <https://doi.org/10.1002/bit.260330712>
24. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685. <https://doi.org/10.1038/227680a0>
25. Macwana SR, Punj S, Cooper J, Schwenk E, John GH (2010) Identification and isolation of an azoreductase from *Enterococcus faecium*. *Curr Issues Mol Biol* 12:43-48. <https://doi.org/10.21775/cimb.012.043>
26. Basharat Z, Yasmin A (2015) *In silico* assessment of phosphorylation and O- $\beta$ -GlcNAcylation sites in human NPC1 protein critical for Ebola virus entry. *Infect Genet Evol* 34:326-338. <https://doi.org/10.1016/j.meegid.2015.06.001>
27. Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* 5(4):725-738. <https://doi.org/10.1038/nprot.2010.5>.
28. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) The I-TASSER Suite: protein structure and function prediction. *Nat Method* 12(1), 7-8. <https://doi.org/10.1038/nmeth.3213>
29. Basharat Z, Yasmin A (2016) Understanding properties of the master effector of phage shock operon in *Mycobacterium tuberculosis* via bioinformatics approach. *BioRxiv* 050047. <https://doi.org/10.1101/050047>
30. Jamroz M, Kolinski A, Kmiecik S (2013) CABS-flex: server for fast simulation of protein structure fluctuations. *Nuc Acids Res* 41(W1):W427-W431. <https://doi.org/10.1093/nar/gkt332>
31. Wang Y, Suzek T, Zhang J, Wang J, He S, Cheng T, Shoemaker BA, Gindulyte A, Bryant SH (2013) PubChem bioassay: 2014 update. *Nuc Acids Res* D1075-82. <https://doi.org/10.1093/nar/gkt978>
32. O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR (2011) Open Babel: An open chemical toolbox. *J Cheminform* 7(3):33. <https://doi.org/10.1186/1758-2946-3-33>
33. Basharat Z, Jahanzaib M, Yasmin A, Khan IA (2021) Pan-genomics, drug candidate mining and ADMET profiling of natural product inhibitors screened against *Yersinia pseudotuberculosis*. *Genomics* 113(1):238-244. <https://doi.org/10.1016/j.ygeno.2020.12.015>

34. Verma P, Madamwar D (2003) Decolorization of synthetic dyes by a newly isolated strain of *Serratia marcescens*. World J Microbiol Biotechnol 19(6):615-618. <https://doi.org/10.1023/A:1025115801331>
35. Mahmood S, Khalid A, Arshad M, Ahmad R (2015) Effect of trace metals and electron shuttle on simultaneous reduction of Reactive black-5 azo dye and hexavalent chromium in liquid medium by *Pseudomonas* sp. Chemosphere 138:895-900. <https://doi.org/10.1016/j.chemosphere.2014.10.084>
36. Karamba I, Yakasai H (2018) Isolation and characterization of a molybdenum-reducing and methylene blue-decolorizing *Serratia marcescens* strain KIK-1 in soils from Nigeria. Bioremed Sci Technol Res 6(1):1-8. <https://doi.org/10.54987/bstr.v6i1.392>
37. Pearce CI, Lloyd JR, Guthrie JT (2003) The removal of color from textile wastewater using whole bacterial cells: a review. Dye Pigment 58:179-196. [https://doi.org/10.1016/S0143-7208\(03\)00064-0](https://doi.org/10.1016/S0143-7208(03)00064-0)
38. Franciscon E, Grossman MJ, Paschoal JAR, Reyes FGR, Durrant LR (2012) Decolorization and biodegradation of reactive sulfonated azo dyes by a newly isolated *Brevibacterium* sp. strain VN-15. SpringerPlus 1(1):1-10. <https://doi.org/10.1186/2193-1801-1-37>
39. Blumel S, Contzen M, Lutz M, Stolz A, Knackmuss H (1998) Isolation of a bacterial strain with the ability to utilize the sulphonated azo compound 4-Carboxy-4'-Sulfoazobenzene as the sole source of carbon and energy. Appl Env Microbiol 64(6): 2315-2317. <https://doi.org/10.1128/AEM.64.6.2315-2317.1998>
40. Hong Y, Xu M, Guo J, Xu Z, Chen X, Sun G (2007) Respiration and growth of *Shewanella decolorationis* S12 with an azo compound as the sole electron acceptor. Appl Env Microbiol 73(1):64-72. <https://doi.org/10.1128/AEM.01415-06>
41. Bheemaraddi MC, Shivannavar CT, Gadda SM (2014) Effect of carbon and nitrogen sources on biodegradation of textile azo dye Reactive Violet 5 by *Pseudomonas aeruginosa* GSM3. Sch Acad J Biosci 2(4):285-289.
42. Manogaran M, Yasid NA, Othman AR, Gunasekaran B, Halimi MIE, Shukor MYA (2021) Biodecolourisation of Reactive Red 120 as a sole carbon source by a bacterial consortium—toxicity assessment and statistical optimisation. Int J Env Res Public Health 18(5):2424. <https://doi.org/10.3390/ijerph18052424>
43. Quan L, Huang J, Qi J, Zhu Y (2018) Isolation of different azo dye decolorizing bacteria and their decolorization mechanisms. Nat Env Pollut Technol 17(3):981-986.
44. Krithika A, Gayathri KV, Kumar DT, Doss CGP (2021) Mixed azo dyes degradation by an intracellular azoreductase enzyme from alkaliphilic *Bacillus subtilis*: a molecular docking study. Arch Microbiol 1-12. <https://doi.org/10.1007/s00203-021-02299-2>
45. Sutherland TD, Horne I, Weir KM, Coppin CW, Williams MR, Selleck M, Russell RJ, Oakeshott JG (2004) Enzymatic bioremediation: from enzyme

- discovery to applications. Clin Exp Pharmacol Physiol 31(11): 817-21.  
<https://doi.org/10.1111/j.1440-1681.2004.04088.x>
46. Rathod J, Dhebar S, Archana G (2017) Efficient approach to enhance whole cell azo dye decolorization by heterologous overexpression of *Enterococcus* sp. L2 azoreductase (azoA) and *Mycobacterium vaccae* formate dehydrogenase (fdh) in different bacterial systems. Int Biodeterior Biodegrad 124:91-100.  
<https://doi.org/10.1016/j.ibiod.2017.04.023>
47. Eslami M, Amoozegar MA, Asad S (2016) Isolation, cloning and characterization of an azoreductase from the halophilic bacterium *Halomonas elongata*. Int J Biol Macromol 85:111-116.  
<https://doi.org/10.1016/j.ijbiomac.2015.12.065>
48. Nisar N, Aleem A, Saleem F, Aslam F, Shahid A, Chaudhry H, Malik K, Albaser A, Iqbal A, Qadri R, Yang Y (2017) Reduction of reactive red 241 by oxygen insensitive azoreductase purified from a novel strain *Staphylococcus* KU898286. Plos One 12(5): e0175551.  
<https://doi.org/10.1371/journal.pone.0175551>
49. Dong L, Zhou S, He Y, Jia Y, Bai Q, Deng P, Gao J, Li Y, Xiao H (2018) Analysis of the genome and chromium metabolism-related genes of *Serratia* sp. S2. Appl Biochem Biotechnol 185(1):140-152.  
<https://doi.org/10.1007/s12010-017-2639-5>
50. Basharat Z, Tanveer F, Yasmin A, Shinwari ZK, He T, Tong Y (2018) Genome of *Serratia nematodiphila* MB307 offers unique insights into its diverse traits. Genome 61(7):469-476. <https://doi.org/10.1139/gen-2017-0250>
51. Suzuki H (2019) Remarkable diversification of bacterial azoreductases: primary sequences, structures, substrates, physiological roles, and biotechnological applications. Appl Microbiol Biotechnol 103(10):3965-3978.  
<https://doi.org/10.1007/s00253-019-09775-2>
52. Correia B, Chen Z, Mendes S, Martins LO, Bento I (2011) Crystallization and preliminary X-ray diffraction analysis of the azoreductase PpAzoR from *Pseudomonas putida* MET94. Acta Crystallogr Sect F Struct Biol Cryst Commun 67(1):121-123. <https://doi.org/10.1107/S1744309110048220>
53. Robinson T, McMullan G, Marchant R, Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Biores Technol 77:247-55. [https://doi.org/10.1016/S0960-8524\(00\)00080-8](https://doi.org/10.1016/S0960-8524(00)00080-8)
54. Dixit S, Garg S (2019) Development of an efficient recombinant bacterium and its application in the degradation of environmentally hazardous azo dyes. Int J Env Sci Technol 16(11):7137-7146. <https://doi.org/10.1007/s13762-018-2054-7>
55. Russ R, Rau J, Stolz A (2000) The function of cytoplasmic flavin reductases in the reduction of azodyes by bacteria. Appl Env Microbiol 66(4):1429-34.  
<https://doi.org/10.1128/AEM.66.4.1429-1434.2000>
56. Dai R, Chen X, Xiang X, Wang Y, Wang F (2018) Understanding azo dye anaerobic bio-decolorization with artificial redox mediator supplement:

- 686 considering the methane production. *Biores Technol* 249:799-808.  
687 <https://doi.org/10.1016/j.biortech.2017.10.072>
- 688 57. Rau J, Knackmuss HJ, Stolz A (2002) Effects of different quinoid redox  
689 mediators on the anaerobic reduction of azo dyes by bacteria. *Env Sci Technol*  
690 36(7):1497-1504. <https://doi.org/10.1021/es010227+>
- 691 58. Meng X, Liu G, Zhou J, Fu QS (2014) Effects of redox mediators on azo dye  
692 decolorization by *Shewanella algae* under saline conditions. *Biores Technol*  
693 151:63-68. <https://doi.org/10.1016/j.biortech.2013.09.131>
- 694 59. Bafana A, Khan F, Suguna K (2020) Purification, characterization and crystal  
695 structure of YhdA-type azoreductase from *Bacillus velezensis*. *Proteins: Struct*  
696 *Funct Bioinform* 89(5):483-492. <https://doi.org/10.1002/prot.26032>
- 697 60. Cao X, Di M, Wang J (2017) Expansion of the active site of the azoreductase  
698 from *Shewanella oneidensis* MR-1. *J Mol Graph Model* 78:213-220.  
699 <https://doi.org/10.1016/j.jmgm.2017.10.020>
- 700 61. Verma K, Kundu D, Kundu LM, Singh AK, Dubey VK (2019) Folding and  
701 stability of recombinant azoreductase enzyme from *Chromobacterium*  
702 *violaceum*. *Enz Microb Technol* 131:109433.  
703 <https://doi.org/10.1016/j.enzmictec.2019.109433>
- 704 62. Romero E, Savino S, Fraaije MW, Loncar N (2020) Mechanistic and  
705 crystallographic studies of azoreductase AzoA from *Bacillus wakoensis* A01.  
706 *ACS Chem Bio* 15(2), 504-512. <https://doi.org/10.1021/acschembio.9b00970>
- 707 63. Maruthanayagam A, Mani P, Kaliappan K, Chinnappan S (2020) *In vitro* and  
708 *in silico* studies on the removal of methyl orange from aqueous solution using  
709 *Oedogonium subplagiostomum* AP1. *Water Air Soil Pollut* 231:1-21.  
710 <https://doi.org/10.1007/s11270-020-04585-z>
- 711 64. Thakuria B, Adhikari S. Bioinformatics based investigation on the assortment  
712 of industrially accessible azo dyes with azoreductase enzyme of *Pseudomonas*  
713 *putida*. In *Current Strategies in Biotechnology and Bioresource Technology*, ed. Soa,  
714 F. C. 92. Book publisher Int. India.
- 715 65. Sun J, Li W, Li Y, Hu Y, Zhang Y (2013) Redox mediator enhanced  
716 simultaneous decolorization of azo dye and bioelectricity generation in air-  
717 cathode microbial fuel cell. *Biores Technol* 142:407-414.  
718 <https://doi.org/10.1016/j.biortech.2013.05.039>.
- 719 66. Mahmood S, Khalid A, Mahmood T, Arshad M, Ahmad R (2013) Potential of  
720 newly isolated bacterial strains for simultaneous removal of hexavalent  
721 chromium and reactive black-5 azo dye from tannery effluent. *J Chem Tech*  
722 *Biotechnol* 88(8):1506-1513. <https://doi.org/10.1002/jctb.3994>
- 723 67. Punj S, John GH (2009) Purification and identification of an FMN-dependent  
724 NAD(P)H azoreductase from *Enterococcus faecalis*. *Curr Issue Mol Biol* 11:59-  
725 66. <https://doi.org/10.21775/cimb.011.059>