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Research paper

A novel recombinant AzrC protein proposed by molecular docking and in silico analyses to improve azo dye's binding affinity

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ABSTRACT

Azo dyes are broadly used in different industries through their chemical stability and ease of synthesis. These dyes are usually identified as critical environmental pollutants and many attentions were performed to degradation of azo dyes using biological systems. In this study, the interactions of an azoreductase from mesophilic gram-positive *Bacillus* sp. B29, AzrC, with four common azo dyes (orange I, orange II, orange G and acid red 88) were investigated. Fifteen points, double, triple and quadruple mutant forms of AzrC were made using Molegro Virtual Docker 6.0 in order to improve the binding affinity of azo dyes to AzrC. The impact of 15 different mutations on azo dye affinity potency of AzrC was computationally analyzed using AzrC-azo dye molecular docking, and each interaction was scored based on AutoDock 4.2 free binding energy. Our results have indicated that Asn 104 (A), Asn 187 (B), and Tyr 151 (A) make stable hydrogen bond between AzrC and azo dyes. The hydrophobic amino acids like Phe105 (A), Phe 125 (B), and Phe 172 (B) in wild type form make hydrophobic interactions. In addition, the presence of more hydrophobic residues F60 (B), I119 (B), I121 (B) and F132 (B) in mutant forms made more powerful hydrophobic pocket in the active site. In conclusion, recombinant AzrC with quadruple mutations was suggested in order to increase the biodegradation capacity of AzrC through improving its affinity to four studied azo dyes. This study would be promising for future experimental analyses in order to produce recombinant form of AzrC.

1. Introduction

Azo dyes are widely used in cosmetics, textile, pharmaceutical, printing, food and many other industries through their chemical stability and ease of synthesis (Saratale et al., 2011). They are the largest group of dyes among all synthetic dyes and are identified by the presence of one or more azo bonds (—N═N—) in their chemical structures (Chen et al., 2003; dos Santos et al., 2007). Azo dyes are generally identified as critical environmental pollutants which don't only affect water quality but also a threat to public health through their carcinogenic and mutagenic potentials (Husain, 2006; Kolekar et al., 2013). They are known as colored compounds which are resistant to fading upon exposure to light, water, microbial attack, and many chemicals. Different methods including physicochemical and biological treatments are used for removing azo dyes from wastewater (Ooi et al., 2007; Bafana et al., 2011). Physicochemical methods are efficient but these methods are expensive due to the use of materials and energy. Therefore, biological treatment methods for biodegradation of azo dyes have been widely studied through some significant upsides including environmental friendliness and cost competitiveness (Robinson et al., 2001; Ryan et al., 2010). A number of microorganisms including bacteria, yeast, fungi and algae, have developed enzyme systems for metabolizing of azo dyes under specific environmental conditions (dos Santos et al., 2007; Anjaneya et al., 2011). Bacterial azoreductases which catalyze the reductive cleavage of the azo bonds, have attracted attention as important enzymes for detoxification and decolorizing of azo dyes (Bafana et al., 2008; Brissos et al., 2014). There are two different types of azoreductases in bacteria including flavin mononucleotide-containing (FMN) azoreductases and FMN-free azoreductases (Matsumoto et al., 2010; Kolekar et al., 2013). The FMN azoreductases are also divided into two groups, NADH-dependent and NADPH-dependent azoreductases, according to the similarities of amino-acid sequence and selectivity of different coenzymes (Liu et al., 2007; Ryan et al., 2010). Three different genes of *Bacillus* sp. B29 encode aerobic azoreductases including AzrA (accession no. AB263756) azrb (accession no. AB471797) and AzrC (accession no. AB471798). AzrC protein is a FMN-containing azoreductase and also NADH-dependent oxidoreductases. It contains a homodimeric structure with two moles of FMN as a non-covalent prosthetic group

Abbreviations: FMN, flavin mononucleotide; PDB, Protein Databank; LGA, Lamarckian genetic algorithm; RMSD, root mean square deviation.

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per mole of dimeric protein (Roberts et al., 2011; Yu et al., 2014). In the present study, a molecular docking analysis to investigate the detailed binding mode between AzrC with four main azo dyes was performed. Good agreement was found between docking scores and experimental results in many studies (Bikadi et al., 2011; Sajitha Lulu, 2013; Wadood et al., 2013). Fifteen points, double, triple and quadruple mutations were also performed in AzrC-azo dye complexes in order to increase the binding affinity. This study provides important clues to identify the candidate residues for replacement in order to improve the AzrC and azo dye interactions for further experimental studies.

2. Materials and methods

2.1. Preparation of protein and ligands structure

The molecular structures of AzrC, Orange I was obtained from RCSB Protein Databank (PDB) (PDB accession code: 3W79). Prior to docking studies, the protein structure was refined in order to get more reasonable and accurate results. The crystallographic water molecules as well as original bound ligand (Orange I) in the active site of the protein were removed, so that other ligands could be docked. This protein has four similar chains in which ligand binding site is characterized by cooperation of residues in two chains (e.g. residues in chains A and B form one of its active binding site). Therefore, the two neighboring chains (chain A and B) were reserved to perform docking simulation. This structure was used as a protein model in order to produce further mutant structures. Four different mutant structures with one specific mutation including W60 (B) F, A119 (B) I, A121 (B) I and P132 (B) F and 11 different AzrC complexes with combination of mentioned point mutation (double, triple and quadruple forms) were made by Molegro Virtual Docker 6.0 (Thomsen and Christensen, 2006). After making point mutations, optimization for target residue as well as neighboring residues was done to set the best dihedral angles for residues. The docking simulation for analyzing the molecular interaction of all mutants as well as wild type proteins in complex with five different azo dyes was performed using the AutoDock 4.2 program (Morris et al., 2009). The structures of five different azo dyes including Methyl Red (MR), Orange I (OI), Orange II (OII), Orange G (OG) and Acid Red 88 (AR) were obtained from NCBI-Pub-Chem Compound database (http://pubchem.ncbi.nlm.nih.gov/) and used for molecular docking analysis with AzrC (mutants and wild type) complex.

2.2. Docking simulations

In order to find the optimal conformation and orientation of ligands when they interact with receptor, the molecular docking simulation was utilized. In this study, it was performed to evaluate the binding affinity of four azo dyes in complex with different AzrC mutants. All molecular docking simulations were performed by AutoDock 4.2. The AutoDock tools were utilized to determine rotatable bonds of the ligands to generate different conformers for the docking. It also create PDBQT format of ligands and AzrC protein in which the Gasteiger charges were calculated for each atom of AzrC molecule in AutoDock 4.2 instead of Kollman charges, which were used in the previous versions of the program. Three-dimensional affinity grids of $80 \times 80 \times 80$ Å^3 with 0.375 Å spacing were set on ligand binding site and the affinity maps for all the atom types present, as well as an electrostatic map, were calculated by AutoGrid. The grid dimensions of the Lamarckian genetic algorithm (LGA) were employed for ligand conformational searching. Important docking parameters for the LGA were selected as follows: the number of GA runs set to 200, the initial population of 150 randomly individuals, 2.50000 energy evaluations, maximum of 27,000 generations, a mutation rate of 0.02 and a crossover rate of 0.80. After docking, cluster analysis based on root mean square deviation values, with reference to the starting structure, was performed and ranked according to the energies of their representative conformations. Additionally, schematic plan of ligand hydrogen bonds and hydrophobic interaction interactions were visualized by LigPlot$^+$ and PyMOL (Laskowski and Swindells; DeLano, 2002). The stability of the AzrC secondary structure was also analyzed by DSSP method to survey the impact of mutations in protein structure.

3. Results

3.1. Validation of the accuracy and performance of AutoDock 4.2

In order to evaluate and validate the accuracy of AutoDock 4.2 as an applicable docking tool for the present study, the co-crystallized OI and AR structures (3W79 and 3W7A, respectively) were docked into the active site of the AzrC protein. Subsequently, the docked position was compared to the crystal structure of the bound ligand–protein complex by calculating RMSD (root mean square deviation) values. According to the method of validation cited in the literature (Wang et al., 2003) if the RMSD of the best docked conformation is ≤2.0 Å from the experimental data, the successful scoring function is achieved. In our study, RMSD values of AR and OI were 2.0 and 1.8 Å respectively, which demonstrate the reliability of our docking methods for the given structures and approve that the docking parameters are reasonable and can be extended for other ligands.

3.2. AutoDock binding affinities of the azo dyes into AzrC protein

After validation the docking method, 64 distinctive docking experiences were done for four different azo dyes in complex to 16 protein structures. These protein structures include wild type, point mutant complexes (m60, m119, m121, and m132), double mutant complexes (m119/132, m60/119, m119/121, m60/121, m121/132, and m60/132), triple mutant complexes (m60/119/121, m60/119/132, m60/121/132, and m119/121/132) and quadruple mutant complex (m60/119/121/132). Two hundred docking conformations for each ligand were clustered based on cluster conformation analysis. RMSD cluster analysis and binding free energy evaluations were used to find the best binding mode of ligand position. The final binding free energy is based on intermolecular energy, internal energy of ligand, and torsional free energy (Lu et al., 2010). Visualization of the docking results by VMD approved that all ligands are docked successfully into the active site. The docked conformation were ranked and sorted from the lowest to highest binding energy. Different docking experiences were categorized to four groups according to their ligand (azo dye). In the first group (AzrC-AR), the free binding energy for wild type was −9.07 kcal/mol and in mutant complexes ranging from −9.62 to −11 kcal/mol. The free binding energies for the second (AzrC-OI), third (AzrC-OII) and forth (AzrC-OG) groups were −8.52, −8.56 and −7.56 kcal/mol for wild type complexes; and ranging from −8.49 to −9.27, −8.57 to −9.30 and −7.73 to −9.13 kcal/mol in mutant complexes, respectively. Details of binding free energies and RMSD values for each complex were indicated in Table 1. The most significant effect was observed in quadruple mutant complexes (Fig. 1). This new structure increases ligand–protein interactions and induces ligand affinity to the receptors. Interestingly, these results indicate that most of the mutations increase the affinity of azo dyes to AzrC protein. In continuation of our study, the Ligplot$^+$ tool was utilized to survey the hydrogen bonds and the hydrophobic interactions of the docked compound and generate molecular level insight to the AzrC and ligands interactions. The secondary structure analyses by DSSP method reveal that target mutations, which are induced in AzrC protein, have no significant effect on protein structure and reserve its functional structure.

4. Discussion

Molecular docking is a powerful tool in structural molecular biology and computer-assisted drug design. The goal of ligand–protein docking

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Table 1
The best docking results based on the binding free energies (ΔG_b) of azo dyes docked into wild type and 15 mutant form of AzrC protein.

AzrC form	AR		OI		OII		OG	
	ΔG_b (kcal/mol)	RMDS (Å)	ΔG_b (kcal/mol)	RMDS (Å)	ΔG_b (kcal/mol)	RMDS (Å)	ΔG_b (kcal/mol)	RMDS (Å)
WT	−9.07	3.607	−8.42	4.008	−8.56	5.600	−7.56	2.802
m60	−10.29	3.530	−8.87	4.614	−8.79	5.840	−7.73	2.818
m119	−11.00	3.585	−9.12	4.450	−8.87	5.670	−7.98	2.824
m121	−10.20	2.880	−8.95	4.066	−8.57	3.878	−8.63	2.840
m132	−10.05	3.580	−8.84	4.476	−9.02	6.000	−8.37	1.060
m119/132	−10.21	3.327	−9.08	4.019	−8.91	5.438	−8.62	2.902
M60/119	−10.12	3.600	−8.55	4.450	−8.73	5.540	−8.06	1.067
m119/121	−9.92	2.95	−9.16	3.990	−8.61	4.926	−8.71	2.775
m60/121	−9.80	4.400	−8.72	4.012	−8.65	5.570	−8.41	2.811
M60/132	−9.62	3.560	−8.52	4.350	−8.63	5.590	−7.99	2.879
m121/132	−10.00	3.369	−8.91	4.030	−8.64	4.119	−8.72	2.920
m119/121/132	−10.07	3.425	−9.27	4.002	−8.88	5.037	−9.13	2.912
m60/121/132	−10.33	3.200	−8.49	4.590	−9.13	5.522	−8.96	2.992
m60/119/121	−10.04	3.328	−8.81	4.069	−8.82	5.512	−8.48	2.798
m60/119/132	−10.12	3.540	−8.92	4.270	−9.18	5.488	−8.41	2.924
m60/119/121/132	−10.75	3.312	−9.12	4.212	−9.30	5.400	−9.10	0.801

is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. It has been generally accepted that azo dyes are major concern as pollutants of the environment. In recent years, the biological treatment of azo dyes has been carried out using dye-degrading enzymes and some of them have been identified so far (Chen et al., 2004; Matsumoto et al., 2010). Some special aerobic bacterial strains can reduce the azo bonds using aerobic azoreductase (Bin et al., 2004; Chen et al., 2005, 2010). The mesophilic gram-positive *Bacillus* sp. B29 contained three aerobic NADH dependent FMN azoreductases including AzrA, AzrB and AzrC. They catalyze the biodegradation of azo dyes using NADH as an electron donor. Yu et al. provided the crystal structures of AzrA and AzrC in complex with azo dyes in order to better understand these azoreductases. Their biochemical studies indicated that all of the azoreductases from *Bacillus* sp. B29 are homodimeric proteins which contain two moles of FMN per mole of dimeric protein. AzrC contains a higher hydrophobicity and a smaller active-site pocket in comparison with AzrA (Yu et al., 2014). In the present study, a molecular docking analysis was performed to evaluate the detailed binding mode between AzrC with four different azo dyes and also to understand the specificity of substrate for AzrC. The molecular interactions of AzrC and ligands were surveyed by LigPlot$^+$ program. Our results indicate that ligand molecules were completely wrapped by amino acid residues at the active site pocket and F105 (A), F125 (B) and F172 (B) have main roles in hydrophobic interactions in all four ligands. These residues belong to hydrophobic pocket in ligand binding site and they have important roles in making appropriate hydrophobic interactions between protein and ligands. There are also some other residues like Trp 60 (A), Ala 119 (B), Ile 120 (B) and others, which participate in hydrophobic interactions in different (but not all) ligands (Fig. 2). The evidences of hydrogen bond interactions indicated that Asn 104 (A), Asn 187 (B), and Tyr 151 (A) make stable hydrogen bond between AzrC and ligands. The presence of both A and B chain residues in ligand binding site implies the cooperation of these two chains in order to make an appropriate active site. Molecular docking has been a focus of attention for analyzing protein–ligand or enzyme–substrate interactions. Different points, double, triple and quadruple mutations were performed to describe the most critical residue substitution that can improve the interaction between AzrC and azo dyes. Point mutations were performed in positions, which are located in hydrophobic pocket of AzrC active-site (according to VMD and LigPlot$^+$ observation). To do this, Trp60 (B), Ala119 (B), Ala121 (B) and Pro 132 (B) amino acids were selected as candidates for point mutations. The mutations were performed based on the hydrophobicity rank of amino acids and properties of their side chains (Pommié et al., 2004). Generally, the mutations were designed to increase the hydrophobicity of amino acids with less conformational changes. In this regard, specific point mutations including W60 (B) F, A119 (B) I, A121 (B) I and P132 (B) F were performed to increase the hydrophobicity and binding affinity of azo dyes. It was supposed that the combination of these mutations in double, triple and quadruple forms would be more effective. In this regard, 11 different AzrC complexes were designed with combination of mentioned point mutation (in double, triple and quadruple forms) and

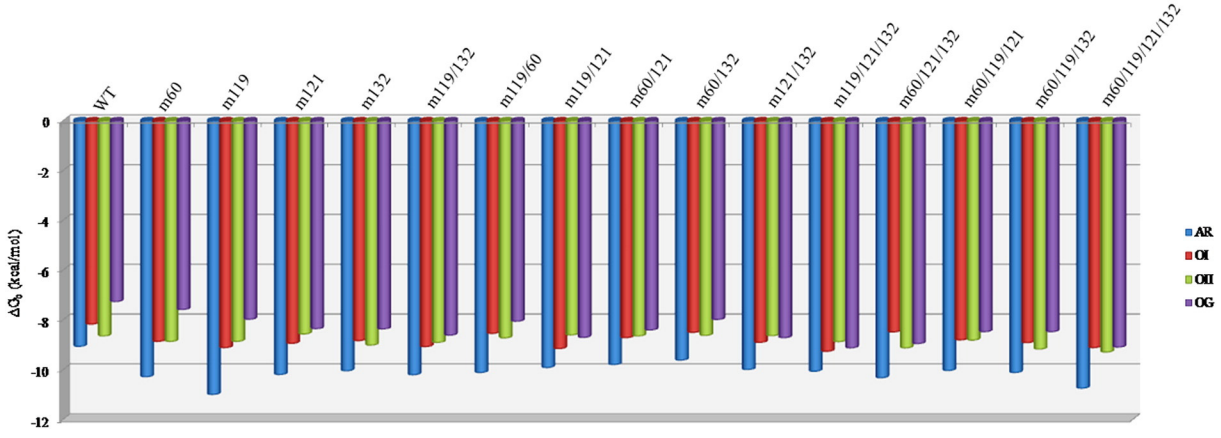

Fig. 1. The effect of different mutations in binding affinity. The quadruple pattern of mutations has most significant effect on binding affinity.

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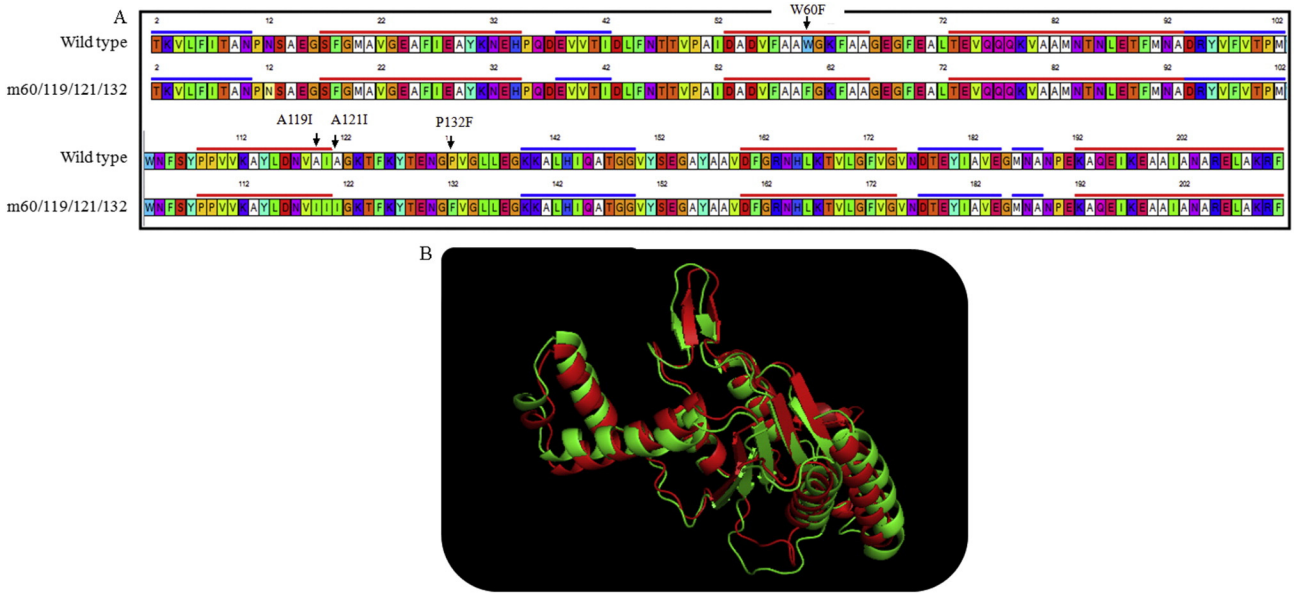

Fig. 2. Structural comparison of wild type AzrC and m60/119/121/132 mutant form of AzrC. A) Sequence alignment between wild type AzrC and m60/119/121/132 mutant. Secondary structures are also shown above the sequences using red lines (α-helix) and blue lines (β-sheet). B) Overall structure comparison. Wild type AzrC is shown in red and m60/119/121/132 mutant form of AzrC is shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

docking analyses were performed for all 16 different complexes. The docking results indicate that all these mutations would increase the ligand affinity, but in different ranges. In the case of point mutations m119, m121 (in AzrC-AR and AzrC-OI), m119, m132 (AzrC-OII), and m121, 132 (AzrC-OG) have the highest effect on free binding energies in order to increase ligand affinity. In order to find a specific pattern of mutations for AzrC protein to improve the affinity of all four ligands in the reasonable manner the combination of these mutations is analyzed (Table 1). Interestingly, the quadruple pattern of mutations could enhance the binding affinities of all ligands (in comparison with the wild type and also other mutation combination) (Fig. 3). In our calculations, AzrC showed higher tendency toward more hydrophobic ligands such as AR88 and OI through the interactions between hydrophobic parts of the ligands and several hydrophobic residues of AzrC. The hydrophobic amino acids like Phe105 (A), Phe 125 (B), and Phe 172 (B) in wild type form make hydrophobic interactions whereas the presence of hydrophobic residues F60 (B), I119 (B), I121 (B) and F132 (B) along with native hydrophobic residues made a powerful hydrophobic pocket in the active site, which play critical roles in increasing the binding affinity of all the four different ligands. In order to define whether combination of these mutations changes the AzrC secondary structure and as a result disrupts its function, the secondary structure of AzrC protein with the presence of four mutations was analyzed by DSSP method. The comparison of secondary structure between wild type and quadruple forms of AzrC has shown no significant difference (Fig. 4A). All the main secondary structures (α-helix, 3–10 helix β-sheet, turn, loop, …) which are reported in the crystallography study were intact and completely similar to the crystallographic structure (Fig. 4B). As a result, this pattern of mutation will not disrupt the protein structure and function. This new pattern of target mutation increases the affinity of four main azo dyes to the AzrC protein. So it would play critical roles to overcome pollution of the environment. These results will be helpful for future studies in order to make a more efficient recombinant AzrC protein for biodegradation of azo dyes. In the present study, the molecular docking methods were employed to gather molecular information about the interactions of various azo dyes with AzrC azoreductase and

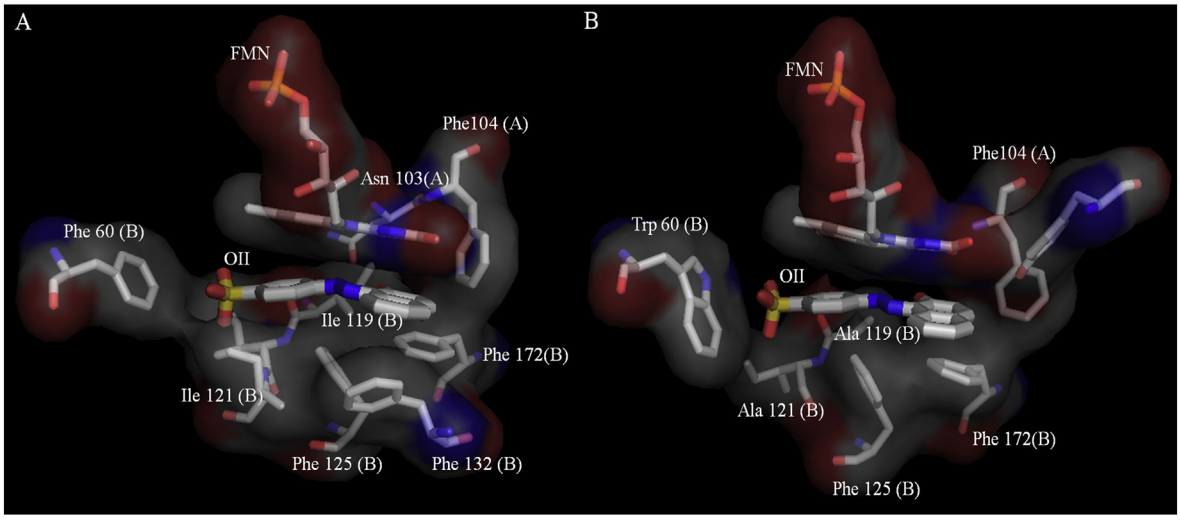

Fig. 3. Quadruple pattern (m60/119/121/132) of mutation. A) The pattern of amino acid residues in the active site of quadruple pattern of AzrC. B) The pattern of amino acid residues in the active site of wild type AzrC.

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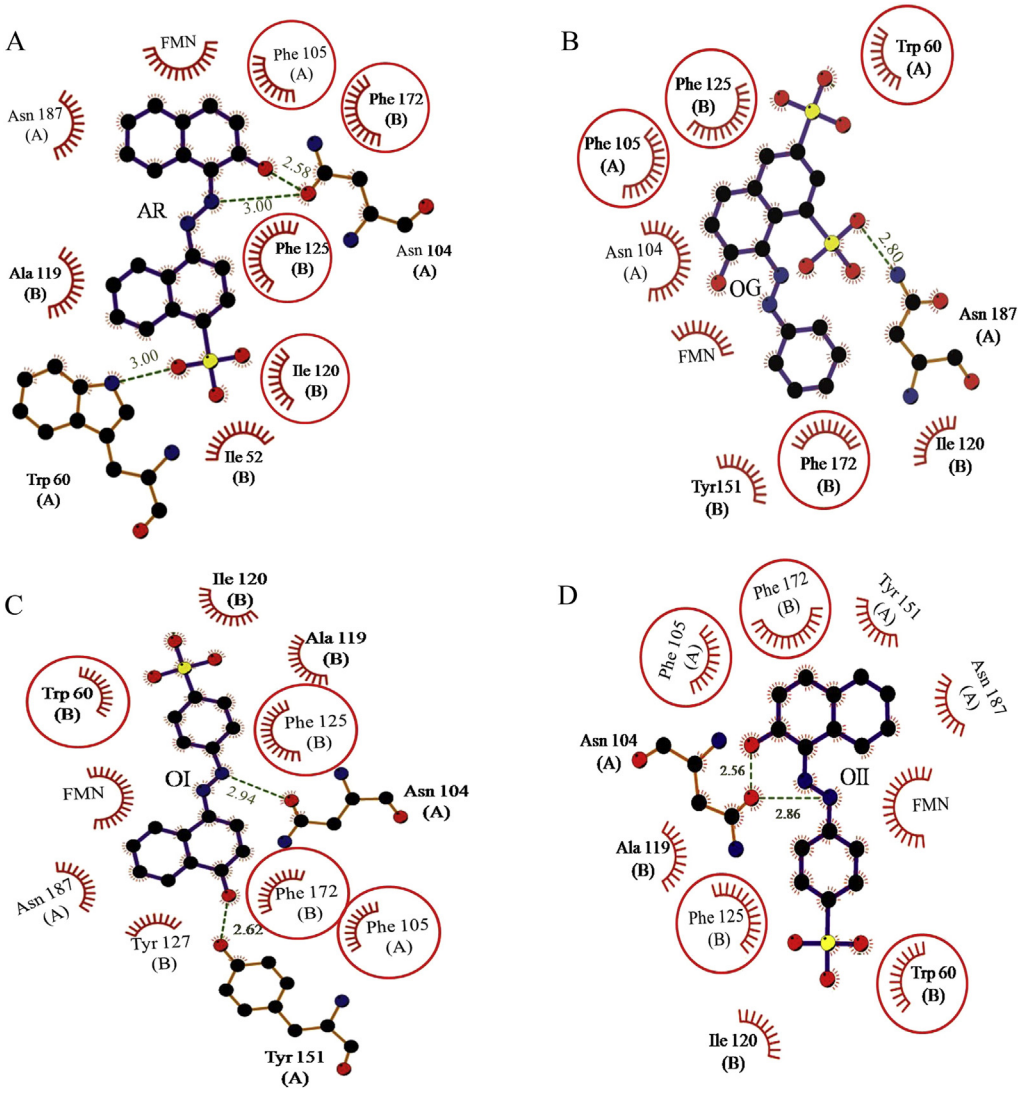

Fig. 4. Ligplot + predictions of AzrC active site residues. A) AzrC-AR B) AzrC-OG C) AzrC-OI and D) AzrC-OII active site residues.

also candidate amino acid substitution which has a potential to improve the ligands and AzrC interaction. Four specific residue substitution (quadruple mutant form) is suggested in order to increase the biodegradation capacity of AzrC by improving its affinity to four azo dyes. Our findings were approved by AutoDock free binding energies and DSSP secondary structure analysis. This new structure of AzrC protein has a potential to use as a key player to degrade Azo dyes as a main pollutant of industrial waste. The results of this study would be helpful for future in vitro experimental studies.

5. Conclusion

In conclusion, a new recombinant AzrC protein with higher affinity to azo dye was suggested. The specific pattern of target mutation improves the affinity of four azo dyes to the AzrC protein. This investigation will be helpful for further studies in order to produce a novel engineered AzrC protein for more efficient biodegradation of azo dyes.

Conflict of interest

The authors declare that they have no financial and non-financial competing interests.

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