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Functional and catalytic active sites prediction and docking analysis of azoreductase enzyme in *Pseudomonas putida* with a variety of commercially available azodyes

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The initial critical step of reduction of azo bond during the metabolism of azo dyes is catalysed by a group of NADH and FAD dependant enzyme called azoreductases. Although several azoreductases have been identified from microorganisms and partially characterized, very little is known about the structural basis of the substrate specificity and the nature of catalysis. Azoreductase enzyme of *Pseudomonas putida* has a wider broad spectrum of substrate specificity and capable of degrading a wide variety of azo dyes. In the present study, the crystal structure of the enzyme from PDB and 10 azo dyes from NCBI PubChem compound were retrieved and their interactions were studied. These azo dyes were then docked with the FMN-dependent NADH-azoreductase enzyme to analyze the binding affinity of the azo dyes with the enzyme and predict the catalytic sites. Consequently, the catalytic residues of FMN-dependent and NADH dependent enzyme were then analysed in terms of properties including function, hydrogen bonding and flexibility. The results suggest that Ala-114, Phe-172 and Glu-174 play a predominant role as catalytic site residues in the enzyme. Furthermore, the approach emphasis on predicting the active sites of this enzyme where substrates can bind in order to give a better understanding of the biodegradation of some of the commercially important azodyes mediated by azoreductase. These results will pave way for further increase in azoreductase activity and for better understanding of the dye degradation pathway.

Key words: Azoreductase, NADH, FMN, chemical properties, docking, active sites.

INTRODUCTION

Azo dyes are known to be widely used class of dyes that are highly toxic and contain carcinogenic compounds. Although lot of research has been carried out for their removal from industrial effluents, very little attention is given to changes in their toxicity and mutagenicity during the treatment processes (Bafana et al., 2008). Azo dyes represents almost 70% of the textile dyestuffs produced and the effluents released into the water system disturbs

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the ecological parameters of mostly water bodies (Knackmuss, 1996). Due to enormous modernization the most problematic environment pollution in the wastewater are the effluents released from production of dyes and the dyeing industries. These residual dyes in industrial effluents are a threat to public health because of its high toxicity and carcinogenicity (Bisschops and Spanjers, 2003; Weisburger, 2002). The uncontrolled release of these compounds in the environment causes severe problems by decreasing light absorption which significantly affect photosynthetic activity of aquatic life and may be toxic due to the presence of aromatics or heavy metals (Banat et al., 1996). Release of such colored compounds in the environment is undesirable not only because of their aesthetic appearance, which may drastically affect photosynthesis in the aquatic ecosystems, but also becausemany of the dyes and/or their breakdown products are mutagenic to life(Chung and Cerniglia, 1992). Unfortunately, azo dyes present in the wastewater are normally unaffected by conventional treatment processes. Their persistence is mainly due to the sulfo and azo groups, which do not occur naturally, making the azo dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla el al., 1983). The persistence of azo dyes as reported could be further decolorized which consequently requires a putative agent for cleavage of azo bonds, after which the resulting aromatic amines can be biodegraded readily under aerobic conditions (Van der Zee and Villaverde, 2005). The cleavage of azo bonds is catalyzed by azoreductase enzyme with the aid of an electron donor. Several bacteria capable of decolorizing azo dyes have been identified, and azoreductase enzyme has been isolated and characterized from some of them (Chen, 2006).

In the recent years, bioremediation of azodyes polluted areas using bacteriaas a potent organism has gained momentum in context to dye effluents and consequently it seems to play a pivotal role in bioremediation activities. Azobenzene reductases, also known as Azoreductase (EC 1.7.1.6) are a family of NAD(P)H and Flavin – dependent enzymes that have been identified from a large number of bacterial species. These enzymes are able to reduce a wide range of substrates including azo dyes/drugs, quinones, metal ions and nitro compounds. They have been found in a number of species including *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas putida* etc. Although various azoreductases may be present in the same organism but the physiological roles of most of these enzymes is generally poorly understood. It has been found that many microorganisms including cyanobacteria can transform these azo dyes into colourless products. Generally, in bacterial system the degradation of azodyes is often initiated by an enzymatic step which involvesa cleavage of azo linkages with the aid of an azoreductase and an electron donor (Hong and Gu, 2010). Several workers have reported on the azo dye degradation in bacterial system(Yeh et al., 2005; Asgher et al., 2007; Delee et al., 1998; Levine, 1991; Walker, 1970). Similarly, azoreductase activities of cyanobacterial species which are known for their ubiquitous occurrence in nature, in response to various mono and diazo compounds have also been reported (Jadhav et al., 2008; Omar, 2008). These azoreductases catalyze the reduction of the azo bond (-N=N-) in both azo pro drugs (for example, balsalazide) and azo dyes (for example, methyl red). They have been shown to reduce azo compounds via a ping pong mechanism (Chan-Ju et al., 2010). In context to the present study, the azoreductase from the *P. putida* has been taken into consideration as a model organism to understand the interaction of several toxic dyes with the enzyme using bioinformatics tools which in turn might probably throw a limelight on the prediction of active sites which could be further exploited for development of effective bioremediation process.

METHODOLOGY

Data set

The FASTA sequence of FMN dependent NADH azoreductase of *P. putida* was retrieved from the PDB database with the PDB ID 4C0W having 203 amino acids (Bernstein et al., 1977). The structures of the commercially important dyes were retrieved from the PubChem compound database namely Azobenzene, p-Aminoazobenzene, Amaranth dye/F.D&C No.2, Methyl Orange, Sudan IV, Amidoschwarz, Congo Red, C.I. Food Yellow 3, Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperdine-3-yl] methanone and Solvent Red 23 with CID numbers 2272, 6051, 5359521, 23673835, 5876571, 54599778, 11313, 6850717, 1530900 and 5809667, respectively, from PubChem compound database (Wang et al., 2009).

Homology model building and evaluation

Homology modeling of the azoreductase enzyme was performed using the CPH model server 3.2 (Nielsen et al., 2010) and the structurewas further validated using Ramachandran Plot.The template generated was 4C0W which had highest similarity percentage and viewed usingUCSFChimera 1.10.1 viewer (Pettersen et al., 2004).

Chemical properties

The various chemical properties of the commercially available and industrially important azo dyes such as the molecular weight, molecular formula, hydrogen bonds (donors and acceptors), rotatable bonds, exact mass, topological polar surface area (A^2), heavy atom count, complexity and covalently bonded unit count were retrieved using PubChem Compound database (Table 2).

Active site analysis

Ligand binding site prediction of the azoreductase enzyme was carried out using DoGsitescorer (Volkamer et al., 2010). The software possesses structure-based method to predict active sites in proteins based on a Difference of Gaussian (DoG) approach

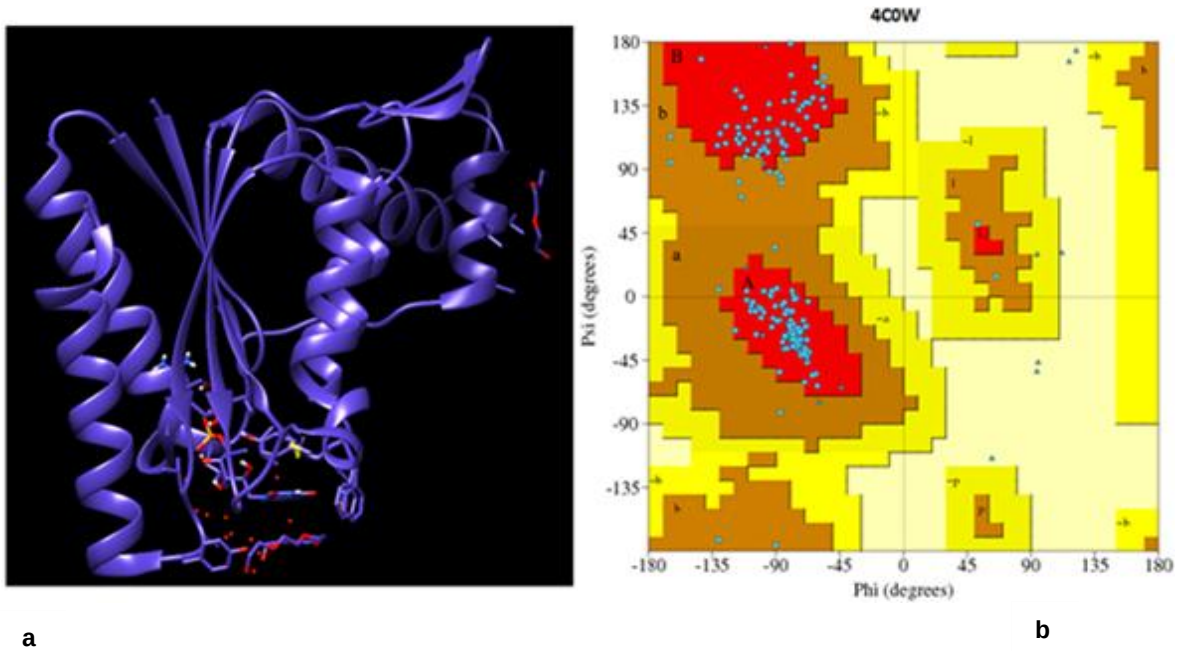

Figure 1. (a) There dimensional structure of azoreductase enzyme from *Pseudomonas putida* (4COW) obtained from PDB with its natural ligands. (b) Ramachandran Plot of 4COW.

which originates from image processing. In contrast to existing methods, DoGSite splits predicted pockets into sub-pockets, revealing a refined description of the topology of active sites. DoGSite correctly predicts binding pockets for over 92% of the PDBBind and the scPDB data set, being in line with the best-performing methods available.

Docking studies

Docking study of the azoreductase enzyme and the commercially important dyes were carried out using Swiss Dock server (Grosdidier et al., 2011), and energy minimization was performed before and after docking by the same in an automated manner.The interpretation of docking results and their integration into existing research pipelines is greatly facilitated by the seamless visualization of docking predictions in the UCSF Chimera molecular viewer which could be launched directly from the web browser.

RESULTS

Structure retrieval

The enzyme, FMN-dependent NADH-azoreductase of *P. putida* has a crystallized structure characterized by three ligands FMN 1201, 12P 1202 and 12P 1203. The structure was obtained from PDB with the PDB ID-4C0W.

Homology modeling and validation

In the present work, the azoreductase enzyme was initially explored for the best homology modeling which basically determines the overall 3D structure of the aminoacids present in the enzyme molecule. The homology modeling using the CPH server depicted the best model of the enzyme molecule which requisite number of strands, helices and loops (Figure 1a). This structure was further validated by the corresponding Ramachandran Plot (Figure 1b) which depictedfewer numbers of amino acids in the disallowed region with maximum number of amino acids molecules in the favorable region. Most favorable regions showing 85.6% having 166 residues, additional allowed region showing 13.9% having 27 residues and generously allowed region showing 0.5% having only 1 residue. There was no residue found in the disallowed region. The Ramachandran Plot analysis further showed 226 as total number of residues, the number of Glycine (Gly) and Proline (Pro) are 16 and 14, respectively, and the number of end residues (excluding Gly and Pro) were 2.

Computation of docking score between the ligands and the enzyme

Protein and other chemical molecule interaction outputs compute putative data which could be an advantage to understand the mechanism. The cumbersome syntax of the docking engine is hidden behind a clean web interface providing reasonable alternative sets of parameters as well as sample input files. All calculations are performed on the server side, so that docking runs do not require any computational power from the user.

Table 1. Detailed docking analysis result showing of azo dyes and azoreductase enzyme. Listed above are the binding affinities, number of hydrogen bond formation of each azo dye with azoreductase. The amino acid residue position of azoreductase interacting each azo dye is also provided along with the azo dye information.

Azo dyes	Common names	Affinity (kcal/mol)	No. of H-bonds	Positions (residues)
2272	Azobenzene	-5.35	0	
5359521	Amaranth	-6.66	2	Glu-174, Phe-172
6051	p-aminoazobenzene	-5.46	2	Gly-141, Phe-98
23673835	Methyl Orange	-5.58	1	Ala-114
1530900	Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone	-3.40	1	Ala-114
587651	Sudan IV	-6.30	0	
54599778	Amiboschwarz	-6.22	1	Ala-114
11313	Congo Red	-7.22	2	Gly-180, Thr-102
6850717	C.I. Food Yellow 3	-6.48	0	
5809667	Solvent Red 23	-6.38	1	Ala-114

Docking study showed the binding affinity, number of hydrogen bonds and the binding residues. It has been noted that the binding affinities have negative values as shown in Table 1 which reveals the high feasibility of this reaction. The docked complexes were analyzed with the molecular visualization tools, Chimera 1.10.1(Volkamer et al., 2010) as shown in Figure 2. The docking analysis showed that seven dyes viz. Amaranth, p-aminoazobenzene, methyl orange, Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone and amidoschwarz formed H-bonds with the enzyme residues Glu-174, Phe-172, Gly-141 and Ala-114. The variation in the docking score indirectly gives the idea about the rate of decolorization.This Comparing the results from DoGsite scorer and docking studies, it indicates that the amino acid residues ALA, ASP, LEU, LYS, PHE and VAL play an important role as catalytic site residues in the azoreductase enzyme of *P. putida*. This docking study also provides information on the binding affinity of the ligands with azoreductase enzyme. The rate of color removal for congo red is higher than any other azo dye and it could be assumed probably that this information would provide a better understanding of the molecular mechanisms involved in catalysis and a heuristic basis for predicting the catalytic residues in enzymes of unknown function. The natural ligands (FMN, 12P) were also found to interact with some of the ligands. In this work, the catalytic residues are reported as well as the binding affinities for some commercially important azodyes. The study made in this project would facilitate researchers a better understanding of enzyme mechanisms and also used to improve the designing strategies of less harmful azodyes

Functional site location

The catalytic or functionally important residues of a protein are known to exist in evolutionary constrained regions. However, the patterns of residue conservation alone are sometimes not very informative, depending on the homologous sequences available for a given query protein. Hence, the prediction of functional sites in newly solved protein structures is a challenge for computational structural biology. Most methods for functional site identification utilize measures of amino acid sequence conservation in homologous sequences, based on the assumption that functional sites are relatively conserved during evolution. Protein structural information has also been used to help identify protein functional sites. Active sites of the target protein were predicted using DoGsite scorer and the output file was viewed under Chimera. Seven active sites were obtained from the study along with the corresponding amino acid residues present in each active site. Each of the sites were analyzed and compared with the amino acids interacting with the ligands in the docking study. The docking result shows that the amino acids such as ALA, ASP, LEU, LYS, PHE and VAL are very much repeated in the interaction with more than one ligand. This reveals that these amino acids are catalytic residues. The active site variations suggest that the enzyme can decolorize a wide range of azo dyes (Figure 3 and Table 3).

Molecular docking interactions showing hydrogen bonding

The hydrogen bonding in the crystal structures were used as a measure of residue flexibility. Analysis shows that the amino acids interacting with the ligands are involved in hydrogen interaction; it can be as a donor or as an acceptor. This shows that catalytic residues have a limited conformational freedom. The docking result shows that the ligands have hydrogen bonding with amino acids and it is illustrated in the docking analysis table.

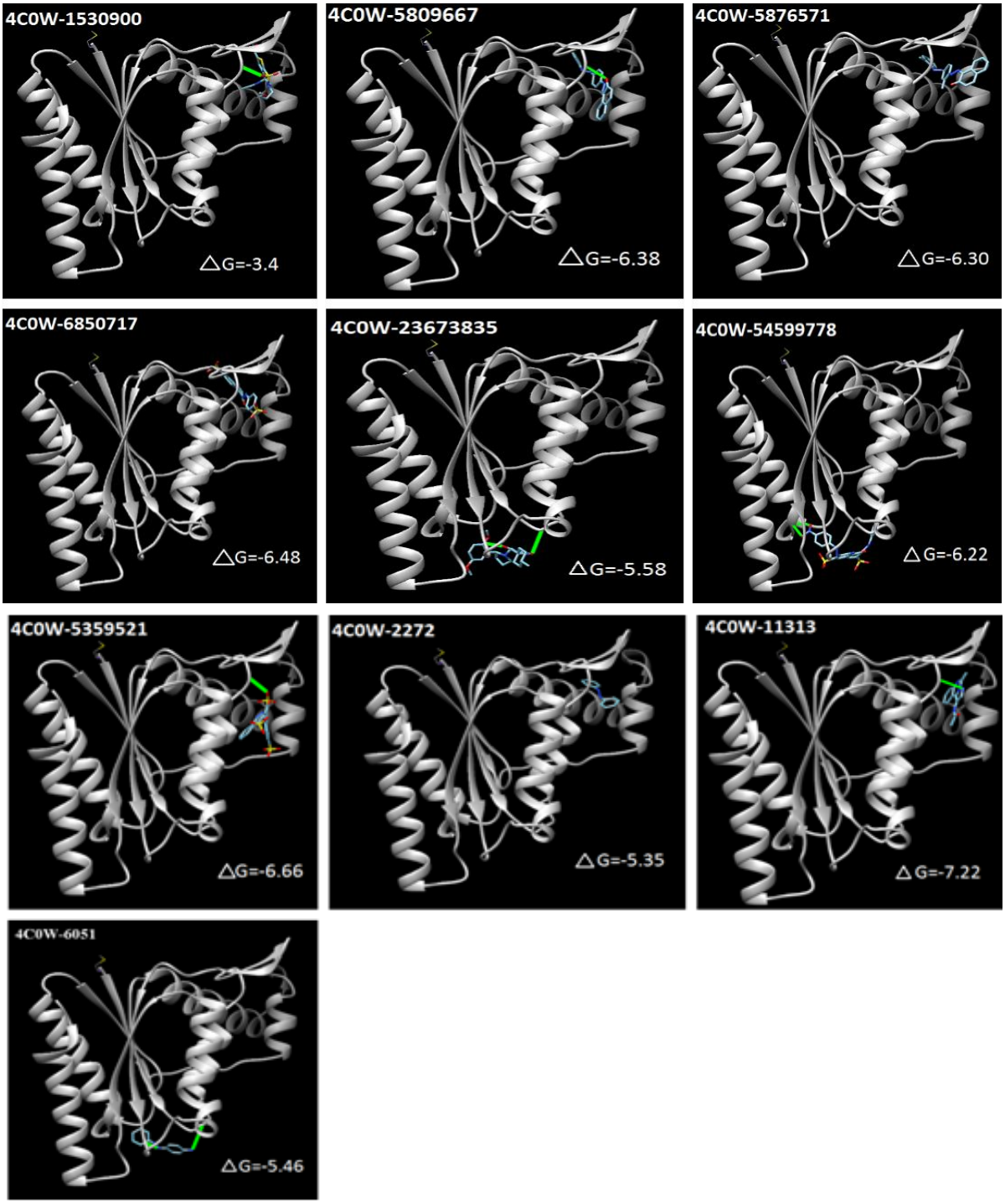

Figure 2. Docked images of the 10 industrially important azo dyes with azoreductase enzyme (PDB ID 4C0W). Figure also shows the respective binding affinities denoted by ∆G. 1530900 indicates Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone, 5809667 indicates Solvent Red 23, 587651 indicates Sudan IV, 6850717 indicates C.I. Food Yellow 3, 23673835 indicates Methyl Orange, 54599778 indicates Amiboschwarz, 5359521 indicates Amaranth, 2272 indicates Azobenzene, 11313 indicates Congo Red, 6051 indicates p-aminoazobenzene.

DISCUSSION

Due to enormous modernization, the most problematic environment pollution is the wastewater where the effluents are being released from production of dyes and the dyeing industries. These residual dyes in industrial

Table 2. Chemical properties of the 10 industrially important dyes from PubChemCompund, showing the Molecular weight, Molecular formula, Hydrogen bond donor count, Hydrogen bond acceptor count, Rotatable bond count, Exact mass, Topological polar surface (A^2), Heavy atom count, Complexity and Covalently bonded unit count of the dyes.

Dye	Molecular wt. (g/mol)	Molecular formula	Hydrogen bond donor count	Hydrogen bond acceptor count	Rotatable bond count	Exact mass (g/mol)	Topological polar surface (A^2)	Heavy atom count	Complexity	Covalently bonded unit count
Azobenzene	182.221	$C_{12}H_{10}N_2$	0	2	2	182.084396	25.0	14	157	1
Amaranth	604.473	$C_{20}H_{11}N_2Na_3O_{10}S_3$	1	5	4	603.92688	65.3	38	1200	4
p-aminoazobenzene	197.236	$C_{12}H_{12}N_3$	2	3	2	197.095291	51.0	15	201	1
methyl orange	327.334	$C_{12}H_{14}NNaO_3S$	0	6	4	327.065357	93.5	22	448	2
Azepan-1-yl[(3S)-[2(chlorophenyl)methyl]piperidin-3-yl]methanone	334.88348	$C_{19}H_{27}ClN_2O$	0	2	3	334.181191	23.6	23	382	1
Sudan IV	380.441	$C_{24}H_{20}N_4O$	1	5	4	380.163711	66.2	29	670	1
Amiboschwarz	595.517	$C_{22}H_{16}N_6NaO_9S_2$	4	14	7	595.031787	264.0	40	1260	2
Congo red	696.663	$C_{32}H_{22}N_6Na_2O_6S_2$	2	12	5	696.083763	233.0	48	1180	3
C.I. Food yellow 3	452.369	$C_{16}H_{10}N_2Na_2O_7S_2$	1	9	2	451.972481	173.0	29	818	3
Solvent red 23	352.388	$C_{22}H_{16}N_4O$	1	5	4	352.132411	66.2	27	597	1

Figure 3. Active sites displayed in three dimensional structure of the azoreductase enzyme predicted with DoGsite scorer and viewed in Chimera.

effluents are a threat to public health because of its high toxicity and carcinogenicity (Bisschops and Spanjers, 2003; Weisburger, 2002). Unfortunately, azo dyes present in the wastewater are normally unaffected by conventional treatment processes. Their persistence is mainly due to the

Table 3. Showing the amino acid composition of the different active sites predicted. The amino acid positions of the sites are listed above.

Active site	Residue
Site 1	SER (15,17), ALA(16,140,145), PRO 94, MET 95, TYR 96, ASN 97, PHE(98, 156), THR(139,149), GLY (141,142,146,176), LEU9143, 157), HIS(144, 153), GLU 154, ASP 155, ARG 158
Site 2	MET 1, LYS (2, 133), LEU 3, VAL 134
Site 3	ALA (70, 73), GLU 72, LYS 74
Site 4	HIS 5, ASP(7, 13), GLY 12, ASN 14, SER 15, ARG (18, 22), GLN 19, VAL (25,37), GLU 26,LYS 29
Site 5	VAL 172, ARG 173, ALA 174, HIS 175, GLY 176
Site 6	ALA (46,48, 106), ILE 47, HIS 49, PHE 50, THR 102, GLN 103, ASP 109
Site 7	ILE (6,108,164,166), PHE (83, 160), VAL (89,111,134), ALA 93, LEU(104,136,157), TRP 107, LYS 132, LEU 161

sulfo and azo groups, which do not occur naturally, making the azo dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla el al., 1983).Although lot of research has been carried out for their removal from industrial effluents, very little attention is given to changes in their toxicity and mutagenicity during the treatment processes (Bafana et al., 2008). Generally, in bacterial systems the degradation of azodyes is often initiated by an enzymatic step involving a cleavage of azo linkages with the aid of an azoreductase and an electron donor (Asgher et al., 2007). These azoreductases catalyze the reduction of the azo bond (-N=N-) in both azo pro drugs (e.g. balsalazide) and azo dyes (e.g. methyl red). They have been shown to reduce azo compounds via a ping pong mechanism (Wang et al., 2009).In recent years, we have seen a number of spectacular discoveries on surprisingly similar structures of proteins whose evolutionary kinship cannot be recognized based on primary sequence analysis alone (Gibrat et al., 1996). Hence, secondary structures allow a simple and intuitive description of 3D structures, which are widely employed in a number of structural studies. Therefore in context to the present the azoreductase enzyme has been subjected for emulating a suitable protein structure for the evaluation of the docking parameters which would be further analyzed with the 10 commercially available dyes as shown in Table 1. The 3D structure that we have predicted using CPH model server 3.2 shows Ramachandran Plot in the favorable regions and it indicates that the structure is applicable to various applications like predicting the active sites and the amino acids involved. Thus, the modeled enzyme structure would provide an insight to the different type of azodyes which are degraded by the azoreductase enzymes and the conformational changes that take place in the enzyme structure which has been furtheranalyzed by biodegradable systems models and docking studies (Nakanishi et al., 2001).

Furthermore, before the adjunct of the docking reactions these modeled structure has been explored for identifying the location of the ligand binding sites and this will help us in comparing the different functional sites of the azoreductase and the aspect of broad substrate specificity. The active sites which are predicted in this study revealed that the azoreductase could bind to any of the sites as shown in Figure 3, but the flexibility of effective binding sites has to be further studied using molecular modeled simulation. Although, the docking interaction predicted the number of stable hydrogen bonds which itself indirectly indicates the binding of these dyes to the active sites of the enzyme molecule. Therefore, it could be predicted that the functional/active site of this enzyme from *P. putida* could probably be explored in further for developing and engineering effective bioremediation tools for detoxification of dyes contaminated areas.

Conflict of interests

The authors did not declare any conflict of interest.

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