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In-silico directed evolution of Laccase enzyme to enhance its stability and catalytic efficiency in the degradation of Aflatoxin B1 and Aflatoxin G1

1) INTRODUCTION

Mycotoxins are secondary metabolites of fungi that cause hepatotoxicity, teratogenicity, and immunotoxicity, and are classified as group I carcinogens. Aflatoxins (AFs) are a type of mycotoxin produced by fungal species such as *Aspergillus flavus*, and *A. parasiticus*.
These commonly infect cereal crops, including wheat, tree nuts, maize, cotton, and peanuts, and can pose serious threats to humans and animals by causing various complications such as hepatotoxicity, teratogenicity and immunotoxicity. The main aflatoxins are B1, B2, G1 and G2, and can be toxic to the body through inhalation, mucous membranes or skin,leading to an overactive inflammatory response.
Various methods such as physical strategies, chemical methods and biodegradation are applied to detoxify aflatoxin. However, many of the existing physical and chemical methods for the detoxification of aflatoxin from the contaminated food and feed are unprofitable for practical application due to loss of nutritional quality, safety concerns, environment pollution, limited efficiency and high marketing costs. In contrast, biological processes involving biodegradation and biosorption are more effective and promising strategies. Microbial and enzymatic applications are moderate, inexpensive and effective with little to no harmful intermediates for humans and animals consumption.
Globally, fungal laccase have better pH/temperature stability, greater tolerance to metals, and can oxidize a wide variety of substrates of different origins. The use of laccase can lead to rapid and significant degradation of various substrates.

Laccases belong to the family of multiple copper (Cu) containing enzymes, which are able to perform the four-electron reduction of dioxygen to water and subsequent oxidation of organic and inorganic compounds by the mechanism of transferring one-electron. The laccase derived from *Myceliophthora thermophilus* has a monomeric active site and differences in the T1-Cu active site topology and polar motifs, amongst different sources of laccases within the taxonomic subgroup, each employ different molecular evolution to serve different functional roles such as in degradation of environmental pollutants, wastewater treatment, and endocrine disruptor chemicals under varying conditions. The conserved histidine residues are found to interact with the copper ions found in the structure which might play a role in degrading aflatoxin through accelerated Redox reactions with the help of copper ions.

Molecular docking is an effective analytical tool to assess the enzyme–substrate interaction. With the development of high technology, the use of computer simulation, and the deep understanding of the three-dimensional structure of laccase, it becomes easy to study the relationship between laccase and its interaction with the substrate. easier and faster.

In-silico **Directed evolution** of enzymes studies hold the potential to develop preventative strategies to reduce AFB1 and AFG1 contamination of food . Our **present study has aimed to** analyze the interaction of AFB1 and AFG1 with the site-directed mutated laccase to find possible differences in enzyme and ligand pocket recognition towards various catalytic sites using different docking simulations and procedures.

2) METHODS

2.1) Structure retrieval

Crystallized structure (X-RAY diffraction structure) of the enzyme under consideration of this study was available on PDB or Protein Data Bank. Structure of Laccase enzyme from the species *Myceliophthora thermophilus* was retrieved with PDB ID:6F5K in the (.pdb) format.

2.2) Cleaning of files using PyMol 2.5

Laccase enzyme pdb file (PDB ID:6F5K) was cleaned up to prepare for molecular docking (rigid docking) using PyMol 2.5; all water molecules, ligands (which included copper ions, calcium ions, N-acetyl glucosamine, nonaethylene glycol and hydroxide ion) and hydrogen atoms were removed. Additionally 11 amino acids had to also be removed from the structure due to conformation errors caused by them in the AutoDock Vina program.
Crystallized ligand of Nonaethylene Glycol (NE) was exported out to be docked for comparison purposes.

2.3) Ligand retrieval

Ligands for the study Aflatoxin B1 (AFB1) and Aflatoxin G1 (AFG1) were retrieved from PubChem in SDF format.

2.4) Control molecular docking of cleaned up laccase with AFB1 and AFG1 using PyRx

Molecular docking was carried out using AutoDock Vina through PyRx, the cleaned up laccase (.pdb file) was imported and converted into an AutoDock Macromolecule and the ligands and NE molecule were imported using OpenBabel. Their energies were minimized using the uff force field algorithm and were converted into pdbqt format for docking procedure. Blind docking was carried out due to lack of proper active site knowledge with maximized grid box feature.

2.5) Mutation predictions using HotSpot Wizard v3.1

Cleaned up structure of laccase enzyme was uploaded to HotSpot Wizard v3.1 and the web-server was run and functional hotspots and stability hotspots were generated from the server. Best results were retrieved from the server. Residues besides recommended for mutation were also taken which were shown to be critical interacting residues in visualization of control clean laccase docking.

2.6) Mutation predictions using CUPSAT server

It is well known that not only functional hotspot mutations but stability mutations improve catalytic efficiency. Cleaned up laccase PDB was uploaded to the server and the results csv file was retrieved with all the possible mutations. The data was sorted and only top 10 results were filtered out with highest values of Delta Delta G (DDG).

2.7) Mutagenesis of proteins using PyMol

Cleaned up laccase PDB was loaded onto PyMol and through the mutagenesis wizard each point mutation was introduced which was predicted by both servers and individual point mutation structures were exported out.

2.8) Molecular Docking of mutants generated

Each point mutated version of laccase was loaded onto PyRx and in the same process molecular docking was performed and results were filtered out to only include the best binding energies, with least RMSD/UB and RMSD/LB values. Best docked conformations were exported for visualization in PDB format from the PyRx console.

2.9) Visualization using BIOVIA Discovery Studio 2021

Control and each of the mutant proteins were loaded in discovery studio with their respective docked conformations and each of their Receptor-Ligand interactions were visualized in 2D and 3D to reveal interacting residues and types of interactions.

2.10) Validation of best mutants and control docking through Molecular Dynamics using Schrodinger Desmond

The two base interaction complexes of cleaned_laccase with AFG1 and AFB1 and best two interacting mutants with their individual complexes were analyzed through Molecular Dynamics analysis using Desmond at 50ns after preparation of protein-ligand complexes using Protein Preparation Wizard. The system builder was utilized to also introduce counter salt ions of Sodium (Na) at 15mM concentration in the system. The dynamics simulation was run and reports were generated.

2.11) Analysis of amino acid residues removed during cleaning of 6F5K laccase pdb using MEGA-X

Mega-X alignment tool was used to align sequences of the base 6F5K laccase structure with the Cleaned_laccase_pdb generated using PyMol. 11 amino acid residues were removed

3) RESULTS

Molecular docking of Wild-type laccase enzyme with AFB1, AFG1 and NE

Binding energies obtained from docking of wild-type laccase enzymes yielded very positive results which were essential in establishing the inert potential of the toxin to get bound and degraded by the enzyme, and proved to further progress the previous findings about laccase enzyme to be very effective at degrading Aflatoxin B1 (-7.8 kcal/mol) and similar binding energy of Aflatoxin G1

(-8.3 kcal/mol) extended those results to indicate that Laccase enzyme from *Myceliophthora thermophilus* possesses potential to degrade all variants of Aflatoxins produced by varying species rather than just one variant of the toxin. The comparison to binding energy of NE (-4.6 kcal/mol) (Nonaethylene Glycol) helped provide a comparison state of binding energy in the analysis.

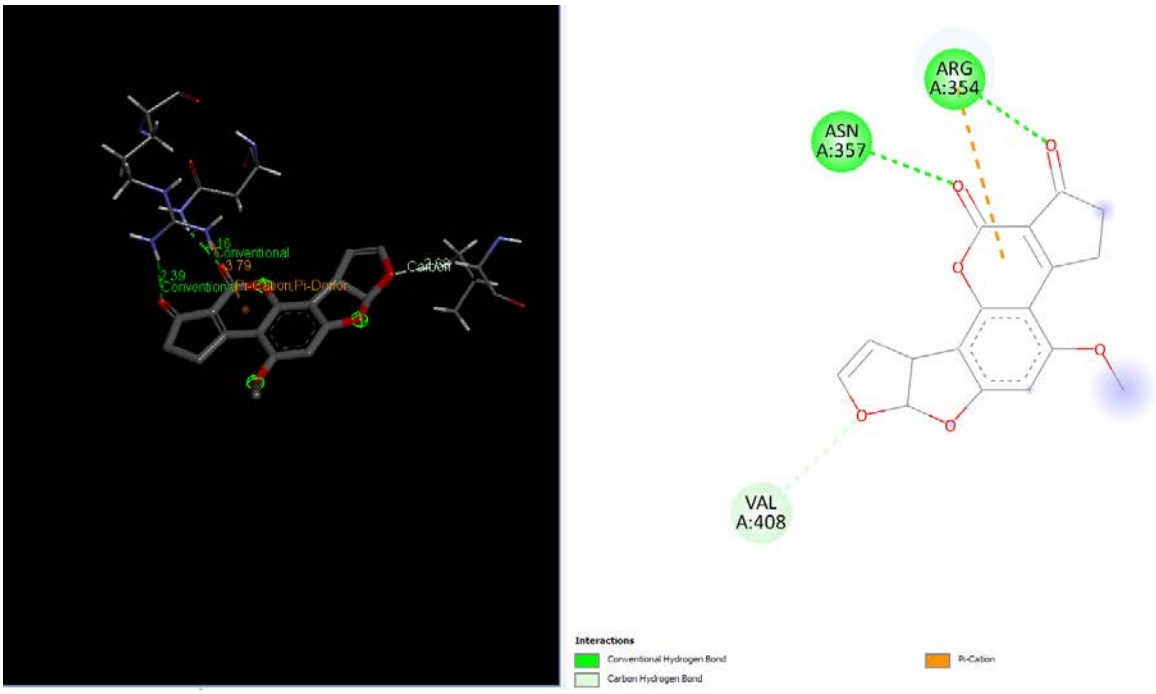


Fig1:-AFB1 interaction with WT Laccase

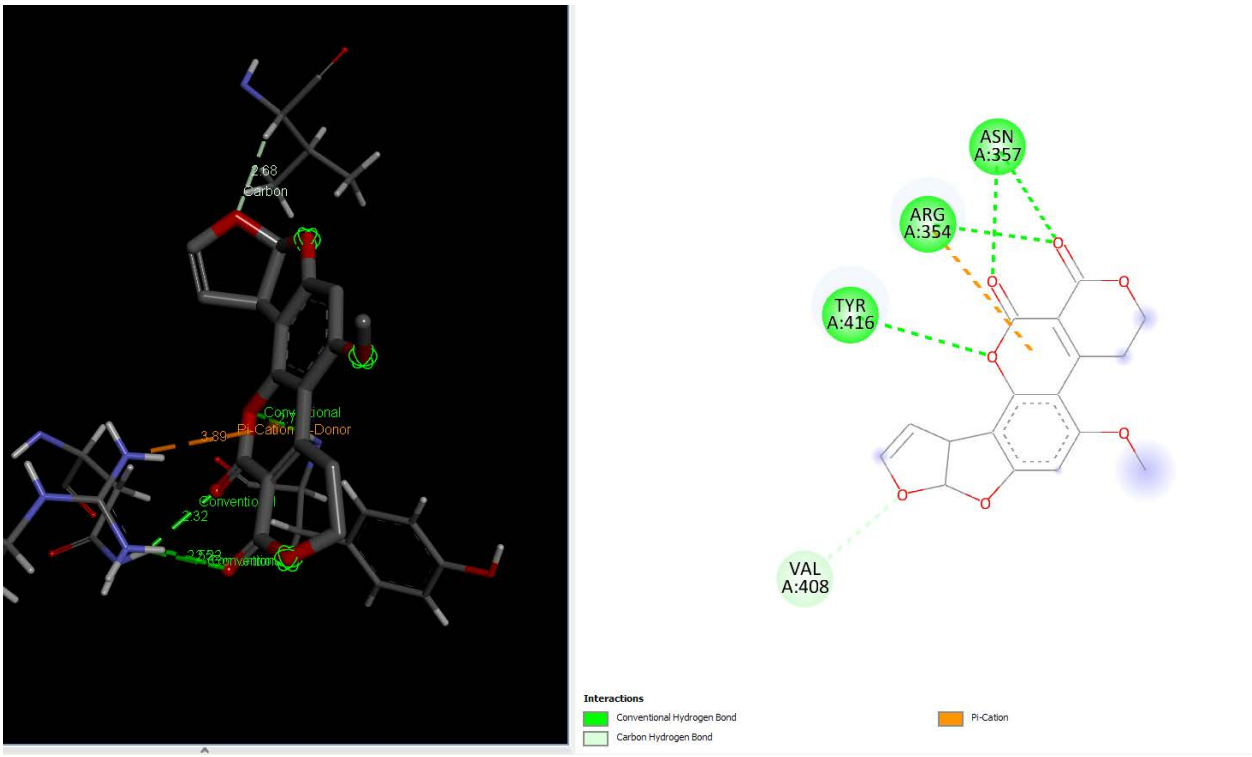


Fig2:-AFG1 interaction with WT laccase

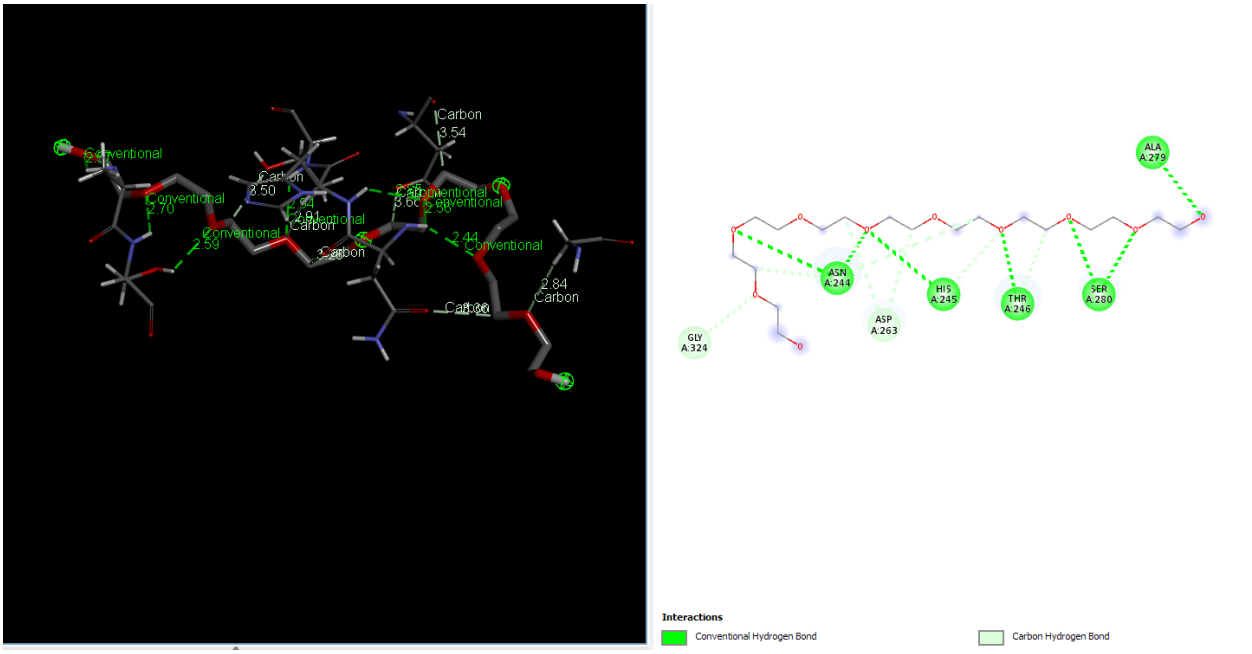


Fig 3:- NE interaction with WT Laccase

Creation of mutants from predictions from HotSpot Wizard and CUPSAT using PyMOL

Total 14 residues were generated from HotSpot wizard of whom point mutations were checked to be stabilizing or destabilizing at stability and functional hotspots. Out of those 14 only 8 of the residues had favorable point mutations and the most stabilizing mutations were introduced in the laccase enzyme (point mutations) and molecular docking was reperformed for each mutant to

check for impact. From CUPSAT the entire list of impact of point mutation on stability for each residue in the structure of laccase provided to the server was checked and top 10 results based on maximum Delta Delta G (DDG) energy were taken to be introduced to laccase and subsequently molecular docking was performed to see the impact of these point mutations on binding energy of laccase to both variants of Aflatoxin. *None of the residues with histidine changes were taken from either servers for mutation due to critical function of histidine in the enzyme structure for interacting with the essential 4 copper ions in the functional enzyme.*

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
1	chain	position	residue	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Lys	Leu	Met	Phe	Pro	Ser	Thr	Val	Trp	Tyr
2	Structural Stability Mutations																						
3																							
4	A	325	Lys	1	0.9	0.1	1.3	2.6	0.4	0.8	1.7	1.2	1	-	0.4	1.1	0.8	11.8	1.2	0.9	1	1.3	0.7
5	A	349	Ser	0.4	-0.3	-0.5	-0.6	1.8	-0.5	-0.2	2.2	-0.3	-0.7	-0.3	-0.6	-0.3	-1.2	12.6	-	-0.3	-0.9	-0.1	-1.2
6	A	126	Arg	0.2	-	0.6	0.9	1.5	-1	-0.3	-	0.1	-2.3	-1	-0.4	-0.7	14.8	-	0.1	-0.5	-1.9	1.6	-0.2
7	A	29	Asp	1	0.1	0	-	2.2	0.1	0.3	3.5	0.4	-	0.1	-0.2	0.4	-0.2	9.2	1.3	0.2	0.2	-	-0.3
8	A	350	Gly	4.1	2.8	0.9	1.7	8.4	2.7	3.6	-	4	8.9	2.8	5.3	3.5	3.4	107.9	3.2	12.9	8.7	-	2.8
9																							
10	Functional mutation stabilities																						
11																							
12	A	143	Ala	-	-	13	15	5.9	18	-	1.4	-	-	-	34.5	67.9	690.2	-	0.8	4.5	19.3	-	-
13	A	179	Ser	0.4	-0.5	2	2.1	1.6	1.3	2.1	1.1	4.9	-	0.7	1	1.4	6.5	1.4	-	-0.7	2.7	-	-
14	A	181	Asp	-0.5	-0.1	-1.4	-	1.3	-2.6	-0.3	0.1	-2.1	-	-0.1	-1.4	-1.4	-2.5	-	-0.7	-0.8	-0.6	-3.9	-1.6
15	A	182	Glu	0.1	0.5	0.1	0.1	3	-0.3	-	0.8	0	-0.3	0.2	-0.3	0.1	-1.7	-	0.5	-0.4	0.1	-	-1.2
16	A	184	Val	1.4	6.8	2.9	3.4	3.8	3.3	3.4	3	2.5	1.6	5.5	2.8	7	11.5	-	2	0.7	-	1.8	20.6
17	A	42	Glu	-0.9	1.3	0.9	1.8	1.5	-0.3	-	-	-	2	0.4	-0.6	-0.9	1.2	-	0.2	-0.4	2.6	-0.7	-0.7
18	A	554	Lys	-0.9	0.3	-1.2	-0.3	-0.1	-2.9	-0.2	-	2.5	-	-	0.3	-2.1	282.9	-	-1.2	-0.8	2.8	-	13.3
19	A	555	Ser	0.4	0.6	-0.9	0.7	2.2	-0.8	0.9	1.6	-1.3	-0.6	-0.8	-1.4	0	-2.5	-	-	-0.4	0.3	-	-1.8
20	A	354	Arg	0.4	-	0	1.7	2.3	0.2	1.1	1.6	-0.3	3	-0.1	0	0.2	-1.6	59.9	1	1.2	1.3	-2.2	-1.4
21																							

HotSpot Wizard mutation predictions

Data Analysis and sorting to reveal most beneficial mutants based on molecular docking results

All binding energy results obtained from docking the 20 point mutation mutants of laccase we tested were placed in a common excel file and sorted to reveal the mutants with highest binding energy for each of the toxins. Top 4 results were filtered out and when compared with docking results of the Wild-type laccase enzyme to reveal that mutation at 480 Met position to Val yielded significant bump in binding energy of AFG1, mutation at 182 Glu position to Tyr yielded significant bump in binding energy of AFG1, mutation at 181 Asp position to Trp yielded significant bump in binding energy of AFB1, and mutation at 106 Asp position to Lys yielded significant bump in binding energy of AFG1. Maximum bumps in binding energy were observed in 106_asp-lys mutation for AFG1 and 181_asp-trp mutation for AFB1.

Ligand	Binding Affinity (kcal/mol)	rmsd/ub	rmsd/lb
6f5k_laccase_clean_14421_uff_E=504.28	-8.3	0	0
6f5k_laccase_clean_186907_uff_E=919.33	-7.8	0	0
6f5k_laccase_clean_4867_uff_E=569.17	-4.6	0	0
126_arg_val_mutated_14421_uff_E=504.28	-8.3	0	0
126_arg_val_mutated_186907_uff_E=919.33	-8	0	0
126_ala_ile_laacase_14421_uff_E=504.28	-8.3	0	0
126_ala_ile_laacase_186907_uff_E=919.33	-8.1	0	0
182_Glu-Tyr_laccase_14421_uff_E=504.28	-8.9	0	0
182_Glu-Tyr_laccase_186907_uff_E=919.33	-8.1	0	0
182_Glu-Phe_laccase_14421_uff_E=504.28	-8.1	0	0
182_Glu-Phe_laccase_186907_uff_E=919.33	-8.1	0	0
181_Asp-Trp_laccase_14421_uff_E=504.28	-8.5	0	0
181_Asp-Trp_laccase_186907_uff_E=919.33	-9.1	0	0
181_asp-gln_laccase_14421_uff_E=504.28	-8.3	0	0
181_asp-gln_laccase_186907_uff_E=919.33	-8.2	0	0
106_asp-lys_laccase_14421_uff_E=504.28	-9	0	0
106_asp-lys_laccase_186907_uff_E=919.33	-8.1	0	0
179_ser_thr_laccase_14421_uff_E=504.28	-7.9	0	0
179_ser_thr_laccase_186907_uff_E=919.33	-8.2	0	0
432_pro-trp_laccase_14421_uff_E=504.28	-8.3	0	0
432_pro-trp_laccase_186907_uff_E=919.33	-8.2	0	0
510_ser_pro_laccase_14421_uff_E=504.28	-8.3	0	0
510_ser_pro_laccase_186907_uff_E=919.33	-8.2	0	0
429_leu_trp_laccase_14421_uff_E=504.28	-8.3	0	0
429_leu_trp_laccase_186907_uff_E=919.33	-8.1	0	0

Ligand	Binding Affinity (kcal/mol)	rmsd/ub	rmsd/lb
42_glu-met_laccase_14421_uff_E=504.28	-8.3	0	0
42_glu-met_laccase_186907_uff_E=919.33	-8.2	0	0
42_glu-tyr_laccase_14421_uff_E=504.28	-8.3	0	0
42_glu-tyr_laccase_186907_uff_E=919.33	-8.2	0	0
555_ser-phe_laccase_14421_uff_E=504.28	-8.3	0	0
555_ser-phe_laccase_186907_uff_E=919.33	-8.2	0	0
554_lys-met_laccase_14421_uff_E=504.28	-8.3	0	0
554_lys-met_laccase_186907_uff_E=919.33	-8.2	0	0
349_ser_phe_laacase_14421_uff_E=504.28	-8.2	0	0
349_ser_phe_laccase_186907_uff_E=919.33	-8.1	0	0
479_thr_cys_laccase_14421_uff_E=504.28	-8.3	0	0
479_thr_cys_laccase_186907_uff_E=919.33	-8.1	0	0
505_ile_asp_laccase_14421_uff_E=504.28	-8.3	0	0
505_ile_asp_laccase_186907_uff_E=919.33	-8.2	0	0
42_glu-met_laccase_14421_uff_E=504.28	-8.3	0	0
42_glu-met_laccase_186907_uff_E=919.33	-8.2	0	0
354_arg-lys_laccase_14421_uff_E=504.28	-7.7	0	0
354_arg-lys_laccase_186907_uff_E=919.33	-8	0	0
354_arg-trp_laccase_14421_uff_E=504.28	-7.7	0	0
354_arg-trp_laccase_186907_uff_E=919.33	-8	0	0

All mutations tested

Ligand	Binding Affinity (kcal/mol)	rmsd/ub	rmsd/lb
6f5k_laccase_clean_14421_uff_E=504.28	-8.3	0	0
6f5k_laccase_clean_186907_uff_E=919.33	-7.8	0	0
6f5k_laccase_clean_4867_uff_E=569.17	-4.6	0	0
480_met_val_laccase_14421_uff_E=504.28	-8.9	0	0
480_met_val_laccase_186907_uff_E=919.33	-8.1	0	0
182_Glu-Tyr_laccase_14421_uff_E=504.28	-8.9	0	0
182_Glu-Tyr_laccase_186907_uff_E=919.33	-8.1	0	0
181_Asp-Trp_laccase_14421_uff_E=504.28	-8.5	0	0
181_Asp-Trp_laccase_186907_uff_E=919.33	-9.1	0	0
106_ASP-LYS_laccase_14421_uff_E=504.28	-9	0	0
106_ASP-LYS_laccase_186907_uff_E=919.33	-8.1	0	0
Top-4_mutations_combined_14421_uff_E=504.28	-8.5	0	0
Top-4_mutations_combined_186907_uff_E=919.33	-8.1	0	0

Top 4 mutations

(14421 → represents AFG1; 186907 → represents AFB1; 4867 → represents NE)

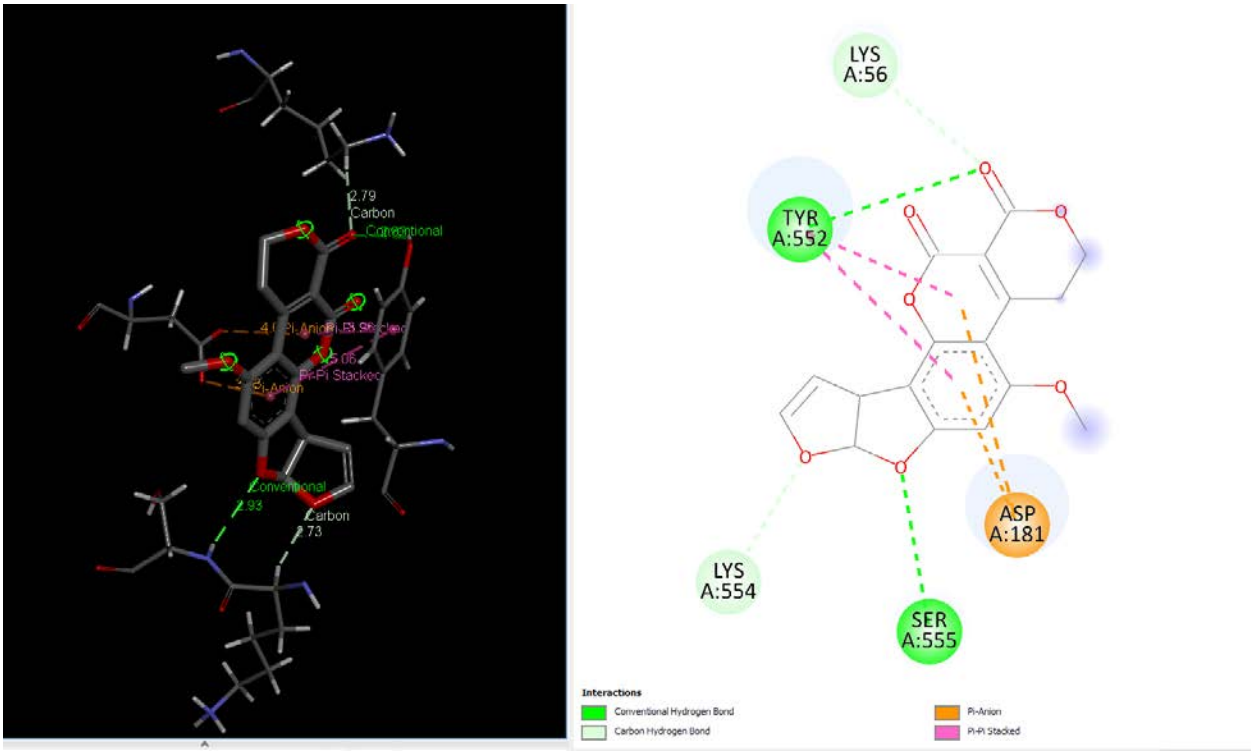


Fig 4:-AFG1 interaction with 106_asp-lys mutant laccase

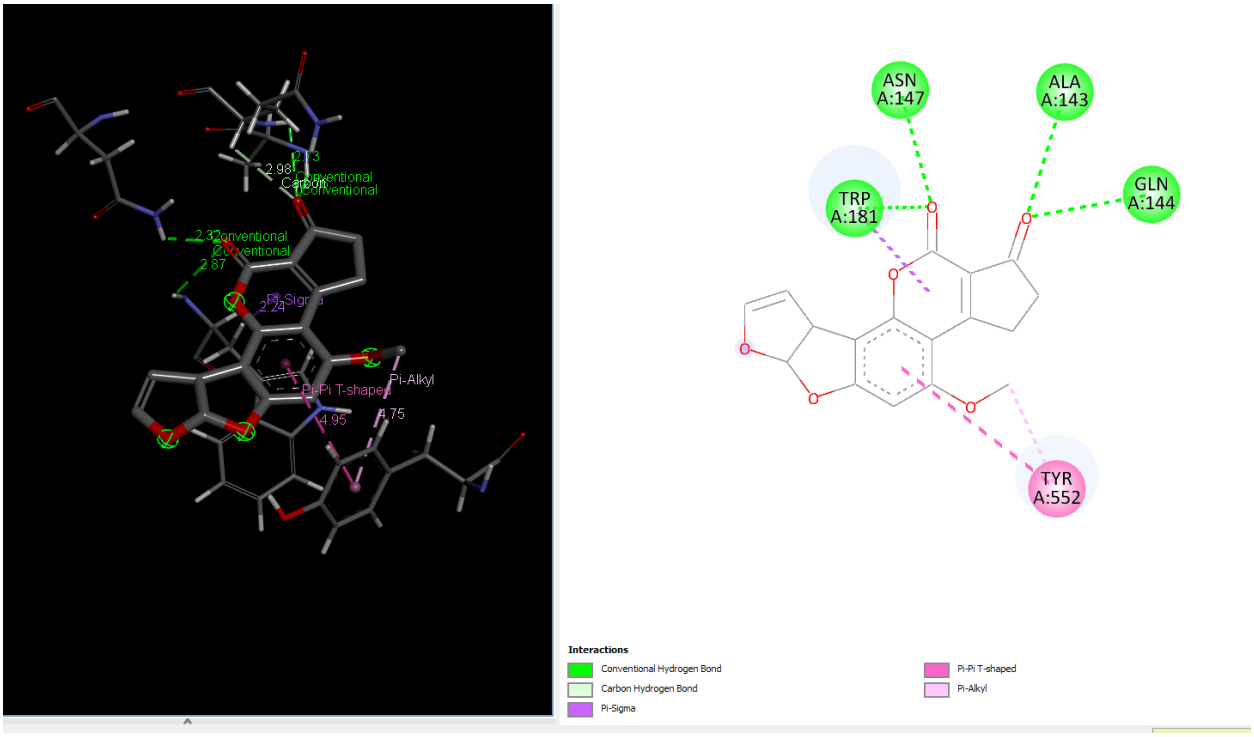


Fig 5:-AFB1 interaction with 181_asp-trp mutant laccase

Multiple mutations in laccase enzyme

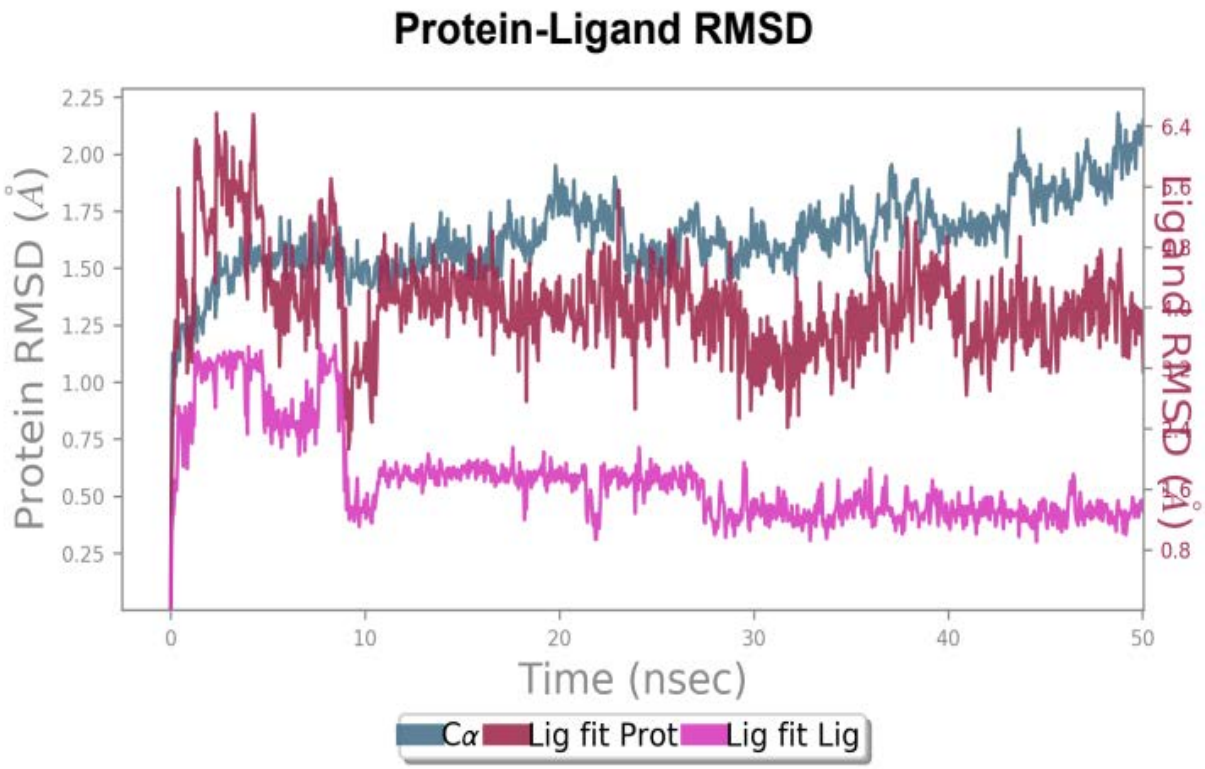
The best 4 results yielding mutations were mutated at the same time in laccase to test the effect cumulative effect of top 4 stabilizing and activity enhancing point mutations, but did not yield good results as the increase in binding energy observed after performing molecular docking of the Top-4_mutations_combined laccase with AFG1 and AFB1, hence multiple mutations were not further considered in this study.

Molecular Dynamics of control and highest binding energy mutants of laccase

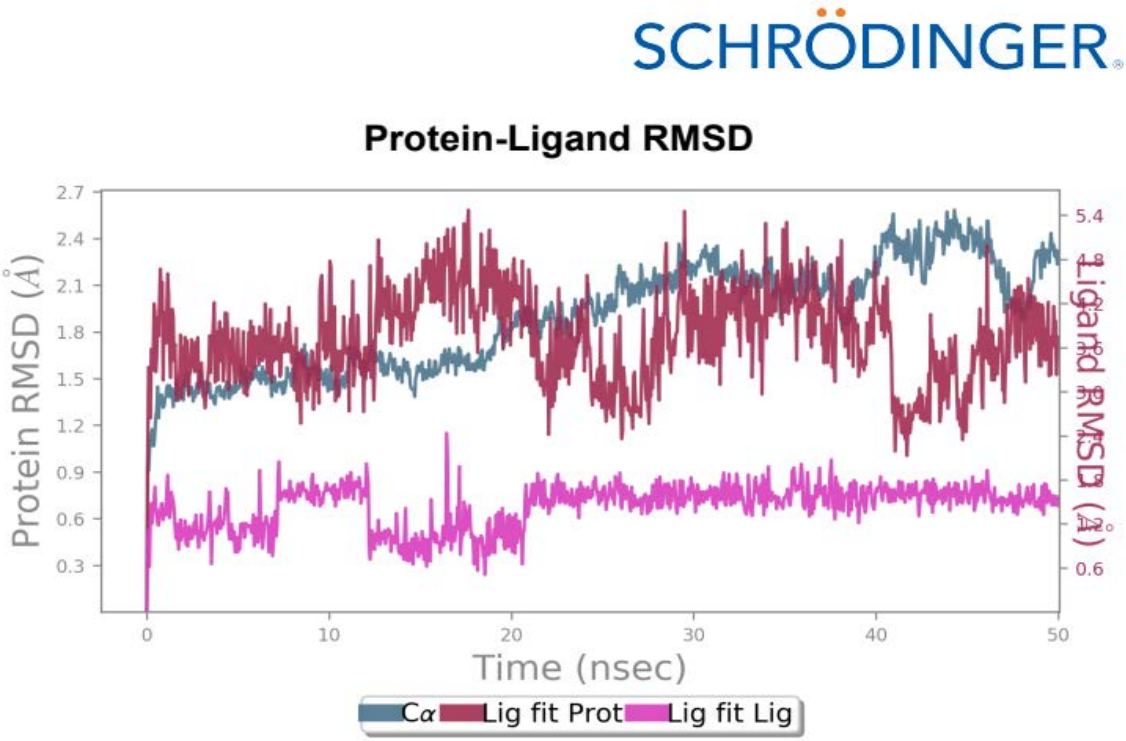
Two of the best mutants i.e. 106_asp-lys for its increased binding energy of AFG1 and 181_asp-trp for its increased binding energy of AFB1 were selected along with control i.e. Wild-type laccase complexes with AFG1 and AFb1 respectively were put through further analysis and validation of binding energy and complex stabilities in a water based system by the means of Molecular dynamics using Schrodinger Desmond and the analysis was run for 50ns.

Interpretation of AFG1 interaction with Wild-type laccase and 106_asp-lys mutant

Protein-Ligand RMSD – Change in RMSD of the Wild-type laccase throughout the 50ns simulation was ~ 1 angstrom whereas change in RMSD of the mutant laccase was ~1.2 angstrom indicating better stability of Wild-type laccase. Maximum RMSD change with value ~ 1.5 angstrom was observed between 0-10ns in the Wild-type laccase whereas Maximum RMSD change with value ~ 1.4 angstrom between 30-40ns of the simulation, indicating slight enhancement in stability of the interaction.

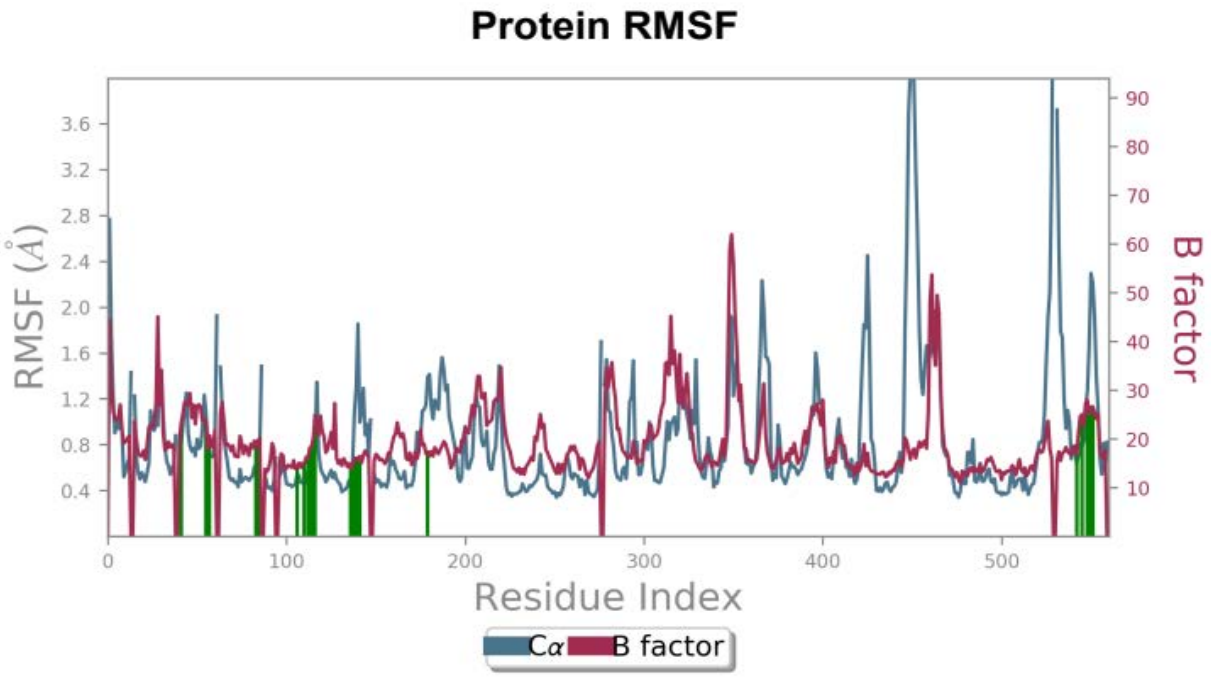


Wild-type Laccase-complex with AFG1

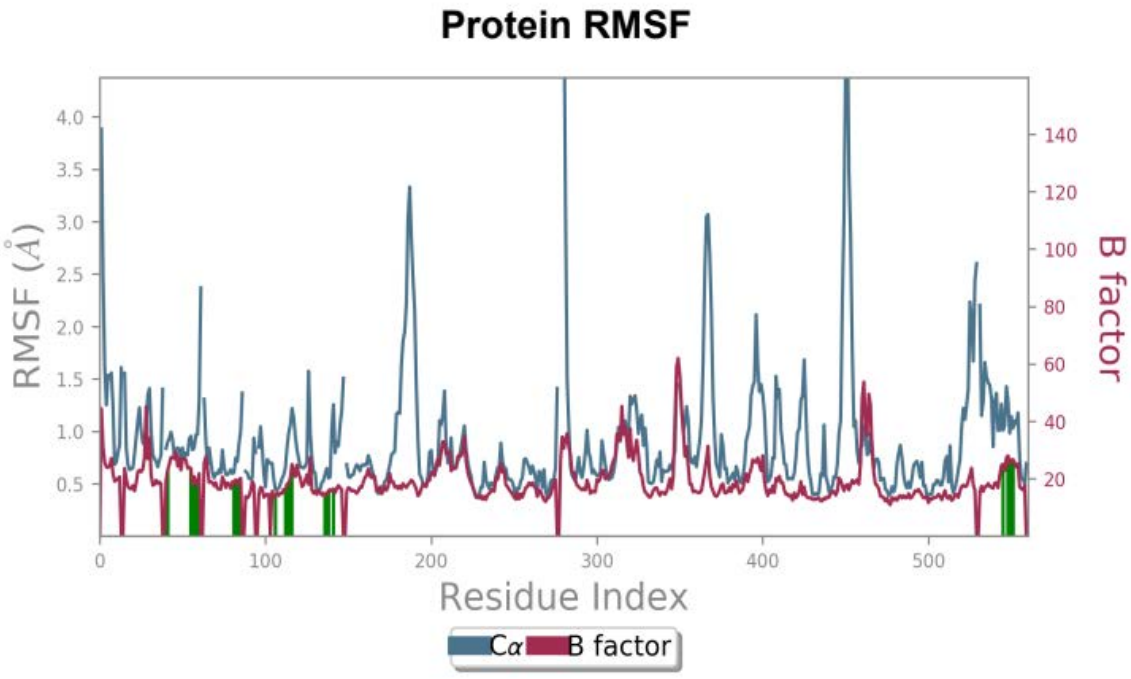


106_asp-lys mutant laccase complex

Protein RMSF – Peaks represent the residues with the highest fluctuations measured in angstroms during the 50ns simulation. Displacement of C-alpha from B-Factor was observed to be greater in the mutant laccase as compared to displacement of C-alpha from B-Factor in Wild-type laccase.



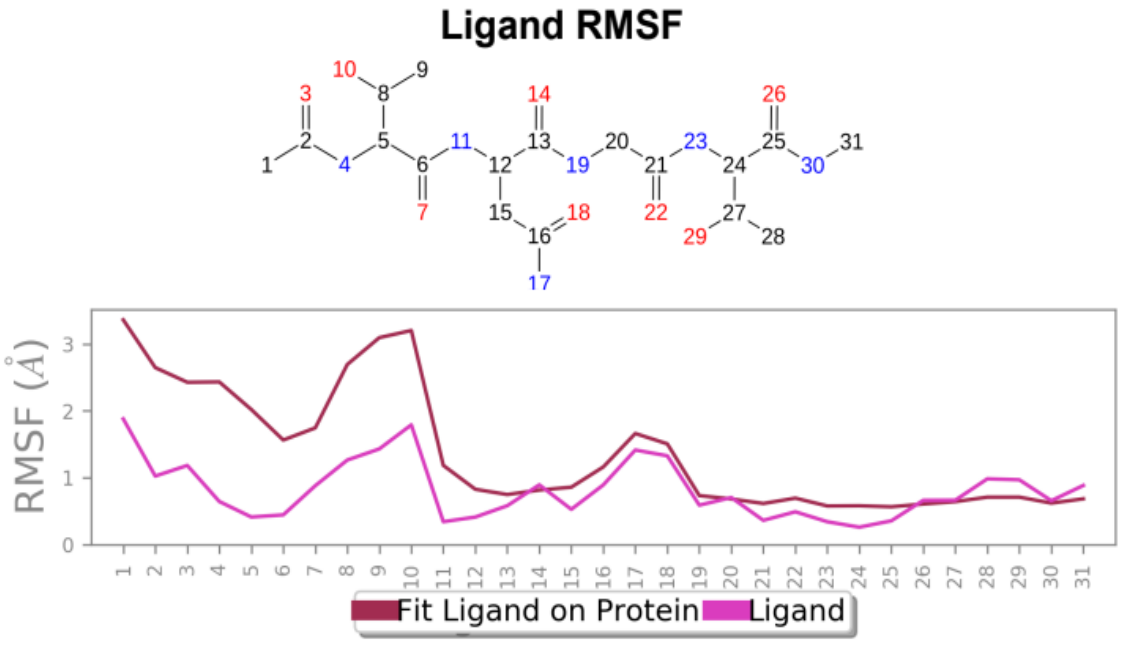
Wild-type laccase



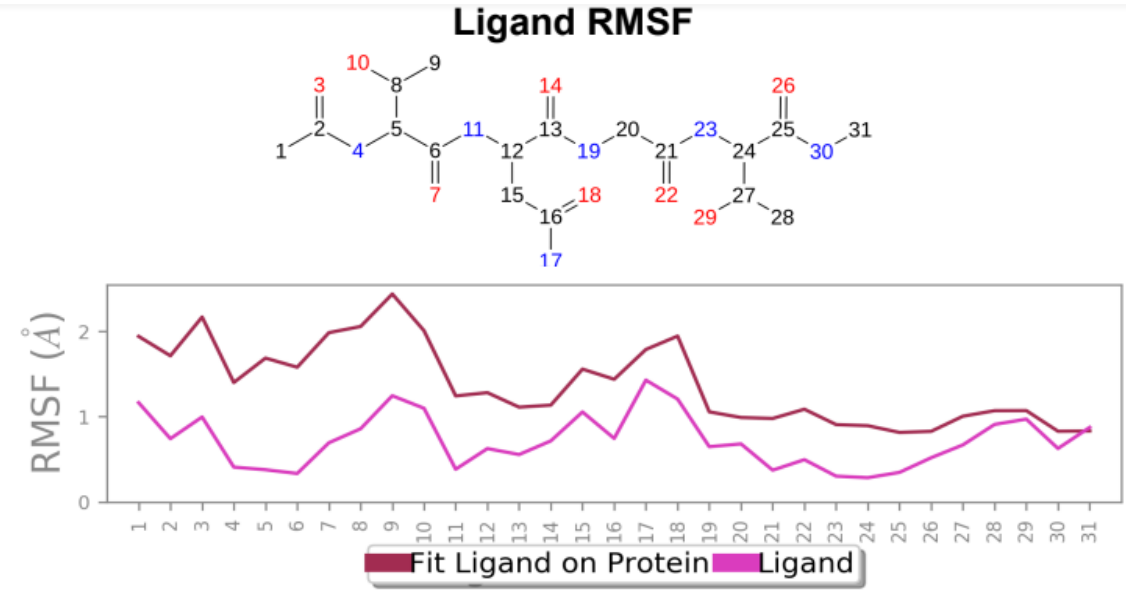
106_asp-lys laccase

Ligand RMSF – Ligand RMSF saw a change in maximum RMSF value ~ 1 angstrom when mutant laccase was compared to wild-type laccase (reduction in maximum RMSF value was present in

mutant) and overall stabilization of fluctuations were present in the ligand bound to mutant laccase when compared to wild-type laccase.



Wild-type laccase

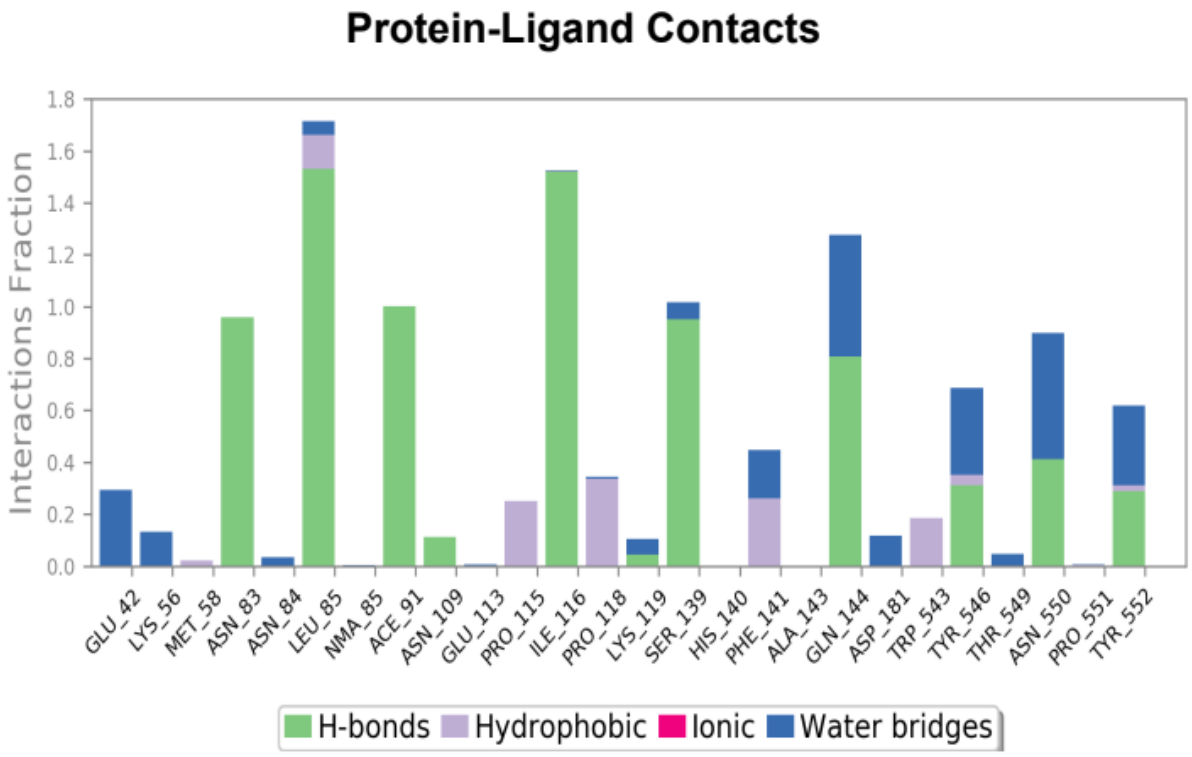


106 asp-lys laccase

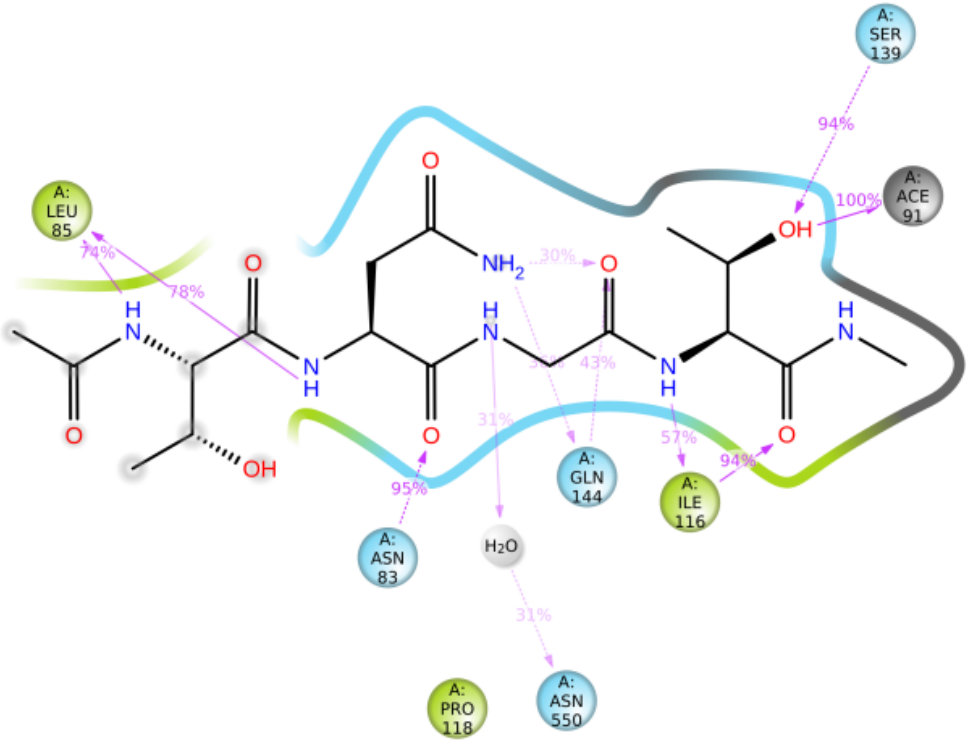
Protein-Ligand Plots and Diagram – It was observed that the wild-type laccase-ligand complex possessed 6 protein-ligand contact residues at which contact was maintained throughout the 50ns simulation of which 3 residues (LEU85, ILE116 & GLN144) possessed more than one type of interactions with the ligand, indicating the residues at which strongest interaction is observed. In the mutant laccase-ligand complex, interactions at 4 residues persisted throughout the entire 50ns

simulation, of which 3 residues (GLU42, ILE116 & TYR546) showed more than a single type of interaction. GLU42 residue in the mutant laccase-complex possessed the highest interaction fraction of the two complexes analyzed with value greater than 2. This indicates very strong interaction at GLU42 residue in the mutant-laccase complex. Intensity of orange color indicates number of contacts of a particular residue with the ligand.

Wild-type laccase P-L contacts and diagrams

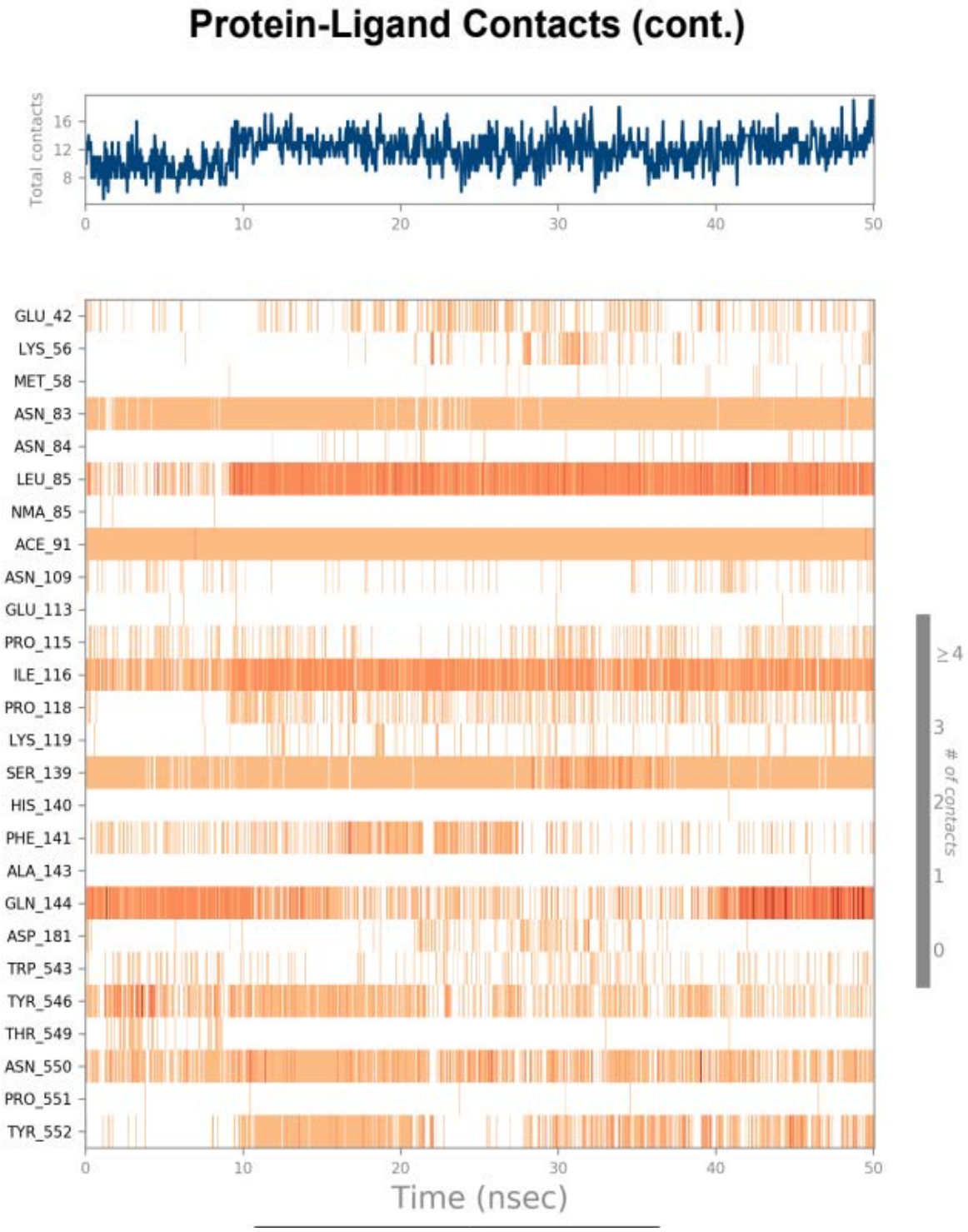


Ligand-Protein Contacts

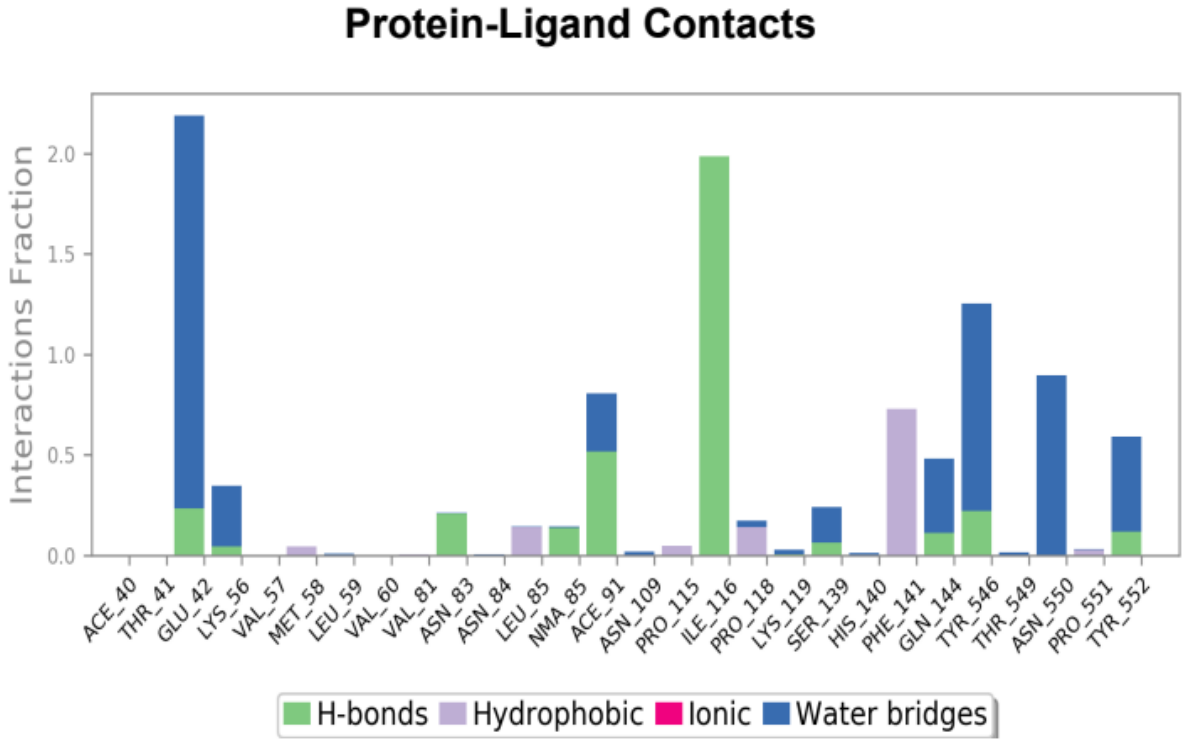


Hydrophobic
Polar
Unspecified residue
Water
Solvent exposure

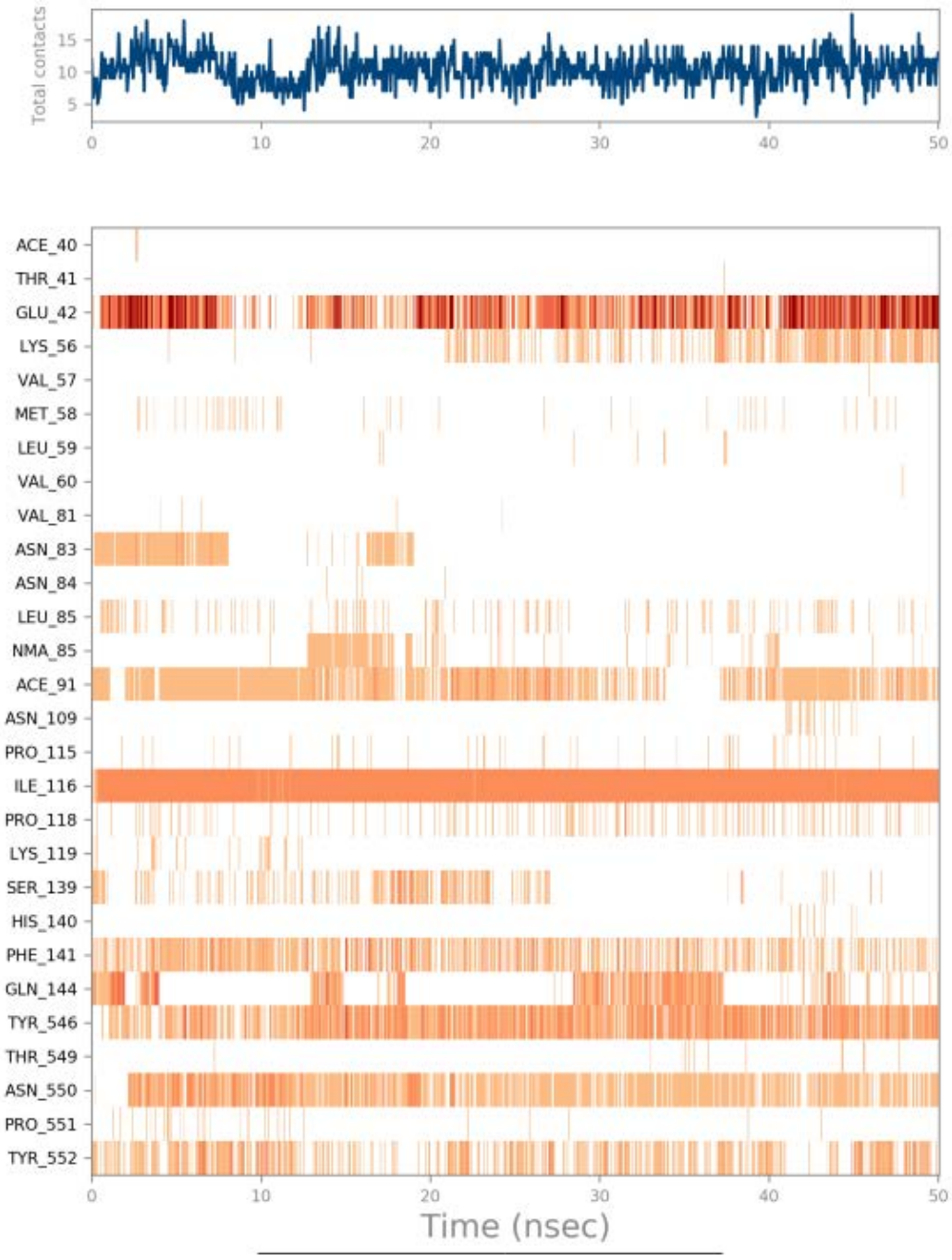
A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than **30.0%** of the simulation time in the selected trajectory (0.00 through 50.05 nsec), are shown.
Note: it is possible to have interactions with >100% as some residues may have multiple interactions of a single type with the same ligand atom. For example, the ARG side chain has four H-bond donors that can all hydrogen-bond to a single H-bond acceptor.

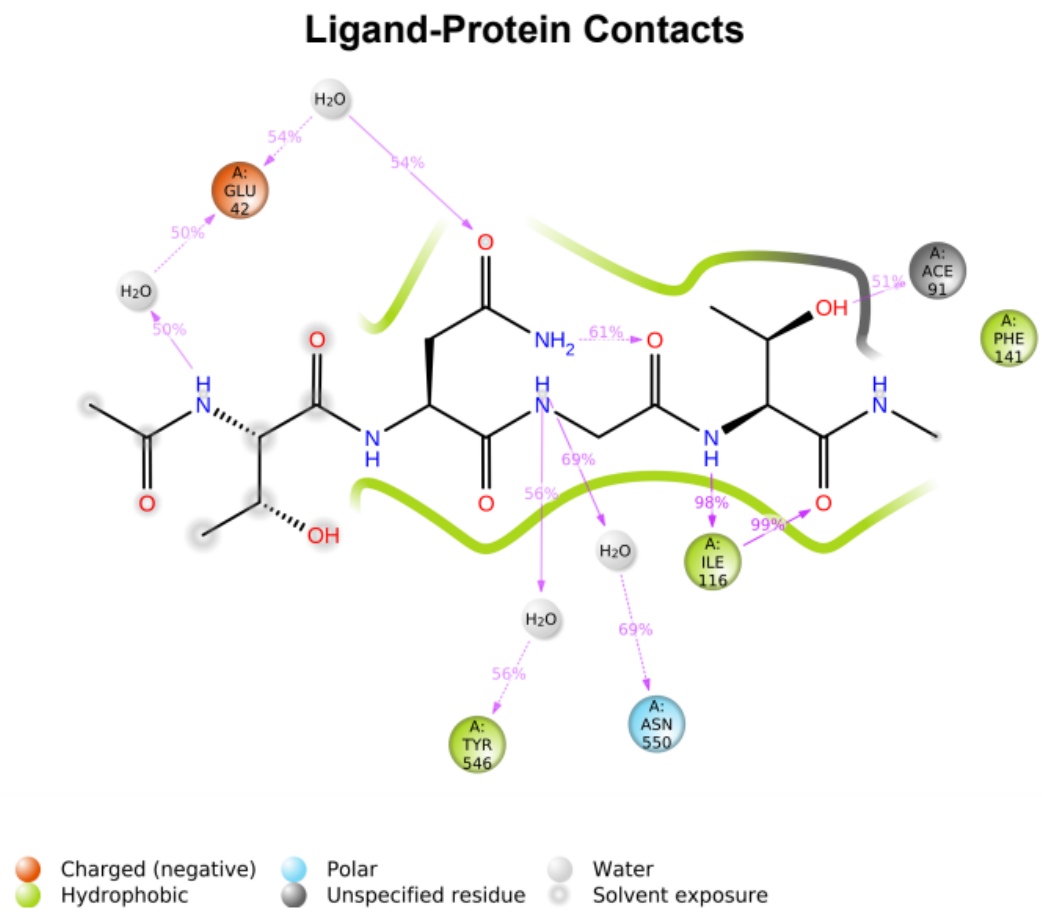


106_asp-lys laccase P-L contacts and Diagram


Protein-Ligand Contacts
Interactions Fraction
2.0
1.5
1.0
0.5
0.0
ACE_40
THR_41
GLU_42
LYS_56
VAL_57
MET_58
LEU_59
VAL_60
VAL_81
ASN_83
ASN_84
LEU_85
NMA_85
ACE_91
ASN_109
PRO_115
ILE_116
PRO_118
LYS_119
SER_139
HIS_140
PHE_141
GLN_144
TYR_546
THR_549
ASN_550
PRO_551
TYR_552
H-bonds
Hydrophobic
Ionic
Water bridges

Protein-Ligand Contacts (cont.)

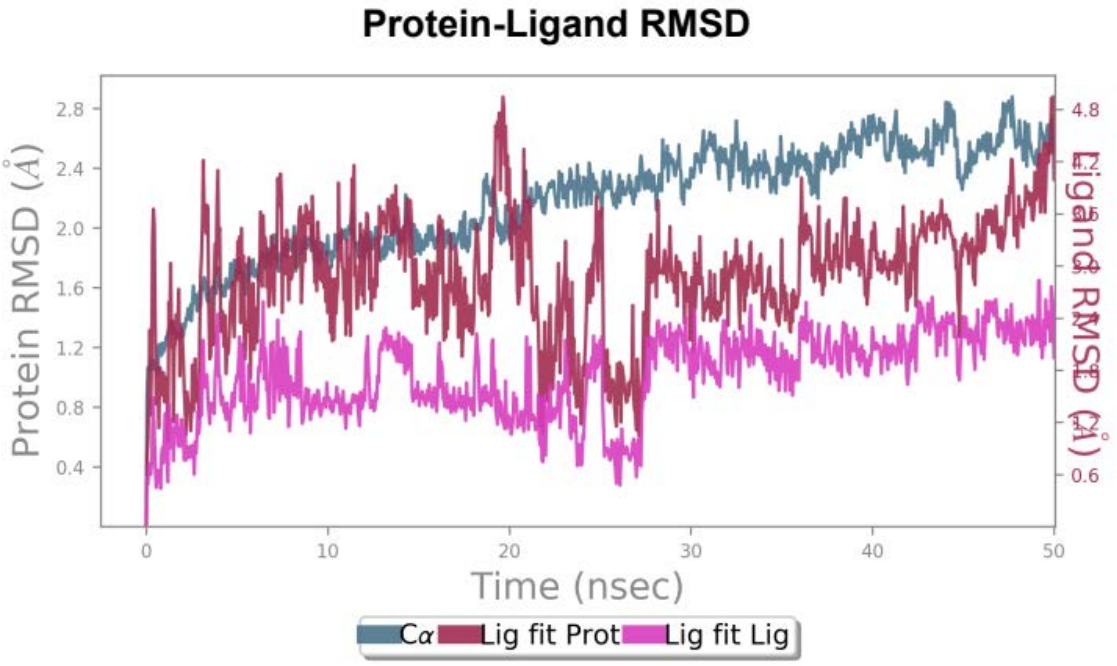




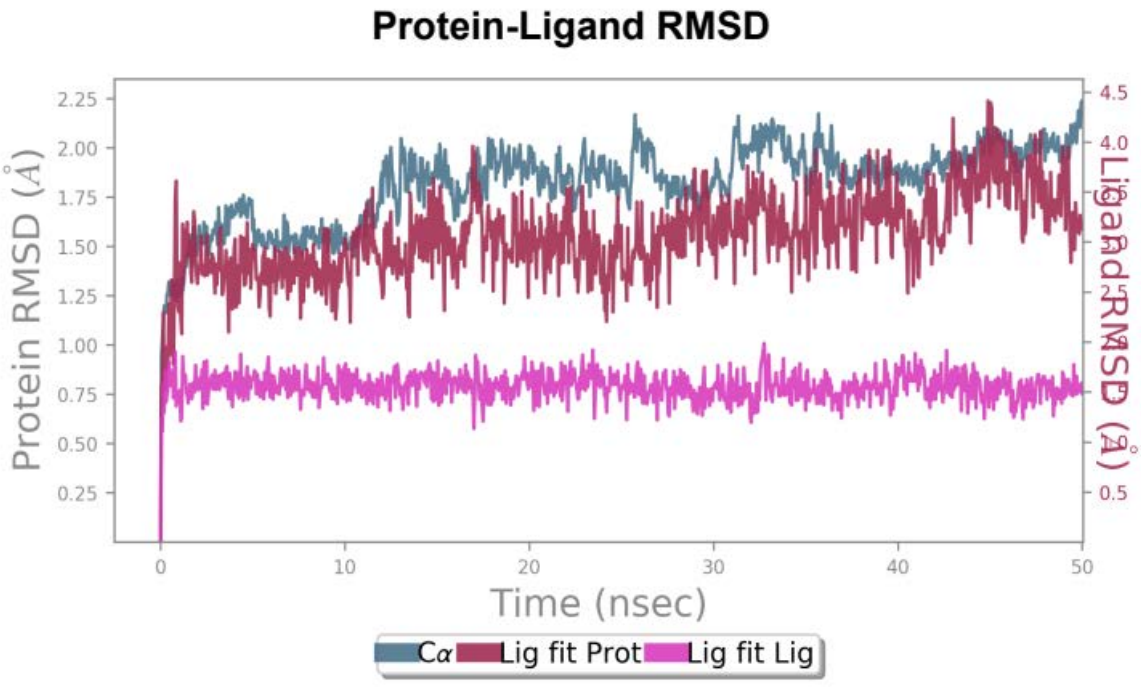
A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than **30.0%** of the simulation time in the selected trajectory (0.00 through 50.05 nsec), are shown.
Note: it is possible to have interactions with >100% as some residues may have multiple interactions of a single type with the same ligand atom. For example, the ARG side chain has four H-bond donors that can all hydrogen-bond to a single H-bond acceptor.

Interpretation of AFB1 interactions with Wild-type laccase and 181_asp-trp mutant

Protein-ligand RMSD - Change in RMSD value of Wild-type laccase throughout the 50ns simulation was nearly ~ 1.6 angstrom, whereas the mutant laccase RMSD change throughout the 50ns simulation was 1 angstrom indicating enhancement in stability of protein structure by point mutation introduced. General fluctuations saw a downward trend in the mutated laccase-AFB1 complex indicating enhanced ability of the ligand to stay in the binding pocket. Net change in ligand RMSD saw a reduction of ~ 0.3 angstrom, indicating enhanced ability to stay bound in the binding pocket of the ligand.



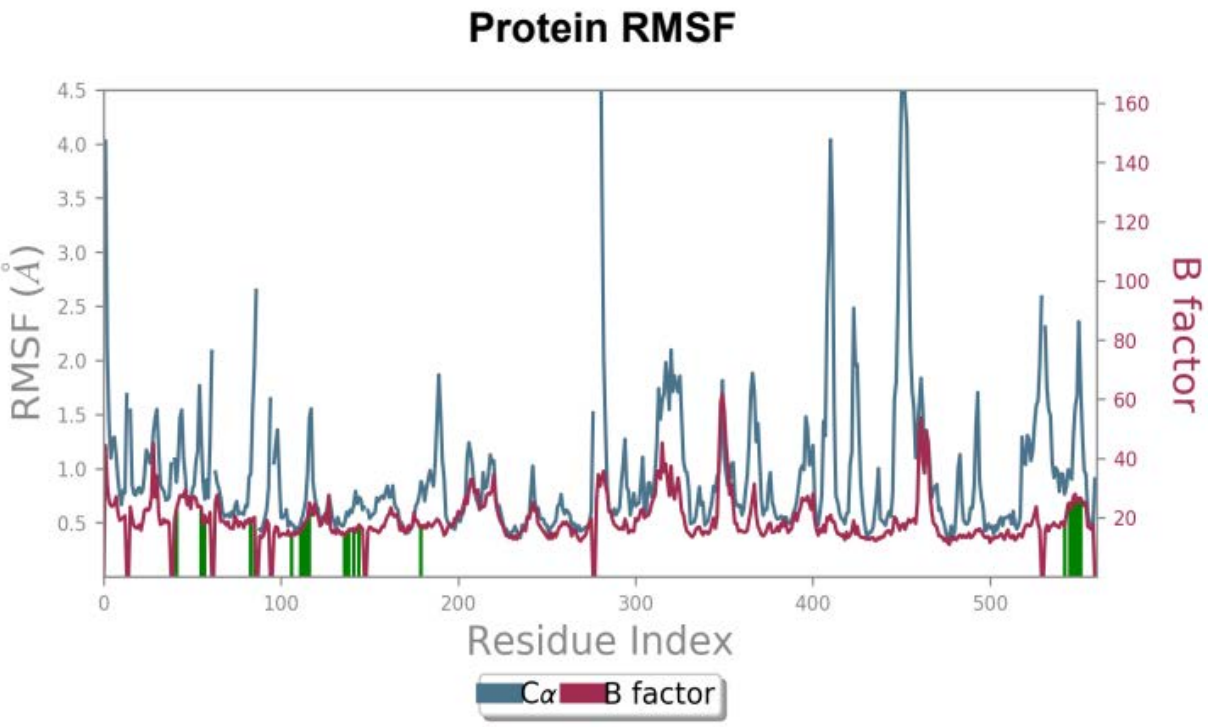
Wild-Type laccase



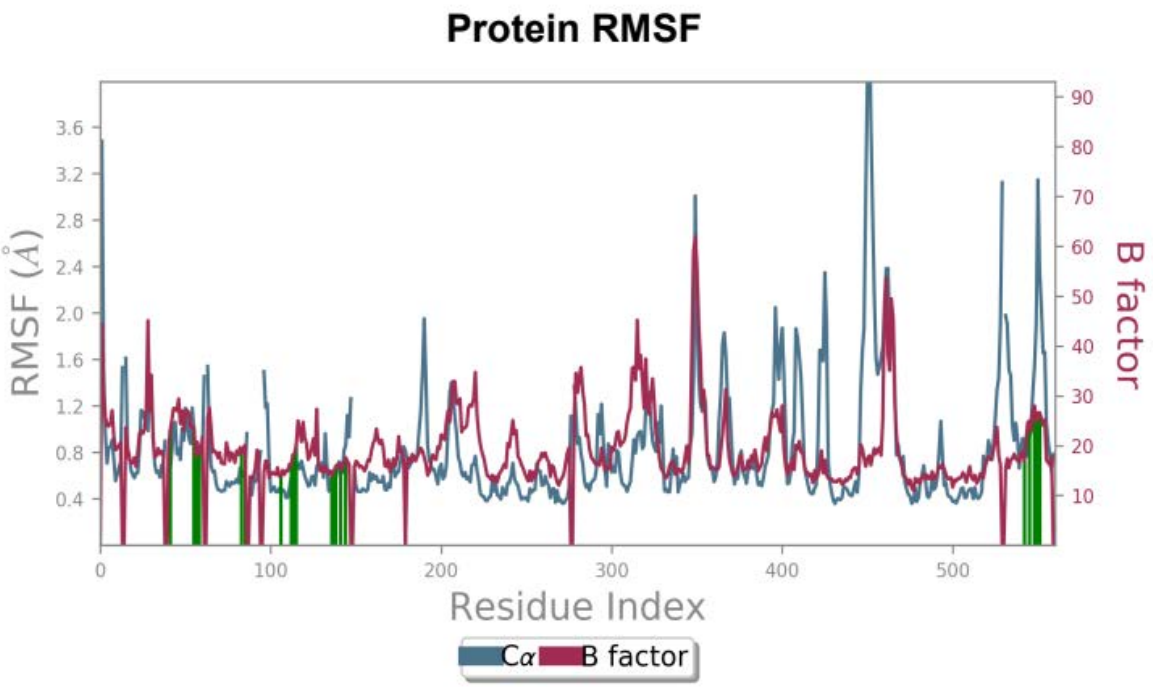
181_asp-trp mutant laccase

Protein RMSF - Peaks represent the residues with the highest fluctuations measured in angstroms during the 50ns simulation. Displacement from C-alpha of the B factor is observed to be less in the mutant

laccase when compared to the Wild-type laccase complex. Maximum fluctuation also saw a dip from 4.5 angstrom to 3.6 angstrom when comparing Wild-type laccase to mutant laccase, indicating enhanced stability of the protein structure.

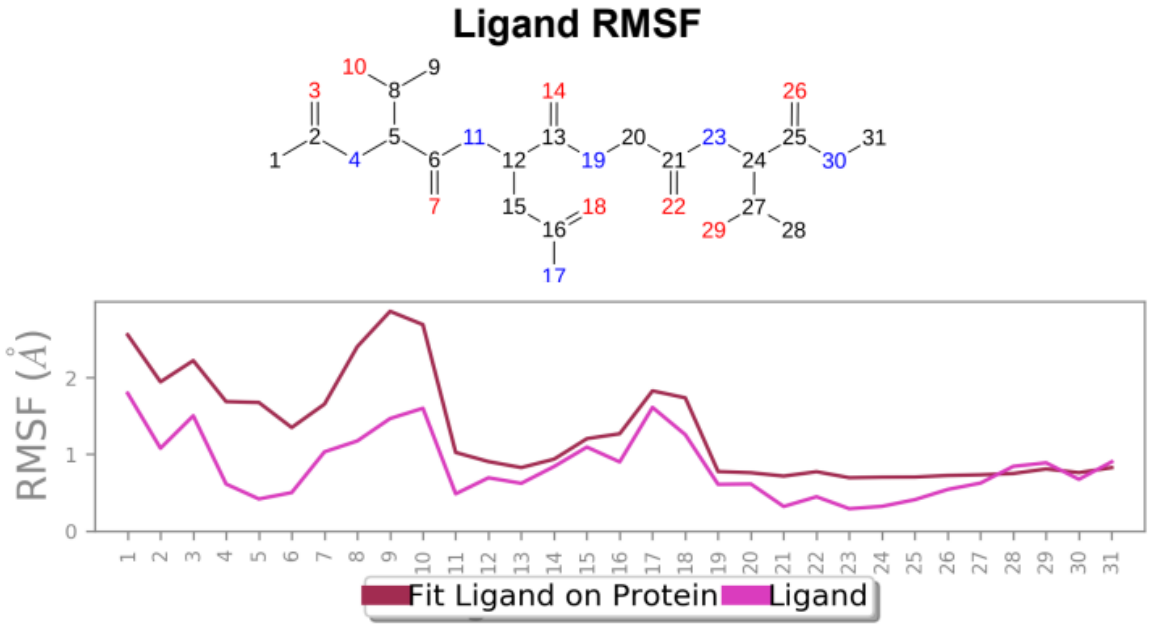


Wild-type laccase

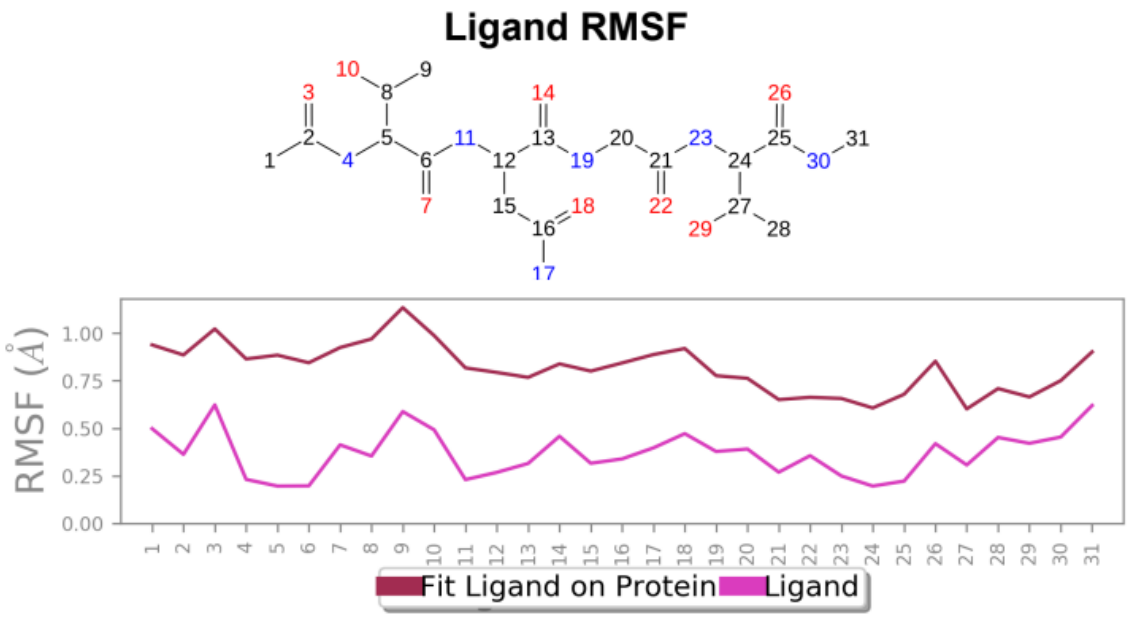


181_asp-trp mutant laccase

Ligand RMSF - Maximum ligand RMSF values saw a reduction of 1 angstrom in the mutant laccase variant, and total fluctuations across the ligand at all atomic positions experienced a reduction in RMSF values.



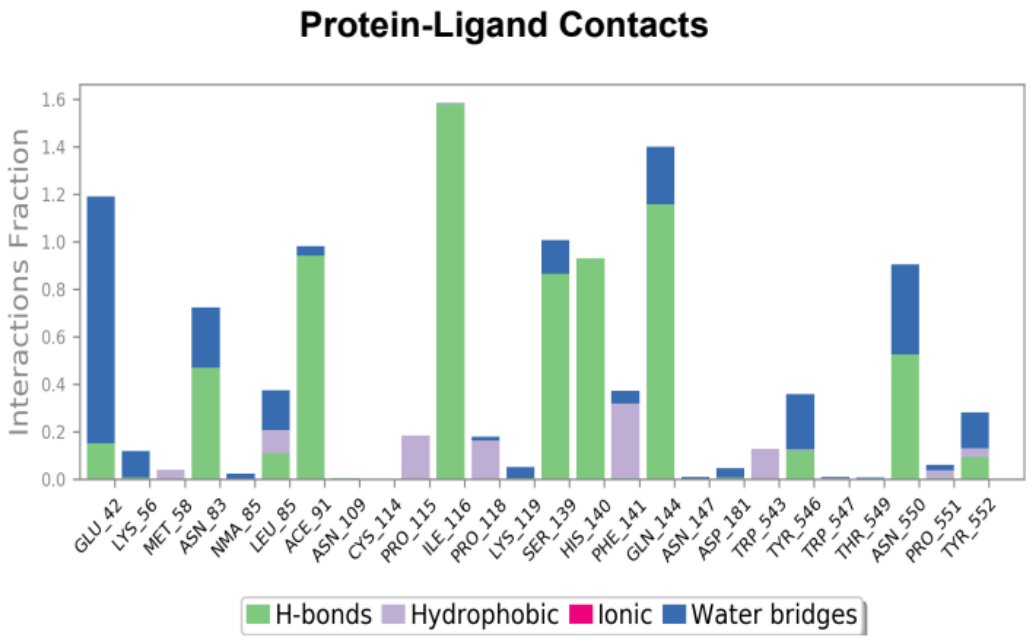
Wild-type laccase



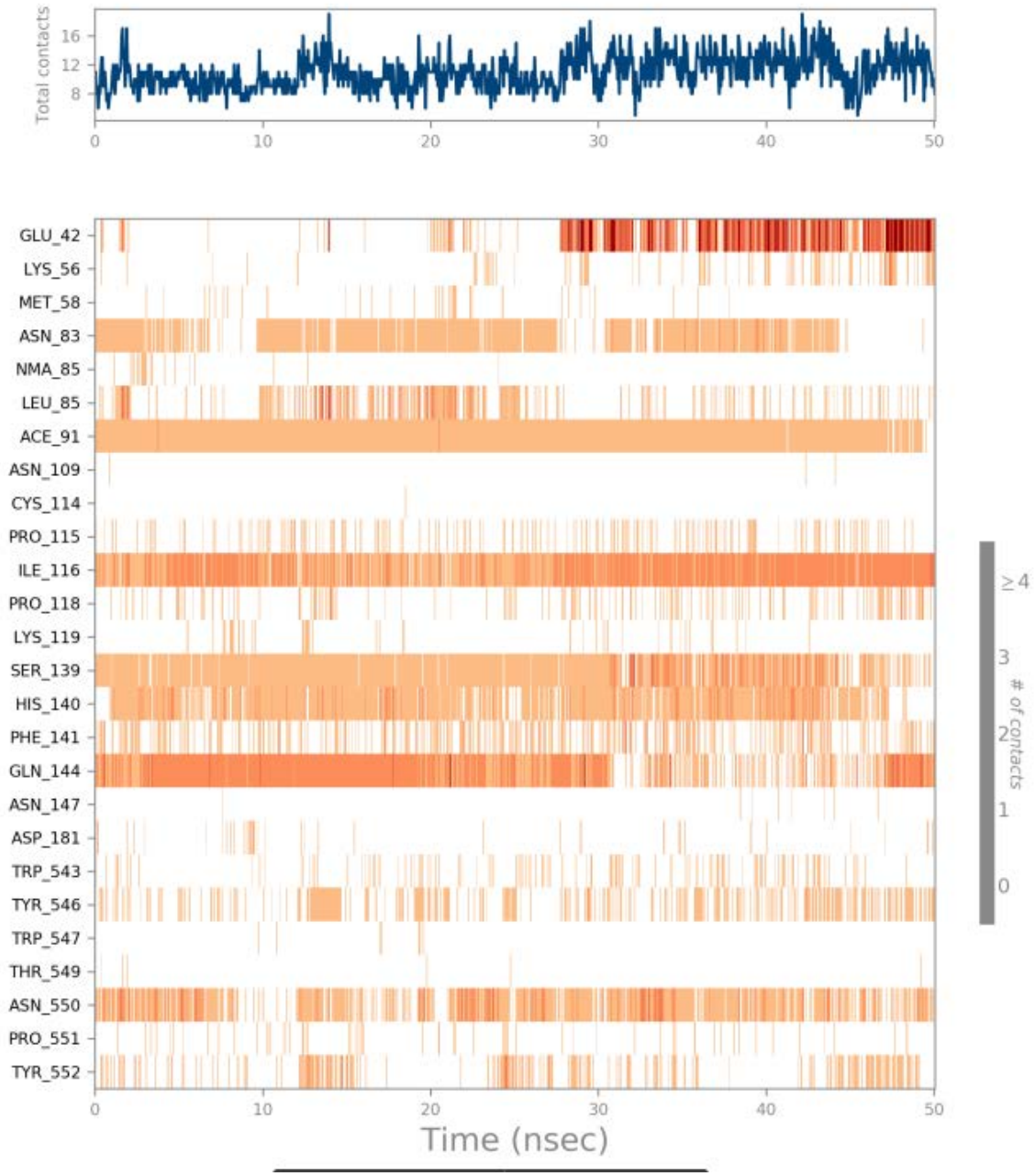
181_asp-trp mutant

Protein-Ligand contact plots and diagrams - It was observed that in the wild-type laccase ligand complex, there are 6 residues which remained in contact with the ligand throughout the 50ns simulation of which 3 residues (GLU42, ILE116 & GLN144) interacting with the ligand in multiple subtypes of interaction. Whereas in the mutant-laccase ligand complex, there are 5 residues which remained in contact with ligand throughout the 50ns simulation, interestingly the same 3 residues (GLU42, ILE116 & GLN144) are the ones interacting with the ligand in more than one type of interaction and hence strengthening the interaction of ligand with the protein. GLU42 residue presents a very strong interaction with ligand, and could be the reason for better binding energy observed in the mutant laccase. Intensity of orange colour indicates number of contacts of a particular residue with the ligand.

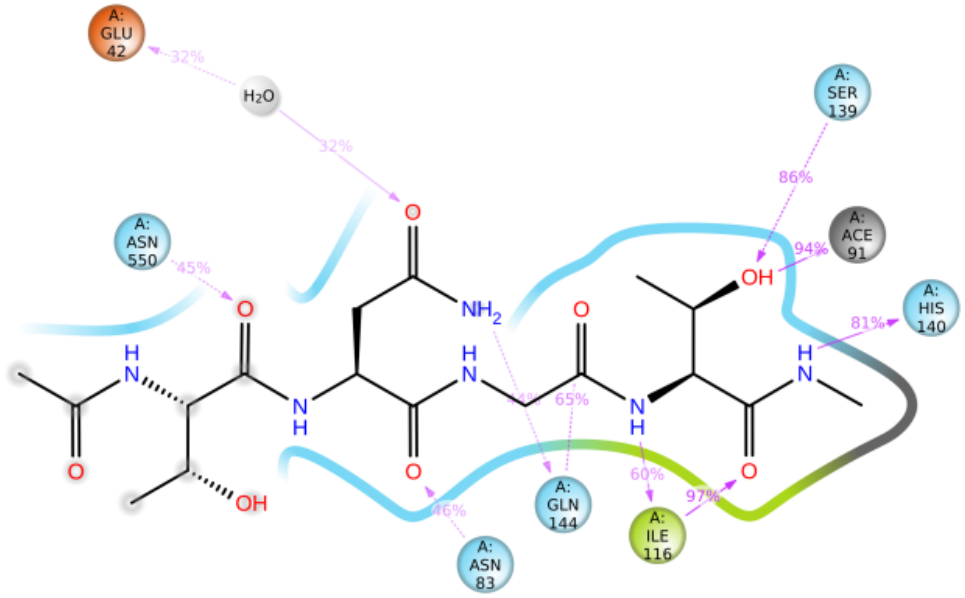
Wild-type laccase



Protein-Ligand Contacts (cont.)



Ligand-Protein Contacts



Charged (negative)
Hydrophobic
Polar
Unspecified residue
Water
Solvent exposure

A schematic of detailed ligand atom interactions with the protein residues. Interactions that occur more than **30.0%** of the simulation time in the selected trajectory (0.00 through 50.05 nsec), are shown.
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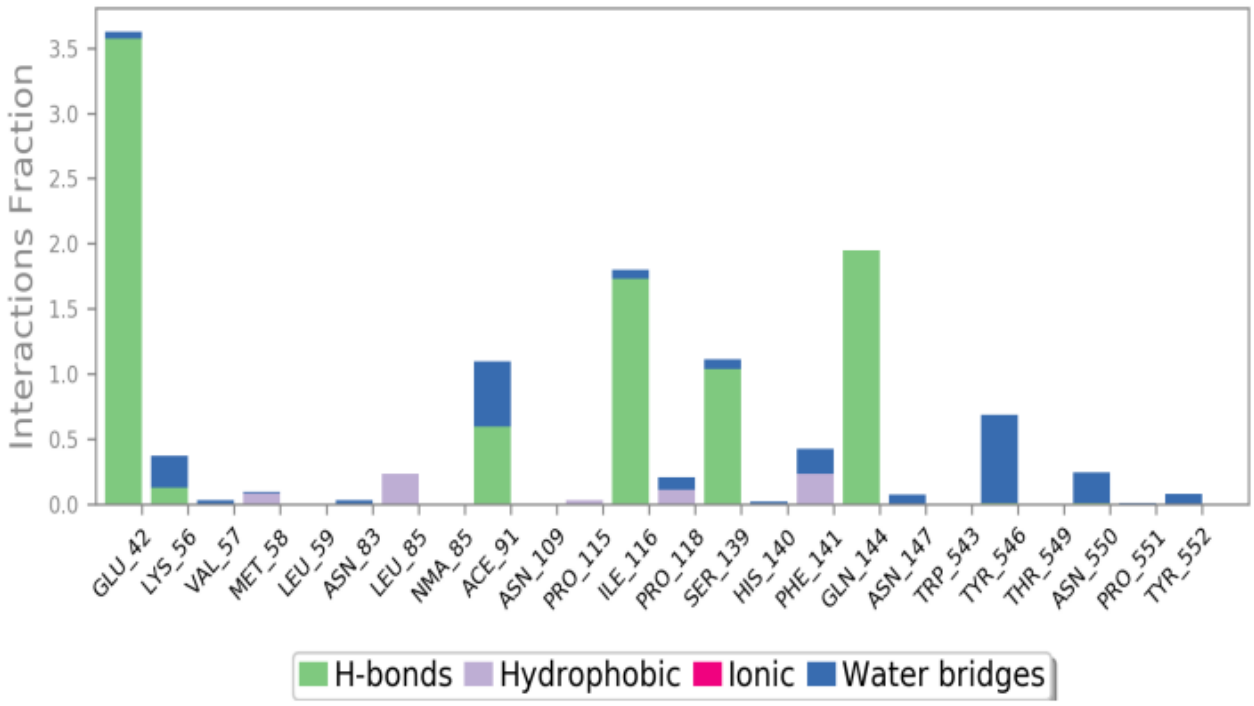
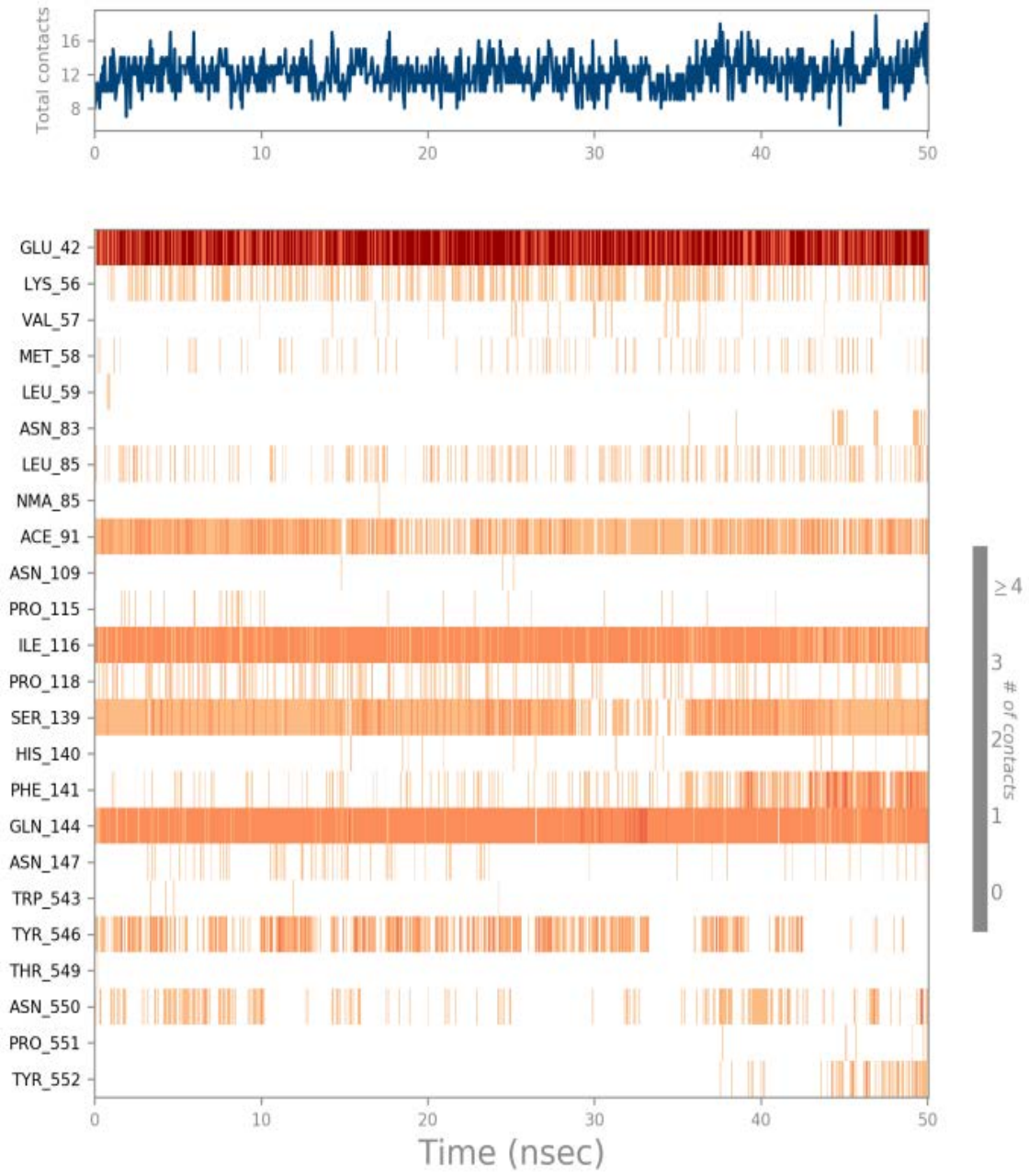
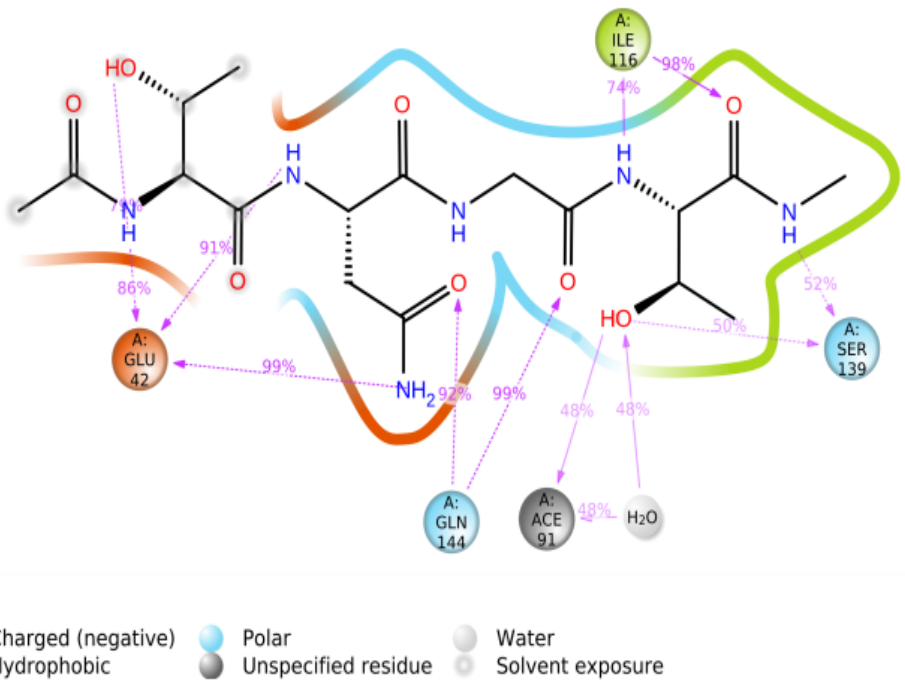
Protein-Ligand Contacts



Protein-Ligand Contacts (cont.)



Ligand-Protein Contacts



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4) DISCUSSION

Laccase enzymes are a type of multi-copper species, which catalyze the oxidation of substrate molecules to corresponding reactive radicals with the simultaneous reduction of oxygen to water at a mononuclear copper center type-1(T1). The catalytic center of these enzymes is composed of four copper atoms. Two histidine residues and one cysteine residue create a metallo-organic link with the T1 copper atom. Additionally, the T1 copper is in close proximity to the side chains of a methionine, leucine, or isoleucine. Two histidine ligands coordinate the trinuclear center's type 2 (T2) copper atom, whereas a total of six histidine ligands coordinate the two types 3 (T3) copper atoms.

The enzyme can oxidize a variety of substances, including polyphenols, methoxy substituted phenols, aromatic diamines, and others. Laccase enzymes are used in a variety of biotechnological processes, such as the delignification of lignocellulosic materials, bio-pulping and bio-bleaching, transformation of textile dyes, removal of phenolics from must and wine, and detoxification of effluent containing phenolic waste. An explicit description of the molecular determinants of their substrate specificity are yet to be deciphered.

Considerable number of crystal structures of fungal laccases have already been determined, hence providing us with a diversity of templates for further study of the molecular interaction between the enzyme and its substrates.

The affinity of laccase toward AFB1 and AFG1 depends on the ability of the enzyme to form hydrogen bonds, ionic interactions, water bridges and hydrophobic contacts with the two toxin variants of Aflatoxin. In the current study, We have performed docking studies of the wild-type (WT) laccases to establish base levels of binding affinity of AFB1 and AFG1, while simultaneously trying to establish if laccases possess the affinity to bind and subsequently degrade different variants of Aflatoxins. Favorable mutations were found using critical interacting residues in the WT molecular docking, using CUPSAT server and HotSpot Wizard. Twenty favorable and stabilizing point mutations found were individually introduced to the laccase enzyme and the impact was tested using molecular docking. From the results generated, 4 best results were filtered and their stacking effect was tested using a multiple mutation approach. The results were not as beneficial as individual mutations. Only two best point mutations were selected namely 106_asp-lys and 181_asp-trp. These were further tested using Molecular Dynamics approach to prove stabilities of their protein-ligand complexes in a water-based system and simultaneously validate our molecular docking results. The 181_asp-trp_afb complex, when compared to the wt-laccase_afb1 complex, was found to possess stronger protein-ligand interactions, and stable structural conformations deduced from reduced RMSF and RMSD values. 106_asp-lys_afg1 complex when compared to wt-laccase-afg1 complex showed an improved protein-ligand interaction despite having a slight increase in protein RMSD values. All experimentation was conducted in a dry lab and further requires wet-lab testing to fortify our findings.

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13.

Tools

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