

## **Enzyme Engineering Term Paper**

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## **Abstract:**

This study explores targeted mutations in *Pichia pastoris* alcohol oxidase (AOX) to improve its selectivity for methanol over methanediol, a common byproduct that competes for the active site. Based on structural data, two mutation targets—Thr617 and Phe98—were selected for site-directed mutagenesis. Mutants T617F, T617L, T617W, and F98W were generated and evaluated through molecular docking using AutoDock Vina. Two approaches were used: one focusing on general binding affinity and the other on catalytically relevant poses. T617W showed the most promising improvement in methanol selectivity, particularly within the constrained active site. Alternative docking tools like YASARA, SwissDock Activated Cavities Method, and CB-Dock2 were explored but had limitations, which prevented further development. Overall, the study highlights T617W as a strong candidate for future experimental validation in improving AOX selectivity for sustainable C1-based biocatalysis.

**Keywords:** Alcohol oxidase (AOX), methanol selectivity, methanediol competition, *Pichia pastoris*, rational enzyme engineering, site-directed mutagenesis, molecular docking, AutoDock Vina

## 1. Introduction:

The gradual shift towards sustainable biofuels for aviation, marine, and industrial sectors requires efficient biological conversion of simple one-carbon (C1) compounds, such as methanol, into more reactive intermediates suitable for downstream processing. In methylotrophic yeasts such as *Pichia pastoris*, this conversion is initiated by the enzyme alcohol oxidase (AOX). The enzyme catalyzes the oxidation of methanol to formaldehyde with the concomitant production of hydrogen peroxide. This reaction represents the first and rate-limiting step in methanol metabolism and plays an important role in the yeast's capacity to utilize methanol as the only carbon and energy source (Koch *et al.*, 2016; Vonck, Parcej and Mills, 2016).

AOX from *Pichia pastoris* is a large homo-octameric flavoprotein, with a total molecular mass of approximately 600 kDa. Each subunit harbors a flavin adenine dinucleotide (FAD) cofactor that is essential for catalytic activity. The enzyme belongs to the glucose-methanol-choline (GMC) oxidoreductase family and is known for its strict substrate specificity toward short-chain primary alcohols, especially methanol. This specificity has been attributed to the architecture of the substrate binding pocket, which is shaped by several bulky aromatic residues that restrict access for larger or structurally divergent substrates(Koch *et al.*, 2016; Vonck, Parcej and Mills, 2016).

A major complication in methanol oxidation arises from the chemical behavior of formaldehyde, the primary oxidation product. Formaldehyde rapidly undergoes hydration to form methanediol (methylene glycol) when they are present in aqueous environments. Even though methanediol is chemically distinct, it retains sufficient similarity to methanol to potentially act as a competing substrate for AOX, thereby affecting catalytic efficiency and selectivity. Interestingly, methanediol does not enter the enzyme via a separate substrate channel but is believed to form spontaneously from formaldehyde within the active site. Therefore, rational engineering efforts to enhance AOX's preference for methanol over methanediol must focus on modifying the active site and immediate surroundings to selectively disfavor the secondary oxidation of methanediol-derived formaldehyde (Hopkins, 1985; Vonck, Parcej and Mills, 2016).

The structural characterization of *P. pastoris* AOX by cryo-electron microscopy (Vonck *et al.*, 2016) gives insights for engineering its substrate specificity. The active site contains conserved residues such as His567 and Asn616, which are critical for catalysis. FAD binding is supported by Glu38 and Asn97, which interact with the ribose and isoalloxazine moieties of the cofactor, respectively. The Trp566 residue, which is roughly positioned 4 Å from the flavin ring, plays a dual role in substrate gating and FAD stabilization. Its large indole side chain may also sterically hinder alternative substrates. Other residues, such as Phe98 and Phe402, constrict the active site volume further, likely contributing to substrate discrimination. Importantly, inter-subunit interactions shape the substrate access tunnel; His515 from a neighboring monomer inserts into the adjacent active site, closely contacting Trp566 and contributing to the narrowing of the entry channel. A hydrophobic substrate access channel is defined by a conserved set of residues, including Ala514 (from the neighboring subunit), Ile83, Pro85, Phe68, Leu61, Met59, Ile96, and

Trp566. These features collectively enforce size and polarity constraints that favor methanol binding.

This project aims to engineer *Pichia pastoris* AOX variants with enhanced selectivity for methanol over methanediol through rational design. By disrupting stabilizing interactions with methanediol and modifying spatial constraints in the active site, the study seeks to shift substrate preference while preserving catalytic efficiency—an outcome that could significantly benefit C1-based bioprocessing platforms for sustainable fuel and chemical production.

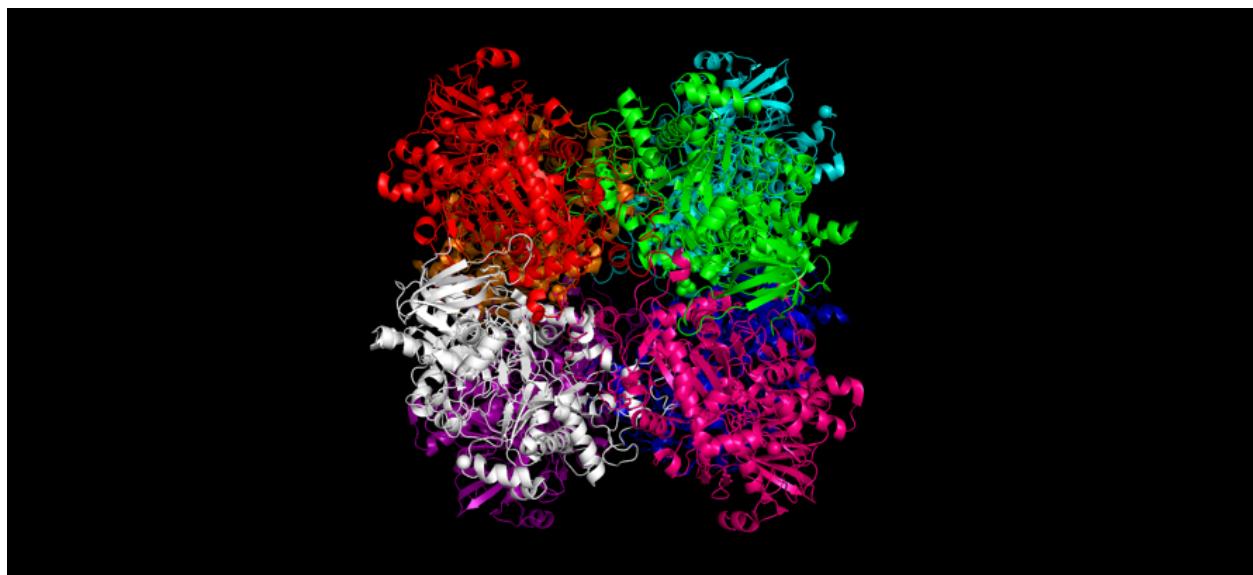


Figure 1: Octamer of AOX from Pymol



Figure 2: Monomer image of AOX from Pymol

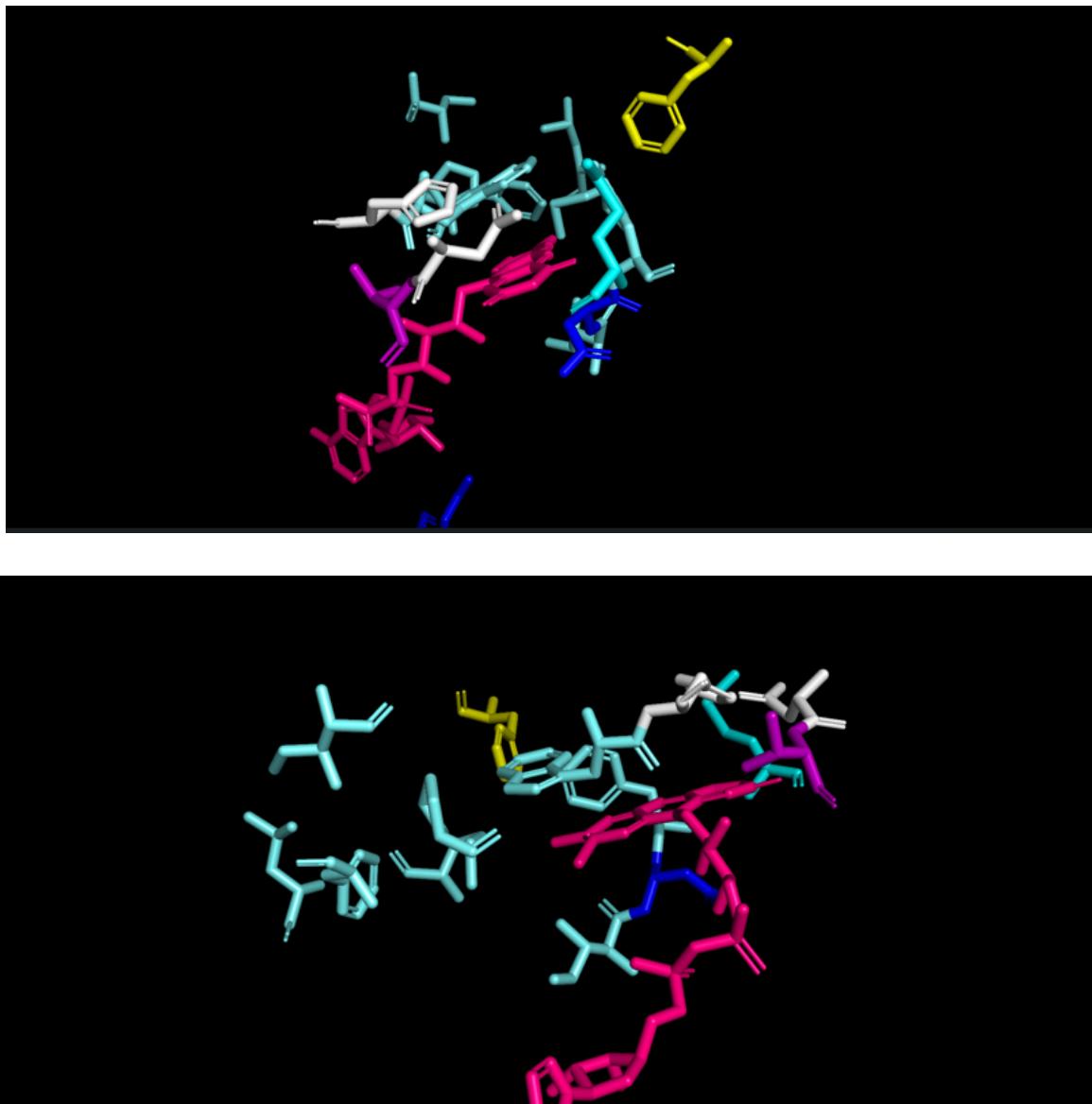


Figure 3 and 4: Pymol Visualisation of the residues (cyan: channel; white: core; yellow: bulky; hotpink: FAD; blue: bind with FAD)

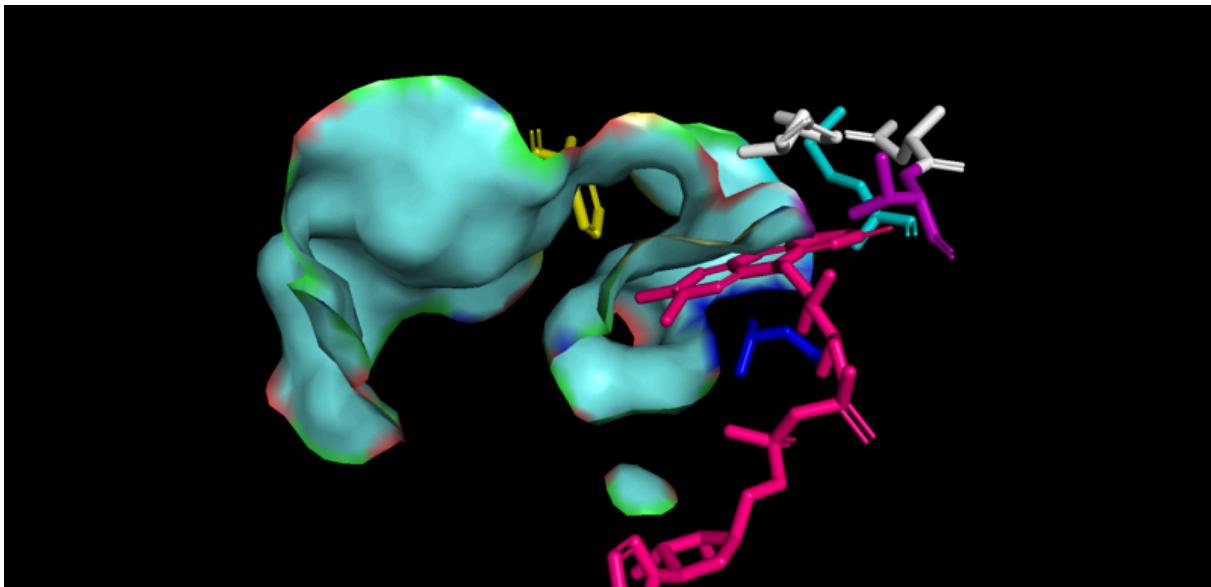


Figure 5: Surface image of channel in PyMol. Notice that the channel leading to FAD is covered with hydrophobic AA

## 2. Hypothesis for Mutation Targets:

Building on the structural insights gained from the cryo-EM model and the substrate interaction profile, the next phase of this study focused on identifying active site residues that could be rationally mutated to enhance selectivity for methanol over methanediol. Given that methanediol forms in situ within the catalytic pocket and may be stabilized through specific hydrogen bonding interactions, we hypothesized that modifying key residues involved in this stabilization could shift the enzyme's substrate preference.

### 2.1.Mutation at site T617:

Two distinct computational strategies were employed to identify optimal sites for mutagenesis:

1. **Proximity- and Architecture-Based Selection:** This method involved identifying all amino acid residues within a 4 Å radius of H567, which is regarded as the primary catalytic residue. Subsequently, these residues were visually inspected to identify those contributing to the active site's pocket architecture, effectively forming its boundary or "wall." These visually selected residues were then systematically analyzed to predict the impact of potential mutations on substrate binding and catalytic efficiency.

Computational Selection Command:

```
Select target, (byres (resi 567 around 4.0)) and not resn FAD
```

**2. Multi-Constraint Proximity Selection:** This more stringent approach sought residues located within a 4 Å radius of *multiple* key components of the active site: H567, N616, the FAD cofactor, and the substrate access channel. This method efficiently filters out residues that might be within 4 Å of H567 but are located outside the immediate active site dome, thereby reducing the need for visual inspection. The substrate access channel to the FAD isoalloxazine ring is notably lined by hydrophobic residues such as Ala514, Ile83, Pro85, Phe68, Leu61, Met59, Ile96, Phe98, and Trp566.

Computational Selection Command:

```
Select target, (byres (resi 567 around 4.0)) and (byres (resn FAD around 4.0)) and (byres (channel around 4.0)) and not resn FAD and not active_site and not channel
```

(NOTE: 'channel' and 'active\_site' are predefined selections in the computational environment)

Both methodologies independently converged on two promising candidate amino acids for mutagenesis: Methionine 100 (M100) and Threonine 617 (T617).

Considering their properties, Methionine is a mildly polar, non-bulky amino acid, while Threonine is a polar, non-bulky amino acid. A working hypothesis posits that during the oxidation of methanol, while one hydroxyl group of the intermediate methane-diol is stabilized by hydrogen bonding with H567, the second hydroxyl group is stabilized through hydrogen bonding with T617. This proposed mechanism of stabilization by T617 renders the active site highly conducive to methane-diol formation and binding. This hypothesis can be later explored and potentially verified through molecular docking simulations, such as those performed using AutoDock Vina.

To disrupt this favorable interaction for methane-diol, a rational design strategy focuses on mutating T617 to a residue that is both bulky and non-polar. This change is hypothesized to sterically hinder and destabilize the binding of methane-diol within the active site dome.

A crucial consideration for these simulations is the inherent rigidity assumption of algorithms like AutoDock Vina, which treats the protein as a static entity. This limitation implies that if the simulation is initiated with an energy-minimized enzyme conformation, a very bulky substituted residue might not be accurately predicted to cause steric clashes, potentially still allowing a bulky substrate (or methane-diol) to seemingly fit within the active site. To account for this, a series of three T617 mutations were suggested, all introducing non-polar residues but varying significantly in their steric bulk: T617L (Leucine, moderately bulky), T617F (Phenylalanine, bulky), and T617W (Tryptophan, very bulky). These three mutants will be subsequently tested to evaluate their impact on enzyme selectivity.

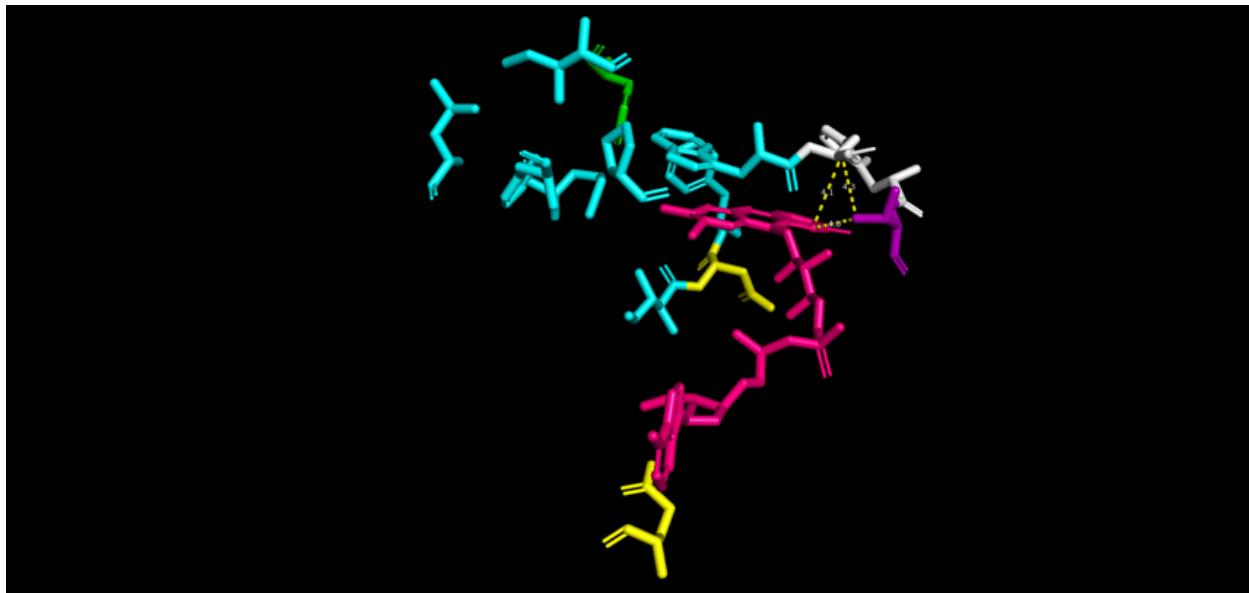


Figure 6: Alignment of T617 (purple) with respect to other residues is close enough to form H-bonds with ligands

## 2.2. Mutation at site F98:

### 2.2.1. Site selection

Mutation data for this class of proteins is available on BRENDA. However, since these proteins originate from different species, the positions of homologous amino acids vary. To identify suitable mutation sites, we first aligned the amino acid sequence from *Arthrobacter globiformis* with that of *Pichia pastoris* using MEGA. In *A. globiformis*, the positions of interest were Ser101, Val355, and Phe357. Based on the alignment (see figure below), the corresponding residues in *P. pastoris* (*Pichia phaffii*) are Phe98, Arg381, and Tyr383.

PROTEIN VARIANTS ▾	ORGANISM ▾	UNIPROT ▾	COMMENTARY ▾	X	LITERATURE ▾
M359R	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13		762910
S101A	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13		762910
S101A/D250G/F253R/V355T/F357R/M359R	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13, with a 20fold increased kcat compared to that of the wildtype enzyme. This variant enables the oxidation of 10 mM hexanol to hexanal in less than 24h with 100% conversion and catalyzes significantly improved oxidation of saturated, unsaturated, aliphatic, cyclic and benzyllic alcohols		762910
S101A/V355T/F357R	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13		762910
S101A/V355T/F357R/M359R	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13		762910
V355T/F357R	Arthrobacter globiformis	Q7X2H8	mutant displays increased activity with hexan-1-ol, reaction of EC 1.1.3.13		762910
G15A	Ogataea angusta	-	mutation in putative FAD-binding domain, prevents enzyme import into peroxisome and assembly		656760
F101N	Phanerodonta chrysosporium	T2M2J4	with enlarged catalytic cavity, increase in activity with substrates 1-propanol, glycerol, (R)-1,2-propanediol		762732
F101S	Phanerodonta chrysosporium	T2M2J4	with enlarged catalytic cavity, retains a high degree of thermostability		762732
M103S	Phanerodonta chrysosporium	T2M2J4	with enlarged catalytic cavity, increase in activity with substrates 1-propanol, glycerol, (R)-1,2-propanediol		762732
additional information	11 entries				

Figure 7. the screenshot of Brenda (<https://www.brenda-enzymes.org/enzyme.php?ecno=1.1.3.13>)



Figure 8. The result of alignment of the Aox from different species

According to the cryo-EM structure described in the paper (Vonck, Parcej and Mills, 2016), F98 is located near the substrate-binding site and is considered a promising candidate for mutagenesis.

## 2.2.2. Variant of F98W

The F98W mutation was selected due to the structural and chemical properties of the involved residues. Tryptophan is bulkier than phenylalanine and contains an indole ring, which introduces greater steric hindrance within the binding pocket. This spatial restriction is expected to reduce binding affinity for methanediol, while the aromatic indole ring may form weak π–hydrophobic interactions with the C–H group of methanol, potentially improving binding specificity.

In summary, the selection of T617 and F98 as mutational hotspots is grounded in both structural proximity to the catalytic core and their potential roles in substrate stabilization. T617 is hypothesized to contribute to methanediol binding via hydrogen bonding, and its substitution with bulkier, non-polar residues aims to disrupt this interaction. Meanwhile, F98, situated near the substrate-binding site, presents an opportunity to modulate spatial constraints and substrate access through the F98W mutation. Together, these targeted modifications form the basis of a rational design strategy to enhance methanol selectivity in *Pichia pastoris* AOX, which will be further evaluated through molecular docking studies.

### 3. Methods and Results:

Molecular docking simulations were performed using AutoDock Vina to investigate the binding of methanol and methanediol to Alcohol Oxidase. Two distinct approaches were employed to address different research objectives.

#### 3.1. General Binding Affinity (Offline AutoDock Vina)

One set of docking simulations was conducted using the offline version of AutoDock Vina. The primary objective of this approach was to assess the overall binding affinity (represented by binding energy) of methanol and methanediol to the enzyme's active site. This provided a general understanding of the energetic favorability of the enzyme environment for these substrates. An exhaustiveness value of 500 was employed to ensure a comprehensive sampling of the conformational space within the defined search box.

##### Autodock Vina usage:

First, you need to use the Autodock Tools to prepare the .pdbqt file of the receptor and the ligand. In this process, the rotating condition of the ligand will be taken into account, and the hydrogen and the electron will be added to the receptor.

Then you need to find the place of the search box in the Autodock Tools.

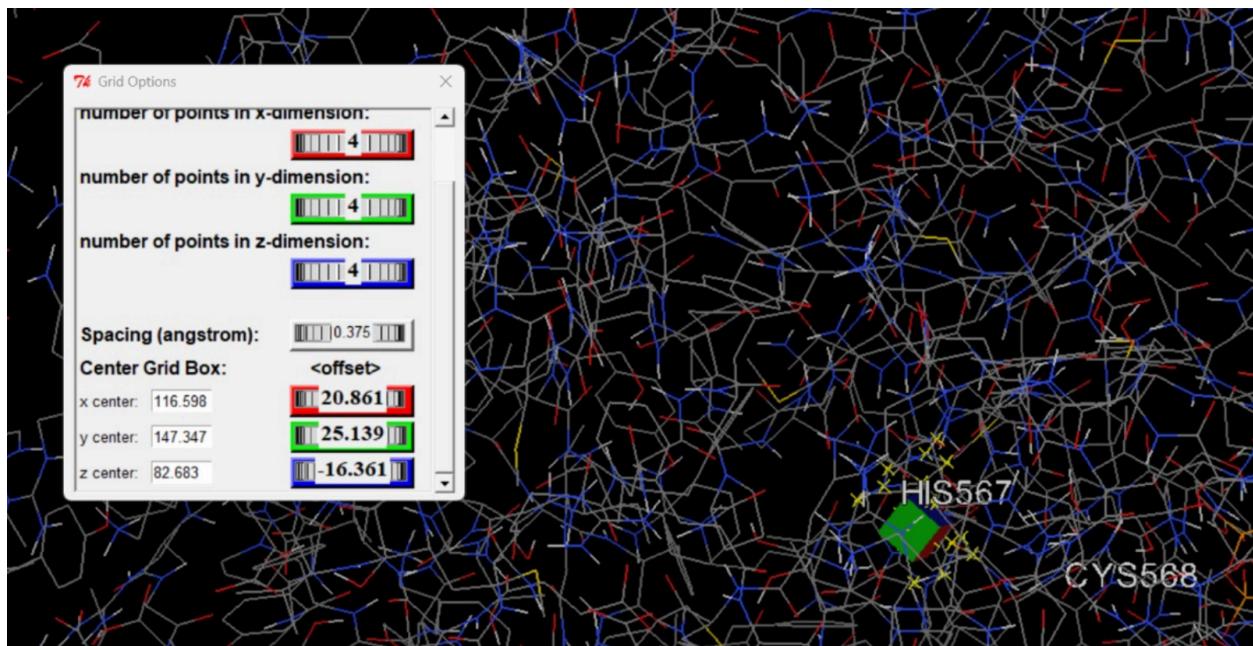


Figure 9. Screenshot of using the Autodock tools to find the binding box

Since the binding site is around the His 567, which will be mentioned in other analytical part, so we will find the place of the box and set the config file of the autodock vina.

```
receptor = Enzyme_minimised.pdbqt
ligand = methanol.pdbqt

center_x = 115.391
center_y = 143.157
center_z = 81.058
size_x = 8.0
size_y = 8.0
size_z = 8.0
out = methanol_out.pdbqt
exhaustiveness = 500
energy_range = 3
num_modes = 9
```

Figure 10. Screenshot of the config file

Then we followed the instructions of Autodock Vina, and ran the program successfully.

Figure 11. Screenshot of using the Autodock Vina successfully

### 3.2. Reactive Site Analysis (Online AutoDock Vina via SwissDock Toolkit)

A second set of docking simulations was performed using the online AutoDock Vina tool, accessible via the SwissDock toolkit. This approach was specifically designed to identify binding

poses that are catalytically relevant for the enzymatic reaction, thereby informing our understanding of the reaction kinetics.

For these targeted simulations, the docking box was meticulously defined based on key catalytic residues and the FAD cofactor. Based on literature, His567 is identified as a crucial amino acid directly involved in the alcohol oxidation mechanism. Furthermore, the N1 atom of the FAD isoalloxazine ring is the primary site of hydride transfer from the substrate during the catalytic reaction. Therefore, the center of the docking box was precisely set at the geometric midpoint between the N1 atom of FAD and the nitrogen atom of His567's imidazole side chain.

A relatively small cubic box of 8 Å x 8 Å x 8 Å was selected to ensure the search was tightly focused on this immediate catalytic environment. This size was chosen to promote favorable hydrogen bonding interactions with His567 while strictly limiting the search space to prevent docking to non-catalytic regions, such as the FAD backbone hydroxyl groups, which had been an issue in preliminary studies. Additionally, this box size was deemed sufficient to accommodate potential hydrogen bonding of methanediol's second hydroxyl group with Thr617, a possibility explored in the hypothesis for Wild Type strains.

The precise coordinates for the box centers were individually calculated for the Wild Type enzyme and each of the designed mutations (F98W, T617F, T617L, T617W). These coordinates, calculated using the centerofmass command in PyMOL for the defined two atoms (FAD N1 and His567 N), are provided in Table 1:

Strain	x	y	z
Wild Type	115.391	143.157	81.058
F98W	114.259	142.714	81.113
T617F	113.587	144.247	81.150
T617L	114.086	142.632	81.441
T617W	113.943	143.053	81.451

Following the docking simulations, the generated ligand configurations were meticulously analyzed. The primary criterion for selecting the most probable reactive configuration was the formation of a direct hydrogen bond between the substrate's hydroxyl group and His567, along with close proximity to the FAD isoalloxazine ring (specifically the N1 atom). An illustration of a representative reactive configuration can be seen in Figure X.

Figure Y presents the selected best configurations for each enzyme strain (Wild Type and mutants) along with their respective calculated binding energies.

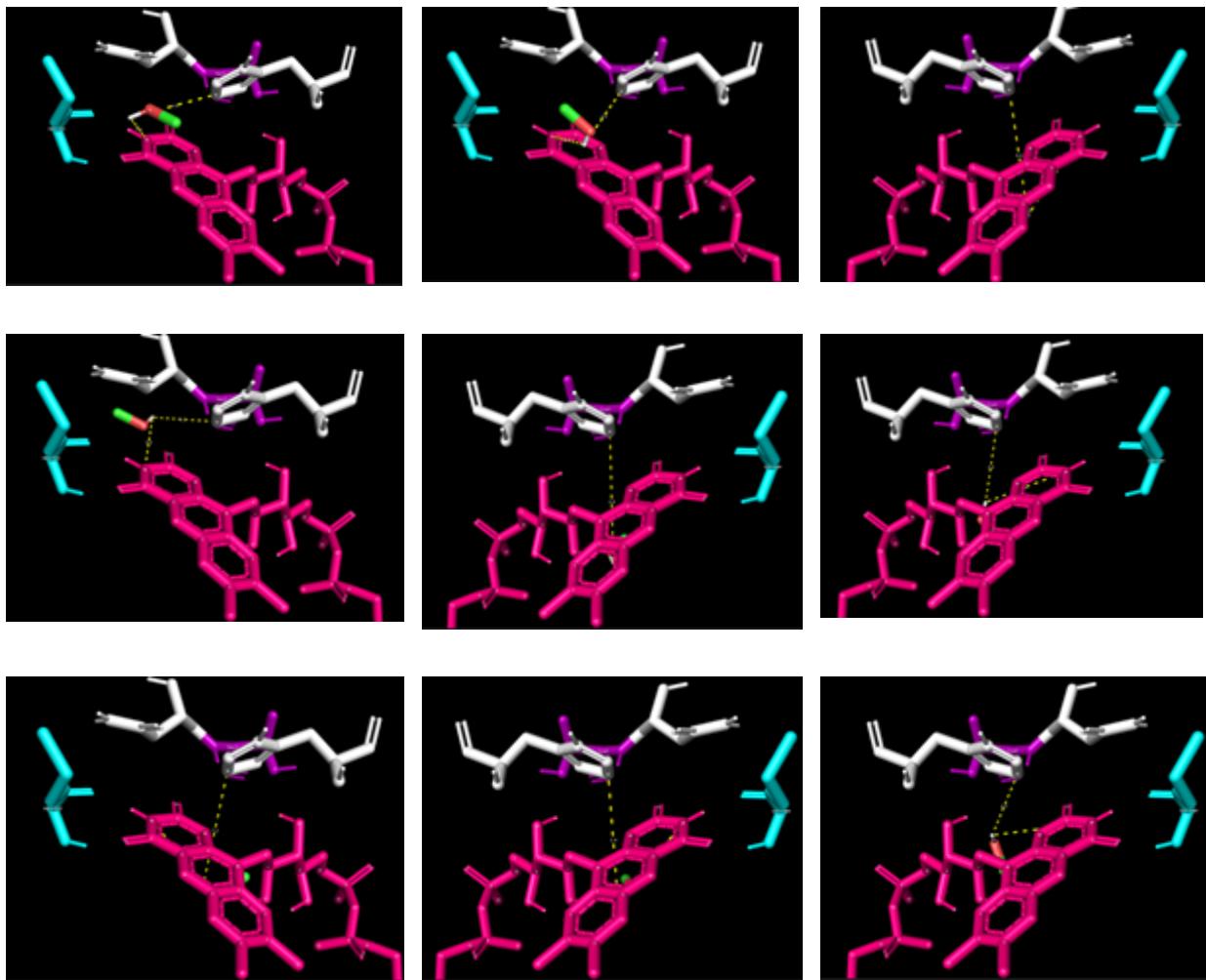


Figure 12. Nine possible configurations for methanol in the Wild type Enzyme. Hot pink is FAD, cyan and purple are the wall (M100 and T617, respectively), white is core (N616 and H567). Configuration 1 is the most probable to react.

Model	Calculated affinity (kcal/mol)
1	-1.313
2	-1.119
3	-0.850
4	-0.715
5	-0.267
6	0.157
7	0.741
8	0.961
9	1.297

Table 2: Binding energies for configurations in Figure 11

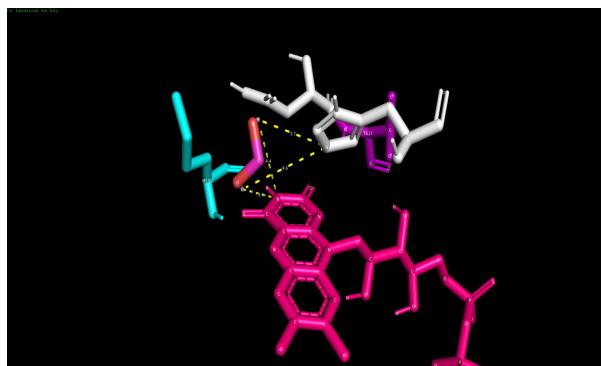


Figure 13. Most reactive config. for Wild type-methanediol

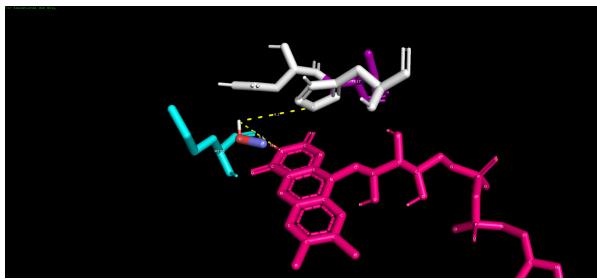


Figure 14: Most reactive config for F98W-Methanol

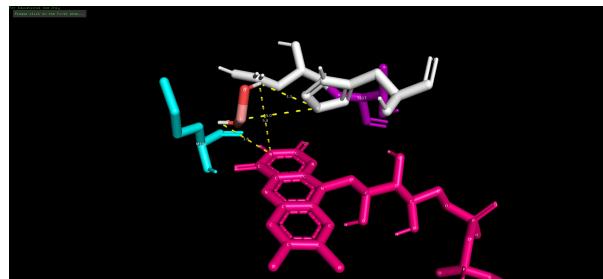


Figure 15: Most reactive config for F98W-Methanediol

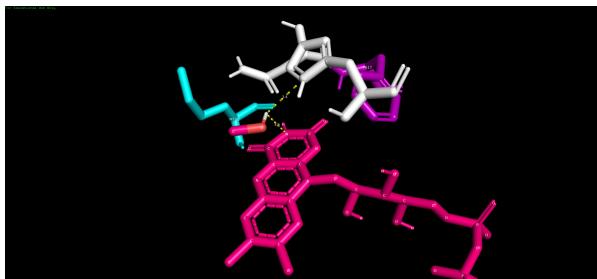


Figure 16: Most reactive config for T617F-Methanol

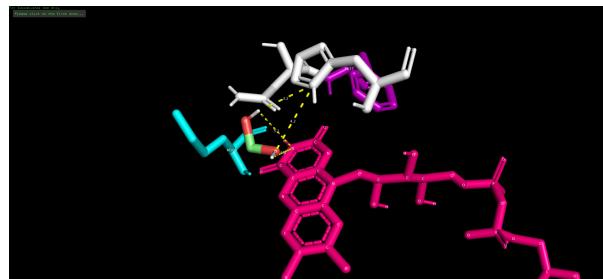


Figure 17: Most reactive config for T617F-Methanediol

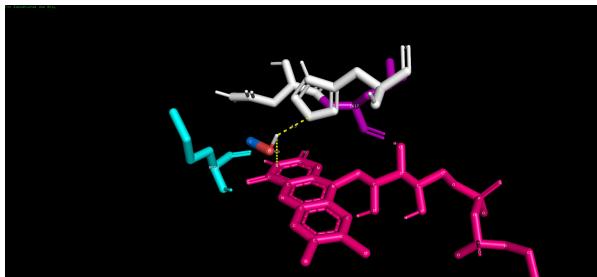


Figure 18: Most reactive config for T617L-Methanol

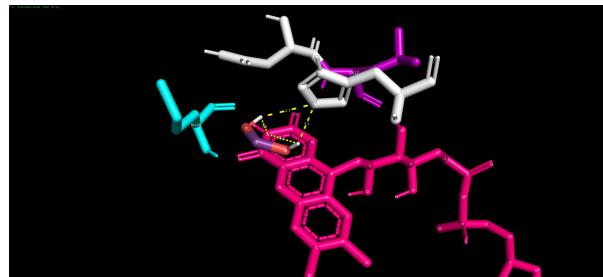


Figure 18: Most reactive config for T617L-Methanediol

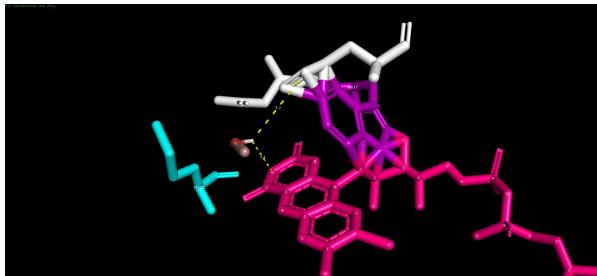


Figure 20: Most reactive config for T617W-Methanol

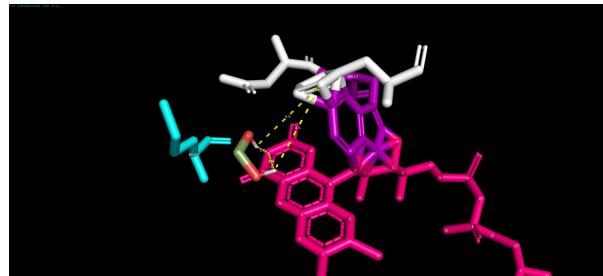


Figure 21: Most reactive config for T617W-Methanediol

### 3.3.Results:

#### Offline AutoDock Vina

Strain	BE – methanol (kcal/mol)	BE-methanediol (kcal/mol)	K-Methanol	K-methanediol	Ratio K (methanol/ methanediol)
WT	-1.378	-2.036	10.2493	31.1389	0.3291
F98W	-1.553	-2.301	13.7736	48.7155	0.2827
T617W	-1.768	-2.386	19.8033	56.2355	0.3521
T617L	-1.486	-1.946	12.3000	26.7481	0.4598
T617F	-1.953	-3.105	27.0661	189.392	0.1429

$K = \exp(-BE/RT)$  where  $R = 1.987 \times 10^{-3}$  kcal/mol/K and  $T = 298K$

Binding Energy Values

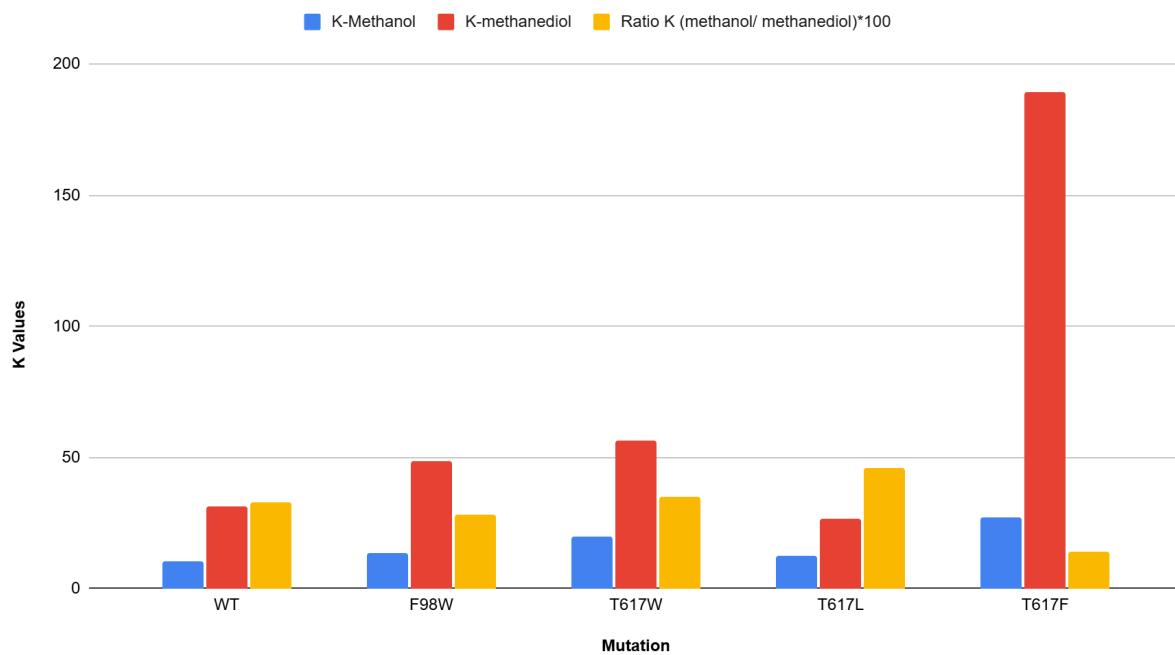


Figure 22: Bar graph for Affinity Constant K

## Online Autodock Vina

Strain	BE – methanol (kcal/mol)	BE-methanediol (kcal/mol)	K-Methanol	K-methanediol	Ratio K (methanol/ methanediol)
WT	-1.313	-1.145	9.18806	6.91796	1.32814
F98W	-0.679	-1.193	3.14860	7.50225	0.41969
T617W	-1.629	-1.020	15.6691	5.60116	2.79747
T617L	-1.503	-1.712	12.6651	18.0274	0.70255
T617F	-1.503	-2.159	12.6651	38.3578	0.33018

Binding Energy (Reactive Configuration)

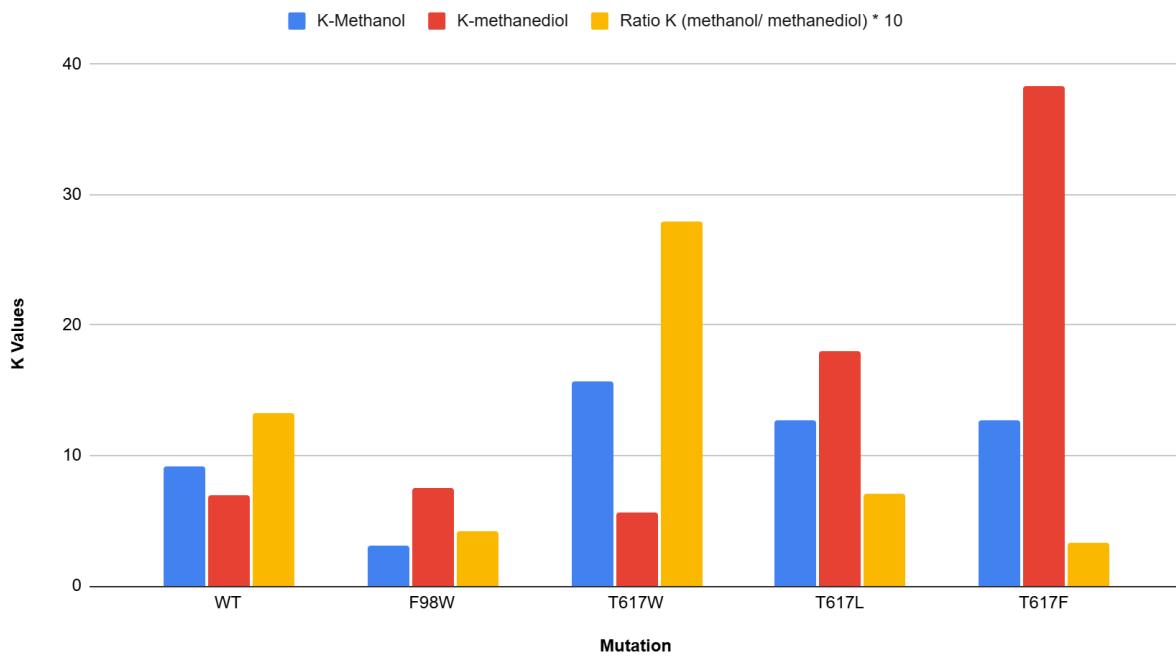


Figure 23: Bar graph for Affinity Constant K for Reactive Configuration

## 3.4. Unsuccessful Docking Attempts: Software Challenges

### 3.4.1. Limitations of the SwissDock Activated Cavities (AC) Method

The SwissDock Activated Cavities (AC) method was initially employed to dock methanol into the active site of Alcohol Oxidase, targeting the N1 atom of FAD and nearby catalytic residues. Despite using a precisely calculated center (115, 141, 81), high exhaustivity, and "buried" cavity prioritization, the method consistently misdocked methanol to non-catalytic regions such as FAD backbone hydroxyl groups. Even with increased RIC values (up to 7), none of the poses aligned

with biologically relevant active site interactions. Additionally, the runs were computationally expensive and failed to prioritize the correct catalytic pocket. Owing to these limitations, SwissDock's AC method was deemed unreliable for this system, and docking was instead carried out using AutoDock Vina.

### **3.4.2.Limitations in CB-Dock2:**

Docking studies were conducted using CB-Dock2, which is a cavity-based blind docking tool that predicts potential binding pockets on the protein surface and performs docking. The rationale for using CB-Dock2 was its automated cavity detection and compatibility with small-molecule ligands. However, several limitations were encountered during its application. Firstly, the software successfully performed docking only when the FAD cofactor was absent, which undermines the biological relevance of the enzyme model, as FAD is essential for catalytic function. Secondly, attempts to use energy-minimized enzyme structures did not give us any results, and the use of enzyme structures without energy minimisation resulted in inaccurate pocket predictions, where CB-Dock2 identified only surface-level cavities and failed to detect the biologically meaningful active site. These discrepancies suggest a poor compatibility of CB-Dock2 with structurally refined (minimized) models and cofactors. Due to these problems faced, CB-Dock2 was deemed unsuitable for this study, and alternative docking platforms were explored for further analysis.

### **3.4.3.Limitations in using YASARA:**

During initial attempts to perform docking using YASARA, we encountered persistent issues that prevented successful execution. The first problem involved an error stating that the receptor file could not be found at the specified path, despite both the `01_ligand.yob` and `01_receptor.sce` files being present in the same directory. It was eventually discovered that the docking script only recognized file names without extensions or full paths, which resolved the initial error. However, a subsequent and unresolved issue related to the incorrect labeling or interpretation of carbon atoms ("the C problem") continued to disrupt the workflow. Even though we have tried to fix the C problem, the error still exists. So we turned to use the Autodock Vina.

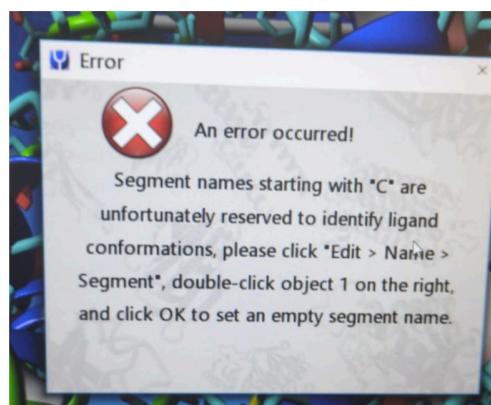


Figure 24. The screenshot of the C name problem

## **4 Discussions:**

The molecular docking simulations reveal distinct selectivity profiles for the designed mutant enzymes depending on the docking approach used (general binding vs. reactive conformations). This divergence in results can be primarily attributed to the different definitions of the docking search space employed by the two methods. The offline AutoDock Vina utilized a broader search box, aiming to identify the most energetically favorable binding pose regardless of its catalytic relevance, whereas the online AutoDock Vina was meticulously targeted to the precise catalytic active site, favoring reactive conformations. Indeed, it is observed that the absolute binding energies for methanol in the reactive conformations (Online Vina) are generally more favorable (more negative) than those in the general binding affinity runs (Offline Vina), with the exception of the F98W mutant (-0.679 kcal/mol), which showed a weaker binding than WT (-1.313 kcal/mol) in the online Vina results. This general improvement in binding affinity for methanol in the reactive conformations highlights the impact of precisely constrained docking on identifying optimal, productive interactions.

In the general binding affinity calculations (Offline AutoDock Vina), where a broader conformational search was performed, the selectivity for methanol over methanediol (indicated by a higher Ratio K (methanol/methanediol)) was most significantly improved by the T617L mutation (Ratio K = 0.4598), followed by T617W (Ratio K = 0.3521). Both of these mutants exhibited a higher selectivity ratio compared to the Wild Type (Ratio K = 0.3291), suggesting that the introduction of moderately bulky non-polar residues at position 617 can reduce the relative binding favorability of methanediol in a broader binding context. Conversely, the F98W and T617F mutations resulted in a decrease in methanol selectivity in this general binding context.

However, when focusing on catalytically relevant, reactive conformations (Online AutoDock Vina), a different selectivity trend emerged. In this more stringent analysis, T617W proved to be the only mutation that significantly improved methanol selectivity (Ratio K = 2.79747) compared to the Wild Type (Ratio K = 1.32814). The other mutants (F98W, T617L, T617F) showed a decrease in methanol selectivity in these reactive conformations relative to the Wild Type. This suggests that while T617L might be effective in generally reducing methanediol binding, T617W's specific steric and electronic properties are more beneficial when the substrate is constrained to the precise catalytic orientation required for reaction.

### **Limitations of Computational Docking**

It is important to acknowledge the inherent limitations of the computational docking methods employed in this study. AutoDock Vina, like many similar algorithms, treats the protein receptor as a rigid entity, neglecting the dynamic nature of protein structures and potential induced-fit conformational changes upon ligand binding. Furthermore, these simulations utilize implicit solvent models, which simplify complex solvent effects and entropic contributions that can significantly influence actual binding affinities and reaction dynamics. The force-field based nature of docking also means it does not model the actual electron transfer process or bond

making/breaking, which would require more computationally intensive quantum mechanical (QM) or hybrid QM/MM calculations. Therefore, the predicted binding energies and selectivity profiles should be interpreted as theoretical estimations that require experimental validation.

## 5. Conclusion:

This study employed a rational design approach combined with molecular docking simulations to investigate strategies for enhancing the selectivity of *Pichia pastoris* Alcohol Oxidase towards methanol over methanediol. Through targeted mutagenesis at the Phe98 and Thr617 positions, we explored the impact of specific amino acid substitutions on substrate binding affinities and selectivity profiles.

Our findings highlight the importance of considering the specific docking methodology and search space definition, as evidenced by the divergent results obtained from general binding affinity (offline AutoDock Vina) and catalytically relevant reactive conformation (online AutoDock Vina) analyses. While the T617L and T617W mutations showed the most significant improvements in methanol selectivity in the broader general binding context, the more stringent analysis of reactive conformations identified T617W as the sole mutation that substantially enhanced methanol selectivity. This suggests that the specific steric and electronic environment created by Tryptophan at position 617 is particularly effective when the substrate is oriented towards catalysis.

In summary, the computational predictions indicate that the T617W mutation holds the most promise for improving methanol selectivity in Alcohol Oxidase by favoring its binding in catalytically productive orientations. While these computational insights provide a strong foundation for enzyme engineering, it is crucial to emphasize that these theoretical predictions require rigorous experimental validation to confirm the observed selectivity enhancements and to fully elucidate the structural and mechanistic basis of these improvements.

## 6. Contributions:

**Dhanush:** Independently identified the Thr617 site through an alternative computational selection method, providing valuable corroboration for its significance as a mutation target. Contributed to initial debugging efforts for YASARA. Contributed to the core molecular docking simulations for reactive site analysis using the online AutoDock Vina platform. Helped in identifying the optimal binding configurations for all strains. Conducted the data analysis of all binding energies, including the calculation of K values and selectivity ratios, and was responsible for generating all figures (e.g., molecular visualizations, plots) for inclusion in the report.

**Jethasri:** Performed the crucial task of energy minimization for all enzyme structures, preparing them for subsequent molecular docking simulations. Explored the utility of CB-Dock2 for preliminary docking studies, identifying its limitations for targeted analysis. Authored the Introduction and Abstract sections of the report, setting the context and summarizing the study's key findings. Furthermore, was responsible for the final formatting of the report, ensuring adherence to academic standards.

**Jixia:** Identified and proposed the Phe98 (F98W) mutation, providing a detailed rationale based on sequence alignment and structural analysis. Conducted molecular docking simulations using an offline installation of AutoDock Vina, generating comprehensive data for general binding affinity comparisons across all enzyme and ligand combinations. Explored the utility and encountered challenges with YASARA for preliminary docking. Contributed foundational reasoning for active site selection, which informed the hypothesis section of the report. Additionally, managed and compiled the References section, ensuring proper citation and formatting.

**Om:** Designed and conceptualized the Thr617 mutations (T617F, T617L, T617W) based on rational design principles. Contributed to the core molecular docking simulations for reactive site analysis using the online AutoDock Vina platform, including the meticulous calculation of precise box centers for all enzyme strains. Debugged and evaluated preliminary docking software, identifying limitations that led to the adoption of AutoDock Vina. Contributed to the identification of optimal binding configurations for all strains. Authored the Conclusion and Discussions sections of the final report.

## Supplementary Data

High Resolution images and PDB files can be found here: [Supplementary Data](#)

## References

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