

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**RATIONAL DESIGN OF L-ALANINE DEHYDROGENASE
VARIANTS FOR ENHANCED CATALYTIC EFFICIENCY**

TUBA OKUR AKSU

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING**

ADVISOR: ASSIST. PROF. DR. ONUR SERÇİNOLU

JULY 2025

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**RATIONAL DESIGN OF L-ALANINE
DEHYDROGENASE VARIANTS FOR ENHANCED
CATALYTIC EFFICIENCY**

TUBA OKUR AKSU

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING**

ADVISOR: ASSIST.PROF.DR. ONUR SERÇİNOĞLU

Co-ADVISOR: ASSOC. PROF. DR. FATİH AKTAŞ

JULY 2025

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**GELİŞTİRİLMİŞ KATALİTİK VERİMLİLİK İÇİN L-
ALANİN DEHİDROGENAZ VARYANTLARININ
RASYONEL TASARIMI**

TUBA OKUR AKSU

YÜKSEK LİSANS TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI

DANIŞMAN: DR. ÖĞR. ÜYESİ ONUR SERÇİNOLU
2. DANIŞMAN: DOÇ. DR. FATİH AKTAŞ

TEMMUZ 2025



MASTER of SCIENCE JURY APPROVAL FORM

A thesis submitted by Tuba OKUR AKSU defended on 04/07/2025 before the jury formed with the 30/06/2025 date and 2025/34 numbered decision of the GTU Graduate Administration Board, has been accepted as a MASTER of SCIENCE thesis in the Department of Bioengineering.

JURY

MEMBER

(THESIS ADVISOR) :ASSIST. PROF. DR. ONUR SERÇİNOĞLU

MEMBER :ASSOC. PROF. DR. FATİH AKTAŞ

MEMBER :PROF. DR. BARIŞ BİNAY

MEMBER : ASSOC. PROF. DR. CEYHUN BEREKETOĞLU

MEMBER :ASSIST. PROF. DR. CANSU ÜLKER TURAN

APPROVAL

Gebze Technical University Graduate Administration Board
...../...../..... date and/..... numbered decision.

SIGNATURE/SEAL

To my beloved Family

ABSTRACT

Amino acids are extensively utilized across diverse industries, a testament to their multifaceted biological roles and unique chemical properties. Among these, L-alanine stands out as one of the smallest chiral amino acids, with broad applications in the pharmaceutical industry, food production, and veterinary medicine.

The enzyme L-alanine Dehydrogenase (L-AlaDH) catalyzes the reversible conversion of pyruvate to L-alanine, utilizing NAD(H) and NAD⁺ as cofactors. Specifically, its reductive amination activity converts pyruvate to L-alanine in the presence of NAD(H). While numerous studies on the oxidative activity of L-AlaDH have been reported, its reductive amination activity remains insufficiently explored. This research presents, for the first time, a comprehensive characterization of the reductive amination activity of L-AlaDH derived from *Amycolatopsis sulphurea* (L-*AsAlaDH*). Furthermore, we identify and characterize L-*AsAlaDH* variants with enhanced catalytic activity generated through protein engineering, evaluating both their reductive amination and oxidative deamination activities.

Within the scope of this thesis, we investigated the design of mutants using rational enzyme engineering methods, followed by their transformation, expression, purification, biochemical characterization, and determination of kinetic parameters. These investigations were performed for both wild-type L-*AsAlaDH* and its mutants during oxidative deamination and reductive amination. Additionally, the metal binding capacity and storage stability relevant to reductive amination were explored. Furthermore, the effects of the mutations were elucidated through active site modeling and Molecular Dynamics (MD) simulation analysis.

Our studies revealed the optimum working conditions for the reductive amination activity of the wild-type and various L-*AsAlaDH* mutants: wild-type, pH 9.0 and 25°C; Tyr14Asn, pH 9.0 and 35°C; Ile97Leu, pH 9.5 and 45°C; and Ser192Ala, pH 10.0 and 40°C. For oxidative deamination, the optimal conditions were pH 10.5 and 25°C for the wild-type; pH 10.0 and 30°C for Tyr14Asn; pH 10.5 and 40°C for Ile97Leu; and pH 10.0 and 35°C for Ser192Ala. All mutants demonstrated an increased catalytic efficiency, ranging from 1.73- to 3.13-fold for reductive amination and 1.80- to 3.04-fold for oxidative deamination. To elucidate the reasons for these changes in catalytic activity, we investigated the active sites of the enzymes and conducted MD simulations. The mutations altered the lengths or types of bonds between the active site amino acids and pyruvate, and also influenced the overall enzyme dynamics, consequently affecting catalytic efficiency. Furthermore, analysis of the metal ion binding capacity and storage stability of the reductive amination activity showed that metal ions significantly inhibited or enhanced the enzyme's reductive amination activity. Storage stability was also influenced by the buffer composition, particularly the presence of metal ions within the protein solution.

As a conclusion, this thesis demonstrates the impacts of site-directed mutations on L-*AsAlaDH* enzyme activity. Comprehending the enzyme's structure-activity relationship is of significant importance for future studies. Specifically, engineered L-*AsAlaDH* emerges as a promising candidate for industrial applications in pharmaceutical, food, and other key sectors.

Keywords: *Amycolatopsis sulphurea*, L-Alanine Dehydrogenase, Molecular Dynamics Simulations, Characterization, Mutation, Active Site Modelling

ÖZET

Amino asitler, hücresel yapının oluşumdan bağışıklık savunma ve onarım mekanizmalarına kadar çeşitli vücut fonksiyonlarının ayrılmaz bir parçası olan temel moleküllerdir. Amino asitler, çok yönlü biyolojik rolleri ve benzersiz kimyasal özellikleri nedeniyle çeşitli endüstrilerde de yaygın olarak kullanılmaktadır.

L-alanin, ilaç endüstrisi, gıda üretimi ve veterinerlik gibi birçok alanda uygulamaları bulunan en küçük kiral amino asitlerden biridir. L-alanin dehidrogenaz (L-AlaDH) enzimi, kofaktör olarak NAD(H) ve NAD⁺ kullanarak pirüvatın L-alanine geri tersinir dönüşümünü katalize eder. Özellikle, indirgeyici aminasyon aktivitesi, NAD(H) varlığında pirüvatı L-alanine dönüştürür. L-AlaDH'nin oksidatif aktivitesi üzerine çok sayıda çalışma bildirilmiş olsa da, indirgeyici aminasyon aktivitesi yeterince araştırılmamıştır. Bu araştırma, ilk kez, *Amycolatopsis sulphurea* (L-AsAlaDH) kaynaklı L-AlaDH enziminin indirgeyici aminasyon aktivitesinin kapsamlı bir karakterizasyonunu bildirmektedir. Ayrıca, protein mühendisliği yoluyla üretilen gelişmiş katalitik aktiviteye sahip L-AsAlaDH varyantları belirlenmiş, ve bu varyantların indirgeyici aminasyon ve oksidatif deaminasyon aktiviteleri karakterize edilmiştir.

Bu tez çalışması kapsamında, rasyonel enzim mühendisliği yöntemleriyle mutantların tasarlanması, dönüşümü, ekspresyonu, saflaştırılması, ve biyokimyasal karakterizasyonu gerçekleştirilmiştir. Ayrıca, oksidatif deaminasyon ve indirgeyici aminasyon sırasında mutantların ve yabancıl tip L-AsAlaDH'nin enzim kinetiği parametrelerinin belirlendi. İndirgeyici aminasyonun metal bağlama kapasitesi ve depolama kararlılığı araştırıldı. Ayrıca, mutantların protein yapısına ve dinamiğine olan etkileri aktif bölge analizi ve klasik moleküler dinamik (MD) simülasyonları gerçekleştirilerek araştırıldı.

Çalışmalar, yabancıl tip, Tyr14Asn, Ile97Leu ve Ser192Ala mutantlarının indirgeyici aminasyon aktivitesi için optimum çalışma koşullarının sırasıyla pH 9,0 ve 25°C, pH 9,0 ve 35°C, pH 9,5 ve 45°C, pH 10,0 ve 40°C olduğunu ortaya koydu. Oksidatif deaminasyon için ise sırasıyla pH 10,5 ve 25°C, pH 10,0 ve 30°C, pH 10,5,0 ve 40°C, pH 10,0 ve 35°C optimum koşullar olarak belirlendi. Tüm mutantlar, indirgeyici aminasyon için katalitik verimliliği 1,73 ila 3,13 kat ve oksidatif deaminasyon için 1,80 ila 3,04 kat arasında artırdı. Katalitik aktivitenin değişmesinin altında yatan mekanizmayı ortaya çıkarmak için, MD simülasyonları ve enzimlerin aktif bölgesi incelendi. Mutantların, aktif bölge aminoasitleri ve pirüvat arasındaki bağların veya bağ tiplerinin uzunluklarını değiştirdiği gözlemlendi. Ayrıca, mutantların global enzim dinamiği üzerinde de etkili oldukları gözlemlendi. Metallerin indirgeyici aminasyon enzim aktivitesi üzerinde önemli bir inhibe edici/artırıcı etkiye yol açtığı belirlendi. İndirgeyici aminasyon aktivitesinin depolama kararlılığının da, metal iyonların varlığından etkilendiği tespit edildi.

Anahtar Kelimeler: *Amycolatopsis sulphurea*, L-Alanin Dehidrogenaz, Moleküler Dinamik Simülasyonları, Karakterizasyon, Mutasyon, Aktif Bölge Modellemesi

ACKNOWLEDGEMENTS

I would like to express my foremost gratitude to my esteemed supervisor, Assist. Prof. Dr. Onur SERÇİNOĞLU, for his immense knowledge, unwavering motivation, exceptional patience, and continuous support. He provided me with the Bioinformatics and Systems Biology infrastructure necessary to conduct the computational aspects of my master's thesis.

I am profoundly grateful to my co-advisor, Assoc. Prof. Dr. Fatih AKTAŞ, for providing me with the invaluable opportunity to work on this project and for accompanying me throughout my MSc journey with his profound knowledge and guidance.

I extend my gratitude to Prof. Dr. Barış BİNAY, whose experience, knowledge, and patience were a constant source of support. He generously provided me with access to the Gebze Enzyme Recognition Center (GERC) infrastructure, enabling me to conduct the experimental portion of my master's thesis.

I am profoundly grateful to my colleagues in the Bioengineering Department at Gebze Technical University for their unwavering support throughout my MSc journey.

Finally, I would like to express my deepest appreciation to my beloved family, especially my mother Perihan OKUR and my father Faruk OKUR. Their endless support, love, and belief have profoundly shaped the individual I am today, and they have always been by my side throughout this journey. My deepest thanks are also extended to my spouse, Mustafa AKSU, for his consistent support and love.

Furthermore, I wish to express my appreciation to the Düzce University Scientific Research Coordinator (DÜBAP) for providing financial support for our project (Project Number: 2023.06.02.1421).

TABLE OF CONTENTS

ABSTRACT	vi
ÖZET	vii
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	ix
LIST OF SYMBOLS AND ABBREVIATIONS	xi
LIST OF FIGURES	xii
LIST OF TABLES	xvi
 1. INTRODUCTION	 1
1.1. Proteins	1
1.2. Enzymes	1
1.2.1. Enzymes in Industry	3
1.3. Enzyme Engineering	5
1.4. L-Alanine Dehydrogenase	7
1.4.1. Applications of AlaDH in Literature	8
1.5. Computational Enzyme Engineering	11
2. PURPOSE OF THE STUDY	14
3. MATERIALS AND METHODS	15
3.1. Materials	15
3.1.1. Laboratory Devices	15
3.1.2. Enzymes and Strains	16
3.1.3. Kits and Purifications Equipments	16
3.1.4. Chemicals	16
3.1.5. Solutions, Buffers and Media	17
3.1.6. Databanks, Programmes, Softwares, Servers and Hardwares	20
3.2. Methods	21
3.2.1. Determination of Mutant Candidates Based on Rational Enzyme Engineering	21
3.2.2. Primer Design and Site Directed Mutagenesis	23
3.2.3. Transformation of L- <i>As</i> AlaDH Plasmids to Competent Cells	24
3.2.4. Preperation of Glycerol Stock and Pellet Stock	25
3.2.5. Expression and Purification of L- <i>As</i> AlaDH Enzymes	25
3.2.6. Optimization of Activity Parameters for L- <i>As</i> AlaDHs	26
3.2.7. Determination of Optimum Working Conditions	26
3.2.8. Determination of Kinetic Parameters of L- <i>As</i> AlaDH Enzymes	27
3.2.9. Storage Stability and Effect of Metal Ions on L- <i>As</i> AlaDH Enzymes for Reductive Amination Activity	27
3.2.10. Molecular Dynamics Simulations	28
3.2.11. Analysis of Molecular Dynamics Simulations	29
3.2.11.1. RMSD and RMSF Profiles of MD Simulations	29
3.2.11.2. Molecular Mechanics Poisson-Boltzman Surface Area (MM-PBSA) Analysis	30
3.2.11.3. Calculation of Non-bonded Pairwise Amino Acid Interaction Energies and Construction of Protein Energy Networks	30
4. RESULTS	32
4.1. Rational Design of L- <i>As</i> AlaDH Enzymes	32

4.2. Mutant Production of L- <i>As</i> AlaDH	44
4.3. Purification of Wild-type and Mutant L- <i>As</i> AlaDH enzymes	47
4.4. Determination of Optimum pH and Temperature for L- <i>As</i> AlaDH Enzymes	49
4.5. Determination of Kinetics Parameters of L- <i>As</i> AlaDH Enzymes	53
4.6. Effects of Metals on L- <i>As</i> AlaDH Enzymes and Statistical Analysis	56
4.7. Storage Stability	57
4.8. Active Site Invetigation	58
4.9. Analysis of Molecular Dynamics Simulations	64
4.9.1. RMSD, RMSF and MM-PBSA Analysis	64
4.9.2. Interaction Energy Network (PEN) Analysis	70
5. DISCUSSION	80
6. CONCLUSIONS	86
REFERENCES	87
BIOGRAPHY	95
PUBLICATIONS AND PRESENTATIONS FROM THE THESIS	96

LIST OF SYMBOLS AND ABBREVIATIONS

Δ	: Difference
ΔG	: Difference in Gibbs Free Energy
2D	: 2-Dimensional
3D	: 3-Dimensional
BCA	: Bicinchoninic Acid Assay
DNA	: Deoxiribo Nucleic Acid
FEB	: Free Binding Energy
gRINN	: get Residue Interaction eNergies and Networks
k_{cat}	: Turnover number of an enzyme
kDa	: kiloDalton
K_M	: Apparent Michaelis–Menten constant
L – AsAlaDH	: <i>Amycolatopsis sulphurea</i> derived L-Alanine Dehydrogenase
L – AlaDH	: L-Alanine Dehydrogenase
LB	: Luria-Bertani
MD	: Molecular Dynamics
mM	: Milimolar
MM/PBSA	: Molecular Mechanics Poisson–Boltzmann Surface
MSA	: Multiple Sequence Alignment
NAD(H)	: Nicotinamide Adenine Dinucleotide Hydrogen
PEN	: Protein Energy Networks
PCR	: Polymerase Chain Reactions
RMSD	: Root Mean Square Deviation
RMSF	: Root Mean Square Fluctuation
SASA	: Solvent Accessible Surface Area
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TIP3P	: Transferable Intermolecular Potential With 3 Points
VMD	: Visual Molecular Dynamics

LIST OF FIGURES

	<u>Page</u>
Figure 1.1: Demonstration of the enzyme-substrate complex formation.	3
Figure 1.2: Enzyme engineering methods.	6
Figure 1.3: The reaction mechanism of L-alanine dehydrogenase catalyzes.	7
Figure 1.4: Active site of L-AlaDH enzyme.	8
Figure 1.5: Representation of the active site of the 3D enzyme structure.	13
Figure 4.1: Multiple sequence alignment result. Conserved residues are shown in red, and active site residues are shown with arrows, respectively (Arg15, Lys75, His96 and Asp270).	33
Figure 4.2: Heatmap of percent sequence identity matrix.	34
Figure 4.3: Superpositioning of <i>Mycobacterium tuberculosis</i> (PDB id: 2VHX, green) and homology model of the <i>Amylocaptosis sulphurea</i> L-alanine dehydrogenase (Purple).	35
Figure 4.4: Ramachandran plot result (a) and model structure (b) of the homology modelling study.	36
Figure 4.5: Active site, ligands and location of computationally mutated residues (a), computationally mutated residues (b).	37
Figure 4.6: $\Delta\Delta G$ – mutated position graph of 1026 mutants. X-axis shows the mutated position, Y-axis shows amino acid in the mutated structure. Each cell shows $\Delta\Delta G$ scores of the mutants in kcal/ mol. Stabilizing mutants (negative values) are shown in red, destabilizing mutants (negative values) are shown in green colors.	38
Figure 4.7: MSA result of <i>Bacillus subtilis</i> 168, <i>Bacillus cereus</i> , <i>Mycobacterium smegmatis</i> MC2 155 and <i>Geobacillus stearothermophilus</i> organisms derived L-AlaDH enzyme sequences.	40
Figure 4.8: B-factor values of each aminoacids in the wild-type protein. Black line shows B-factor values, red line shows the average B-factor value.	42
Figure 4.9: Analysed properties of primer designed for Tyr1Asn mutant.	44
Figure 4.10: Analysed properties of primer designed for Ile97Leu mutant.	44
Figure 4.11: Analysed properties of primer designed for Ser192Ala mutant.	45
Figure 4.12: Visualization of mutants and wild-type on agarose gel electrophoresis. M: Molecular Weight Marker, WT: Wild-type plasmid, Ser192Ala: Ser192Ala mutant plasmid, Ile97Leu: Ile97Leu Mutant, Tyr14Asn: Tyr14Asn mutant, <i>DpnI</i> Ser192Ala: <i>DpnI</i> manipulated Ser192Ala mutant, <i>DpnI</i> Ile97Leu: <i>DpnI</i> manipulated Ile97Leu mutant, <i>DpnI</i> Tyr14Asn: <i>DpnI</i> manipulated Tyr14Asn.	46
Figure 4.13: Alignment of sequencing results compared to wild-type.	47
Figure 4.14: After purification, SDS page image belongs to Ile97Leu and wild-type. 1, 12, 23 and 31: Protein marker, 2: Flowthrough elution fraction belongs to Ile97Leu, 3: Buffer A elution fraction belongs to Ile97Leu, 4, 5, 6: 100mM imidazol	48

including buffer A elution fractions belong to Ile97Leu, 7, 8, 9, 10,11: 200mM imidazol including buffer A elution fractions belong to Ile97Leu, 13, 14,15: 400mM imidazol including buffer A elution fractions belong to Ile97Leu, 16: Buffer B elution fraction belongs to Ile97Leu, 17: Flowthrough elution fraction belongs to wild-type, 18: Buffer A elution fraction belongs to wild-type, 19,20,21: 100mM imidazol including buffer A elution fractions belong to wild-type, 22, 24, 25,26,27: 200mM imidazol including buffer A elution fractions belong to wild-type, 28, 29, 30: 400mM imidazol including buffer A elution fractions belong to wild-type, 32: Buffer B elution fraction belongs to wild-type.

- Figure 4.15:** After purification, SDS page image belong to Ser192Ala and Tyr14Asn. 1, 12, 23 and 31: Protein marker, 2: Flowthrough elution fraction belongs to Ser192Ala, 3: Buffer A elution fraction belongs to Ser192Ala, 4, 5, 6: 100mM imidazol including buffer A elution fractions belong to Ser192Ala, 7, 8, 9, 10,11: 200mM imidazol including buffer A elution fractions belong to Ser192Ala, 13,14,15: 400mM imidazol including buffer A elution fractions belong to Ser192Ala, 16: Buffer B elution fraction belongs to Ser192Ala, 17: Flowthrough elution fraction belongs to Tyr14Asn, 18: Buffer A elution fraction belongs to Tyr14Asn, 19, 20,21: 100mM imidazol including buffer A elution fractions belong to Tyr14Asn, 22, 24, 25, 26, 27: 200mM imidazol including buffer A elution fractions belong to Tyr14Asn, 28, 29, 30: 400mM imidazol including buffer A elution fractions belong to Tyr14Asn, 32: Buffer B elution fraction belongs to Tyr14Asn. 49
- Figure 4.16:** Effects of different pH (Citric Acid-Sodium Citrate: pH 3.0-5.5, Sodium Phosphate: pH6.0-7.5, Tris-HCl: pH8.0-9.0, Glycine-NaOH: pH 9.0-10.0, Sodium carbonate-Sodium bicarbonate: pH9.5-11.0) on reductive amination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala. 50
- Figure 4.17:** Effects of different pH (Citric Acid-Sodium Citrate: pH 3.0-5.5, Sodium Phosphate: pH6.0-7.5, Tris-HCl: pH8.0-9.0, Glycine-NaOH: pH 9.0-10.0, Sodium Carbonate-Sodium Bicarbonate: pH9.5-11.0) on oxydative deamination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala. 51
- Figure 4.18:** Effects of different temperatures (25-55°C) on reductive amination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala. 52
- Figure 4.19:** Effects of different temperatures (25-55°C) on oxydative deamination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala. 53
- Figure 4.20:** Michealis-Menten graphs of reductive amination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala. 54

Figure 4.21: Michaelis-Menten graphs of oxidative deamination catalyzed by L- <i>AsAlaDH</i> : (a) wild-type, (b) Tyr14Asn, (c) Ile97Leu, and, (d) Ser192Ala.	54
Figure 4.22: Metal binding statistics of reductive amination catalyzed by L- <i>AsAlaDH</i> : a) wild-type b) Tyr14Asn c) Ile97Leu and, d) Ser192Ala.	57
Figure 4.23: Storage stability of reductive amination catalyzed by L- <i>AsAlaDH</i> : a) wild-type b) Tyr14Asn c) Ile97Leu and, d) Ser192Ala.	58
Figure 4.24: (a) 2D and (b) 3D active site representation of wild-type enzyme during reductive amination activity (yellow dashes represent the bonds).	59
Figure 4.25: (a) 2D and (b) 3D active site representation of Tyr14Asn enzyme during reductive amination activity (yellow dashes represent the bonds).	59
Figure 4.26: (a) 2D and (b) 3D active site representation of Ile97Leu enzyme during reductive amination activity (yellow dashes represent the bonds).	60
Figure 4.27: (a) 2D and (b) 3D active site representation of Ser192Ala enzyme during reductive amination activity (yellow dashes represent the bonds).	61
Figure 4.28: (a) 2D and (b) 3D active site representation of wild-type enzyme during oxidative deamination activity (yellow dashes represent the bonds).	61
Figure 4.29: (a) 2D and (b) 3D active site representation of Tyr14Asn enzyme during oxidative deamination activity (yellow dashes represent the bonds).	62
Figure 4.30: (a) 2D and (b) 3D active site representation of Ile97Leu enzyme during oxidative deamination activity (yellow dashes represent the bonds).	63
Figure 4.31: (a) 2D and (b) 3D active site representation of Ser192Ala enzyme during oxidative deamination activity (yellow dashes represent the bonds).	63
Figure 4.32: RMSD (Å)-time profiles of wild-type and mutant L- <i>AsAlaDH</i> -NAD(H) complex.	65
Figure 4.33: RMSF (Å)-Residue profiles of wild-type and mutant L- <i>AsAlaDH</i> -NAD(H) simulations.	66
Figure 4.34: FEB (kcal/mol)-time profile of L- <i>AsAlaDH</i> -NAD(H) simulations.	67
Figure 4.35: RMSD (Å)-Time profiles of L- <i>AsAlaDH</i> -NAD(H)-Pyr simulations.	68
Figure 4.36: RMSF (Å)-Time profiles of L- <i>AsAlaDH</i> -NAD(H)-Pyr simulations.	68
Figure 4.37: FEB (kcal/mol)-time profile NAD(H)molecule on L- <i>AsAlaDH</i> -NAD(H)-Pyr simulations.	69
Figure 4.38: FEB (kcal/mol)-time profile pyruvate molecule on w L- <i>AsAlaDH</i> -NAD(H)-Pyr simulations.	70
Figure 4.39: Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction	72

of the Tyr14Asn mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H)-Pyr simulations.

- Figure 4.40:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ile97Leu mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H)-Pyr simulations. 73
- Figure 4.41:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ser192Ala mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H)-Pyr simulations. 74
- Figure 4.42:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Tyr14Asn mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H) simulations. 75
- Figure 4.43:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ile97Leu mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H) simulations. 76
- Figure 4.44:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ser192Ala mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H) simulations. 77
- Figure 4.45:** Betweenness centrality levels that changed most significantly upon mutations. 78
- Figure 1.46:** Residues with changes in their highest betweenness centrality values. 79

LIST OF TABLES

	<u>Page</u>
Table 1.1: Enzyme classification based on reaction mechanism.	3
Table 1.2: Mutagenesis studies on L-alanine dehydrogenases from different organisms.	10
Table 3.1: Laboratory devices, their brands and purpose of their use during the thesis study.	15
Table 3.2: Purification equipments and kits used in the thesis studies.	16
Table 3.3: Chemicals used in the experimental process.	16
Table 3.4: Media used in the experimental process.	17
Table 3.5: Solutions and buffers	17
Table 3.6: Solutions used to prepare competent cells.	18
Table 3.7: Solutions used in SDS-PAGE gel electrophoresis.	19
Table 3.8: Utilized softwares, servers and hardware for mutation scanning and MD simulation performing and analyzing.	20
Table 3.9: Organisms from which L-alanine dehydrogenases were obtained and characterized previously in literature.	22
Table 3.10: PCR components.	24
Table 4.1: Experimental activities of AlaDH from <i>Bacillus subtilis</i> 168, <i>Bacillus cereus</i> , <i>Mycobacterium smegmatis</i> MC2 155 and <i>Geobacillus stearothermophilus</i> , and amino acids found in positions identified from MSA and computational saturation mutagenesis results.	41
Table 4.2: SASA values of position 14 and 97 detected for mutants and wild-type.	43
Table 4.3: Changing the pKa values of active site residues on Tyr14Asn and Ile97Leu compared to wild-type L- <i>As</i> AlaDH.	43
Table 4.4: Optimized PCR steps during mutat creation process.	45
Table 4.5: Concentration and 260/280 ratio of wild-type, Tyr14Asn, Ile97Leu and Ser192Ala.	46
Table 4.6: Kinetics values belong to reductive amination and oxidative deamination catalysis of wild-type and mutant L- <i>As</i> AlaDH.	55
Table 4.7: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Tyr14Asn mutation into the wild-type L- <i>As</i> AlaDH enzyme in L- <i>As</i> AlaDH-NAD(H)-Pyr simulations.	73
Table 4.8: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Ile97Leu mutation into the wild-type L- <i>As</i> AlaDH enzyme in L- <i>As</i> AlaDH-NAD(H)-Pyr simulations.	73
Table 4.9: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Ser192Ala mutation into the wild-type L- <i>As</i> AlaDH enzyme in L- <i>As</i> AlaDH-NAD(H)-Pyr simulations.	74
Table 4.10: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon	75

introduction of the Tyr14Asn mutation into the wild-type L- <i>AsAlaDH</i> enzyme in L- <i>AsAlaDH</i> -NAD(H) simulations.	
Table 4.11: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Ile97Leu mutation into the wild-type L- <i>AsAlaDH</i> enzyme in L- <i>AsAlaDH</i> -NAD(H) simulations.	76
Table 4.12: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Ser192Ala mutation into the wild-type L- <i>AsAlaDH</i> enzyme in L- <i>AsAlaDH</i> -NAD(H) simulations.	77
Table 5.1: Reported optimum working conditions and kinetic parameters of L-AlaDH enzymes.	81

1. INTRODUCTION

1.1. Proteins

Proteins are biological macromolecules synthesized in living organisms, performing a multitude of vital activities. For instance, enzymes catalyze reactions, hormones regulate bodily functions, carrier proteins transport molecules throughout the body, antibodies form part of the immune defense system, and receptor proteins act as signal receivers. Furthermore, some proteins contribute to the structural integrity of an organism. Consequently, protein synthesis is a crucial process for all living organisms.

Protein synthesis requires essential components: amino acids. Each amino acid comprises two main parts: a backbone and a side chain. The side chain, or R-group, is unique for each of the 20 common amino acids (Blanco, 2017). The diverse properties of amino acids, dictated by their unique side chains, enable their use across various industries, including food, pharmaceuticals, animal feed, medicine, and cosmetics. Beyond their role in protein synthesis, amino acids enhance food bioavailability, regulate body adiposity, promote growth and immunity, and control muscle protein metabolism. Given their critical importance in bodily processes, amino acid deficiencies can lead to various health problems for organisms. Consequently, amino acids are often supplemented in diets to prevent such issues (Tuo et al., 2023).

Amino acids can be synthesized through several approaches, including extraction from natural sources or protein hydrolysates. Another method for amino acid production involves biological processes (Wendisch, 2020). The increasing trend towards biological processes has led to the widespread use of enzymes for amino acid production, owing to their numerous industrial advantages (D'Este et al., 2018; Tuo et al., 2023).

1.2. Enzymes

Enzymes are specialized proteins that accelerate reaction rates during catalysis within various cellular pathways of organisms. Each enzyme, therefore, plays a distinct role in cellular processes such as metabolism, immune system reactions, gene expression,

and cell division. Enzymes consist of both proteinaceous and non-proteinaceous regions; the latter are known as cofactors and are composed of organic molecules (like vitamins) or metal ions.

Enzymatic reactions convert a substrate into a product. Each reaction proceeds through a specific energy profile. To form a product, an enzyme-substrate complex must first be established, and this complex must then overcome a specific energy barrier. Enzymes reduce this required energy barrier, known as the activation energy, thereby increasing the reaction rate.

Enzymes are highly specific to their target substrates, binding them to facilitate conversion into products. The substrate binding site, a small region of the enzyme, is known as the active site (**Figure 1.1**). This active site, typically composed of fewer than 10 amino acids, ensures the specific binding of appropriate substrates.

Due to enzyme specificity, various enzyme-substrate interaction models have been proposed. In the lock-and-key model, the ligand and enzyme binding site are thought to perfectly match, implying rigidity in both the enzyme and substrate. This rigidity was once believed to be the source of enzyme specificity. However, it is now understood that biological macromolecules, including enzymes, are flexible. Consequently, enzymes can recognize their substrates and enhance ligand binding energy by altering their active site—an approach termed the induced-fit model. While the induced-fit model also describes enzyme-substrate binding, it is believed that enzymes undergo conformational changes to create a more stable complex. This is crucial because enzyme stability, sequence, and three-dimensional (3D) structure are all determinants of its activity and function. Enzymes are classified according to their functions (**Table 1.1**).

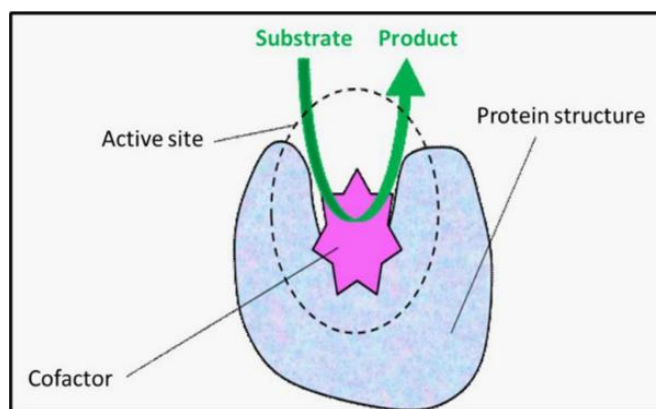


Figure 1.1: Demonstration of the enzyme-substrate complex formation (Buaki-Sogó et al., 2020)

Table 1.1: Enzyme classification based on reaction mechanism (Di Cera, 2020; Porto de Souza Vandenberghe et al., 2020).

Enzyme Comission (EC) Number	Enzyme Classes	Reaction Mechanism
EC1	Oxidoreductases	Catalyze oxidation-reduction type reactions in the presence of a cofactor. Covers one-third of whole enzymatic activities.
EC2	Transferases	Catalyze group transfer from substrate to product.
EC3	Hydrolases	Catalyze hydrolysis reactions.
EC4	Lyases	Catalyze addition or removal of chemical groups, leading to the formation of double bonds or new rings, or the addition of a new group across a double bond.
EC5	Isomerases	Catalyze rearrangement of intramolecular changes and produces isomers of the substrate.
EC6	Ligases	Catalyze attachment reactions involve the joining of two molecules.
EC7	Translocases	Catalyze movement of molecules across the cell membrane.

1.2.1. Enzymes in Industry

Bioprocess technology, which leverages living organisms or their derivatives to yield valuable chemicals, is gaining prominence in industry. This technology is progressively replacing traditional chemical production methods due to its inherent advantages. Specifically, bioprocesses frequently utilize enzymes for industrial

production. Enzymes are highly environmentally friendly, as they generate fewer, non-toxic by-products and can be readily inactivated. Furthermore, their reusability and superior catalytic properties confer significant economic advantages (Victorino da Silva Amatto et al., 2022a). The increasing demand for enzymes is significantly boosting their presence in the global marketplace. Projections indicate that the global enzyme market is estimated to reach approximately USD 20.31 billion by 2030

(*Industrial Enzymes Market Trend, Size, Global, Share | MRFR*, 2025). Today, enzymes find applications in diverse fields, including pharmaceuticals, agriculture, fuel production, cosmetics, food and beverages, textiles, waste management, and leather processing. The majority of industrial enzymes are derived from bacteria and primarily belong to the hydrolase enzyme family. Notably, 70% of these enzymes are proteases and carbohydrates.

Enzymes play a crucial role in various industrial applications. For instance, detergents incorporate enzymes for the breakdown of lipids, carbohydrates, and proteins. In the textile industry, cellulases are employed to soften fabrics and impart specific colors. The paper and pulp industry heavily relies on amylases to control properties like viscosity, softness, and brightness in paper products. The food industry utilizes diverse enzymes; for example, lipases and laccases are crucial in dairy processing, including the production of lactose-free products. Additionally, cellulases are essential in the bioethanol production process.

Despite their advantages, enzymes also present certain drawbacks for industrial application. Industrial processes often operate under conditions harsh for enzymes, including high temperatures, extreme pH levels, and the presence of various organic solvents. These demanding environments can lead to undesirable changes in enzymatic properties, such as altered activity or denaturation. Additionally, enzymes typically have a short half-life, limiting their stability over time. Furthermore, they may not be naturally adapted to non-natural substrates, and their inhibition mechanisms by substrates or products may not be optimized for industrial efficiency.

Therefore, enzymes are required to exhibit substantial stability throughout industrial processing steps. The stability range for most naturally occurring enzymes is often narrow. However, given their inherent advantages, it is essential for wild-type enzymes to be adapted for industrial applications. Fortunately, enzyme properties can be

effectively improved through various engineering methods (Fasim et al., 2021; Mesbah, 2022).

1.3. Enzyme Engineering

The revolution brought about by the development of PCR technology in the 1970s significantly transformed methods within the pharmaceutical industry. This breakthrough provided new application areas for enzymes and simplified production processes. Moreover, PCR technology enabled the engineering of enzymes to regulate their thermostability, substrate specificity, and activity. Specifically, PCR was applied to the enzyme's DNA sequence to alter its amino acid sequence (Victorino da Silva Amatto et al., 2022b). Various methods can be used to perform enzyme engineering (Ali et al., 2020; Ferreira et al., 2022).

Directed evolution is one of the enzyme engineering techniques that is used to tailor properties of an enzyme such as catalytic or functional properties. Directed evolution is a method based on random mutagenesis. During PCR amplification, cloned regions of DNA can be mutated using polymerases with high mutations rates, and conditions of PCR can be changed. Directed evolution then takes place via a process that produces mutants in repetitive rounds with the purpose of creating a mutant library. There are some advantages and disadvantages of creating mutant libraries with directed evolution. Directed evolution does not require any knowledge for enzyme structure and function. However, it requires generation of a large mutant library. Thus, intensive library screening is required for colony selection. Because of this, directed evolution is a costly process, requires labor-intensive studies, and thus also time-consuming (Nielsen et al., 2023; Sharma et al., 2021a).

Semi-rational design is an enzyme engineering approach employed to mitigate the disadvantages of directed evolution, particularly the extensive library screening, intensive laboratory work, and lengthy time commitment. This method leverages computational tools to theoretically predict critical residues or regions for desired enzyme properties, such as enhanced activity or thermostability, or improved substrate specificity and selectivity. Consequently, semi-rational design facilitates the creation

of smaller, more focused, and logically constructed libraries (Nielsen et al., 2023; Sharma et al., 2021).

Rational design is an enzyme engineering method that utilizes the enzyme's structural information to elucidate the structural properties and functional role of its active site. (Sharma et al., 2021). During the rational design process, candidate residues for mutation are identified using the enzyme's structural information. Various methods exist to determine these residues. One prominent approach is enzyme structure analysis, which involves examining the enzyme's crystal structure. This method allows for the precise identification of amino acids located within the substrate binding pocket, as these residues directly interact with the substrate. Consequently, mutating amino acids within the binding pocket can be highly beneficial for engineering enzymes with desired properties.

Designing proteins using computational methods largely builds on evolutionary sequence information to create a library (Sharma et al., 2021). Moreover, structure information can be utilized. Modern approaches to computational enzyme engineering utilize both sequence and structure information to decrease the number of variants that will be tested (**Figure1.2**).

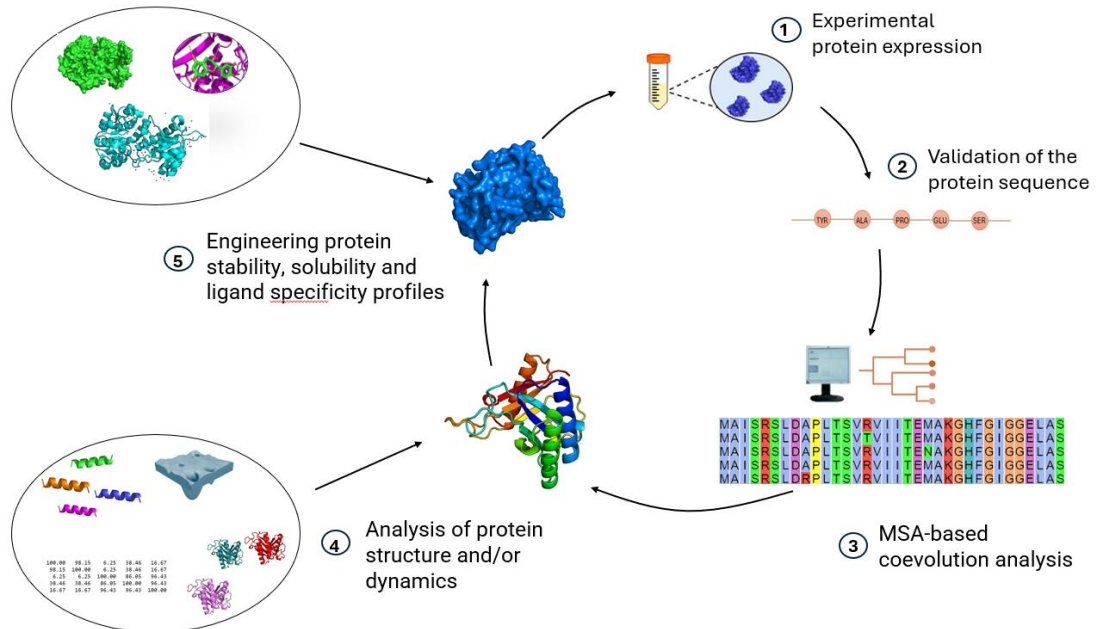


Figure 1.2: Enzyme Engineering Methods (Okur & Serçinoğlu, 2025).

1.4. L-Alanine Dehydrogenase

L-alanine dehydrogenase (L-AlaDH) is a microorganism-derived oxidoreductase enzyme that plays a crucial role in the life cycle of organisms. As an intracellular enzyme, it is primarily isolated from the cellular membrane. L-AlaDH catalyzes the reversible conversion of L-alanine, one of the smallest amino acids, to pyruvate (**Figure 1.3**). In the oxidative deamination reaction (the forward reaction), L-alanine serves as a substrate for conversion to pyruvate, while the NAD(H) molecule is utilized as a cofactor in the presence of ammonium, undergoing oxidation to NAD⁺.

This reaction is crucial for pyruvate production within the cell, particularly during the tricarboxylic acid (TCA) cycle. In the reductive amination reaction (the backward reaction), the pyruvate molecule is converted to the L-alanine amino acid. During this process, NAD⁺ is utilized as a cofactor and is reduced to NAD(H). This provides alanine for cellular needs, especially for protein synthesis. The reversible nature of the L-AlaDH reaction not only fulfills cellular alanine and pyruvate requirements but also maintains the NAD(H)/ NAD⁺ balance within the cell. Moreover, these reactions are significant for nitrogen assimilation (Dave & Kadeppagari, 2019a).

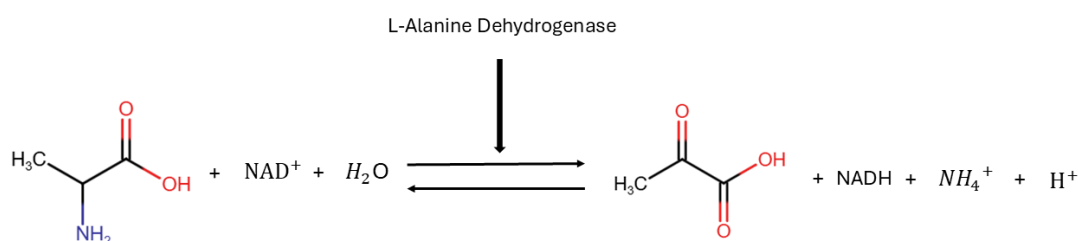


Figure 1.3: The reaction L-alanine dehydrogenase catalyzes.

L-alanine production via the reductive amination reaction catalyzed by L-AlaDH plays a significant role in industry due to the wide range of applications of L-alanine. Its main uses span pharmaceuticals, cosmetics, food and beverages, veterinary medicine, and feed supplements. For instance, sweet taste of L-alanine makes it valuable in food and beverage products and food supplements. In the pharmaceutical industry, it serves as a chiral precursor for various drugs and can be administered parenterally before or after surgical operations.

1.4.1. Applications of AlaDH in Literature

Given the industrial significance of both L-alanine and pyruvate, the L-AlaDH catalyzed reaction has been extensively studied in the literature (**Table 1.2**). These investigations primarily aim to understand its reaction mechanism and enhance its activity. He et al. (2018) analyzed the conserved regions in the protein sequence of the L-AlaDH enzyme in *Phormidium lapideum*, *Mycobacterium tuberculosis*, *Thermus thermophilus* and *Bacillus pseudofirmus* OF4 organisms, and it was reported that the pyruvate molecule binds to the highly conserved catalytic region with the amino acids Arg-15, Lys75, His-96 and Asp-270, especially in the sequencing study of *M. tuberculosis* organism (**Figure 1.4**).

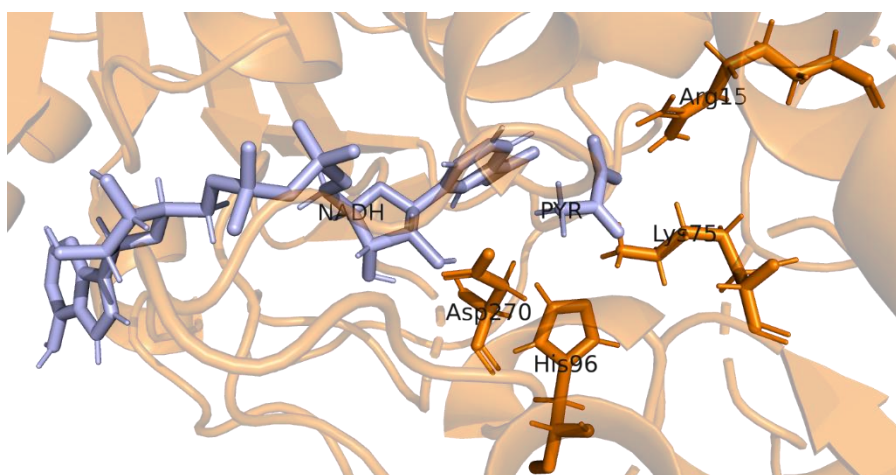


Figure 1.4: Active site of L-AlaDH enzyme.

In this study, the Lys73Ala mutation in *Bacillus pseudofirmus* OF4 was reported to increase L-AlaDH enzyme activity compared to the wild-type. Based on the activity data, it was concluded that the conserved Lys73 may play a role in catalytic activity (He et al., 2018). In another study, a molecular dynamics simulation of the L-AlaDH enzyme from *Mycobacterium tuberculosis* was performed. The study reported that changes and mutations in the active site's highly conserved and stable residues, such as His96 and Asp270, would lead to enzyme inactivity. Specifically, Asp270 was found to maintain the stability of the NAD(H) sugar and nicotinamide rings, while His96 plays a role in the protein's conformational change through its interaction with Gln271. It was also determined that Met301 prevents the rotation of the NAD(H) nicotinamide ring by fixing it in place (Ling et al., 2012). In another study, it was

determined that Asp270 and His96 of AlaDH originating from *Mycobacterium tuberculosis* are involved in catalytic activity within the active site (Tripathi & Ramachandran, 2008). AlaDH from *Mycobacterium tuberculosis* comprises two distinct subunits. One subunit, encompassing amino acids 128 and 309-371, is responsible for substrate binding. The other subunit, spanning amino acids 129-308, constitutes the NAD(H)-binding region. Within the scope of that research, mutations His96Ala, Asp270Ala, and Asp270Asn were introduced into the conserved amino acids His96 and Asp270, which are known to be part of the catalytic region. The result was a complete loss of enzyme activity in all three mutations (Ågren et al., 2008a). In another study where mutations were made at Phe94 and Leu130 points on L-AlaDH (PDB: 2VHX) from *Mycobacterium tuberculosis*, considering that larger amino acids would affect binding, the binding site was examined by replacing phenylalanine and leucine amino acids with relatively smaller alanine and serine amino acids at Phe94 and Leu130, and the effect on substrate binding and the diversity were investigated. As a result, it was reported that Phe130Ala and Phe130Ser mutations decreased the solubility of the enzyme. Position 94 was preferred by amino acids with more small, branched and hydrophilic side chains, while position 94 was preferred by hydrophobic, unbranched side chains. In addition, Phe94Ser mutation was reported to confer activity against L-norleucine, L-leucine, L-methionine, L-homophenylalanine and L-allysine ethylene acetal, L-norvaline amino acids, which the wild-type AlaDH enzyme lacks. Leu130, Met133, Tyr117, Leu133 were also subjected to mutations as well. While an F94S-like effect was observed in mutations in Leu130, no higher activity was obtained than wild-type with Met133 and Leu313. Tyr117 mutations also showed activity against L-norleucine, L-norvaline and L-leucine amino acids. Phe94Ser/Tyr117Leu double mutation showed an increase in activity against L-norleucine, L-leucine, L-norvaline, L-methionine and L-homophenylalanine amino acids, but a decrease in activity against alanine, the main substrate, was reported in all mutations (Fernandes et al., 2015). In another study, cofactor specificity of reductive amination reaction was changed from NAD(H) to NADPH and a double mutation (Asp196Ala/Leu197Arg) were examined in *Bacillus subtilis* organism. However, neither mutation nor cofactor change did not significantly alter catalytic efficiency (Lerchner et al., 2016).

Table 1.2: Mutagenesis studies on L-alanine dehydrogenases from different organisms.

Mutation	Organism	Effect	References
Lys73Ala	<i>Bacillus pseudofirmus</i> OF4	6-fold increase in catalytic efficiency.	(He et al., 2018)
Arg15Ala		Complete activity loss.	
Lys75Ala			
His95Ala			
Asp269Ala			
His96Ala	<i>Mycobacterium tuberculosis</i>	Complete activity loss.	(Ågren et al., 2008b)
Asp270Asn			
Asp270Ala			
Asp196Ala/Leu197Arg	<i>Bacillus subtilis</i>	Changing cofactor specificity together with dual mutation application did not significantly changed catalytic efficiency.	(Lerchner et al., 2016)
Phe94Ser/Tyr117Leu	<i>Mycobacterium tuberculosis</i>	Increased activity toward different substrates, however decreased activity with its original substrate. Also, Phe94Ser/Tyr117Leu showed better activity than single Phe94Ser and Phe94Ala mutant.	(Fernandes et al., 2015)
Phe94Ser		Increased activity toward different substrates, however decreased activity to its original substrate.	
Phe94Ala			
Leu130Ser		Protein found in insoluble lysate fraction	
Leu130Ala			

Table 1.2 presents experimental studies detailing the enhancement of L-AlaDH enzyme activity through various enzyme engineering approaches. Creating mutations in an enzyme can alter its substrate specificity, cofactor specificity, and either increase or decrease its activity. These findings contribute to our understanding of the enzyme's structure-activity relationship, ultimately enabling the development of superior mutants with improved properties, particularly for industrial applications.

1.5. Computational Enzyme Design

Enzyme stability is a crucial phenomenon for industrial applications. The structural integrity of an enzyme is maintained by various non-covalent interactions, including salt bridges, Van der Waals forces, hydrogen bonds, and hydrophobic interactions. Consequently, enzymatic stability can be enhanced by introducing new interactions within a specific distance from the binding site. Substrate stability also influences enzyme activity by contributing to the rigidity of the substrate at the active site (Xie et al., 2014).

Creating new interactions necessitates understanding the protein structure; thus, various methods exist to enhance enzymatic stability, including mutation-based design using computer-aided methods. Root Mean Square Fluctuations (RMSF) calculations from molecular dynamics (MD) simulations and B-factor analysis are computer-aided methods employed to understand the dynamics of amino acid residues within a protein structure. Every atom within a structure fluctuates to reach equilibrium. Flexible residues exhibit greater fluctuation than other protein regions and contribute less to protein stability due to fewer contacts with other residues. For enzyme engineering, identifying highly fluctuating residues is a method for determining which residues impact stability. Increasing overall rigidity of protein may require increasing the rigidity of specific residues at certain positions (Xie et al., 2014; Xing et al., 2021).

Creating desired enzyme variants with enhanced stability, altered substrate selectivity or specificity, or modified catalytic properties often require scanning a mutant library using computational methods. Computational saturation mutagenesis is a valuable technique for this virtual screening, enabling the systematic substitution of a single amino acid with all 19 other standard amino acids within a computational environment. For each resulting mutant, the Gibbs free energy (ΔG) difference between the mutant and wild-type protein, termed the $\Delta\Delta G$ score, can be calculated. Various software tools exist to predict protein stability improvements, with FoldX being a prominent example. FoldX not only facilitates the design of stable or thermostable enzymes but also offers visualization capabilities through its integrated tools. It calculates the $\Delta\Delta G$ score, where negative values indicate stabilizing mutations. However, mutants identified via saturation mutagenesis must be further validated using complementary methods, such as solubility prediction programs (Aalbers et al., 2020; Buß et al., 2018; Christensen & Kepp, 2012a; Zhao et al., 2021).

Enzyme activity is directly influenced by its solubility; for instance, insoluble proteins typically exhibit low activity, whereas soluble proteins demonstrate higher activity. Insoluble proteins may possess issues with their tertiary structure or even incorrect folding. Low solubility often leads to the formation of aggregates in aqueous media. Especially when engineering more potent enzymes through mutation, generating variants with lower solubility than the wild-type is undesirable. The degree of variant solubility can be assessed using web-based tools that analyze protein sequence information. An example is the SODA server, which compares the solubility of a variant to that of the wild-type (Paladin et al., 2017a).

An enzyme solubility, stability, structure, and function are all influenced by the charge of its ionizable groups. There are seven amino acids possessing ionizable groups, and alterations in their ionization states can significantly affect enzyme stability. This is because changes in charge or charge strength impact bond formation within the enzyme and can even alter the ionization state of other ionizable groups. The degree of ionization is measured as pKa, which can be determined using computational methods and web servers. For instance, the PropKa web server is a tool commonly used for pKa determination of ionizable groups.

Protein structure can be elucidated using experimental methods such as X-ray crystallography, electron microscopy, and NMR. However, if an experimental structure is unavailable, homology modeling stands as the most successful and reliable computational alternative. Homology modeling operates on a template-target approach, requiring a suitable template to build and validate a model. Sequence identity and the determination of conserved regions between the target protein and its template are crucial parameters in enzyme engineering. For this purpose, Multiple Sequence Alignment (MSA) is employed to identify evolutionarily significant positions. During homology modeling, the chosen template must meet specific criteria: its resolution should not be higher than 2-2.5 Å, its sequence identity with the target must exceed 50%, and ideally, only a single chain should be used. Popular software tools for homology modeling include SWISS-MODEL, I-TASSER, and MODELLER. The development of computational biology in enzyme engineering has revolutionized the field. It has deepened our understanding of enzymes, facilitated enzyme engineering approaches beyond traditional experimental methods, and aided in novel enzyme identification (Fiser, 2010; Qu et al., 2009).

Molecular modeling is employed to tailor the geometry, electrostatic properties, or binding affinity of enzyme active sites (**Figure 1.5**) for specific purposes, such as altering substrate specificity or improving catalytic properties. The molecular-level investigation of mutation effects can be simulated using molecular dynamics (MD) simulations that mimic environmental conditions. By analyzing simulation data, changes in binding energy, conformational alterations, and flexibility of amino acids within mutated enzymes can be detected. This approach allows for a comprehensive understanding of the enzyme-substrate complex's stability (Kim et al., 2024).

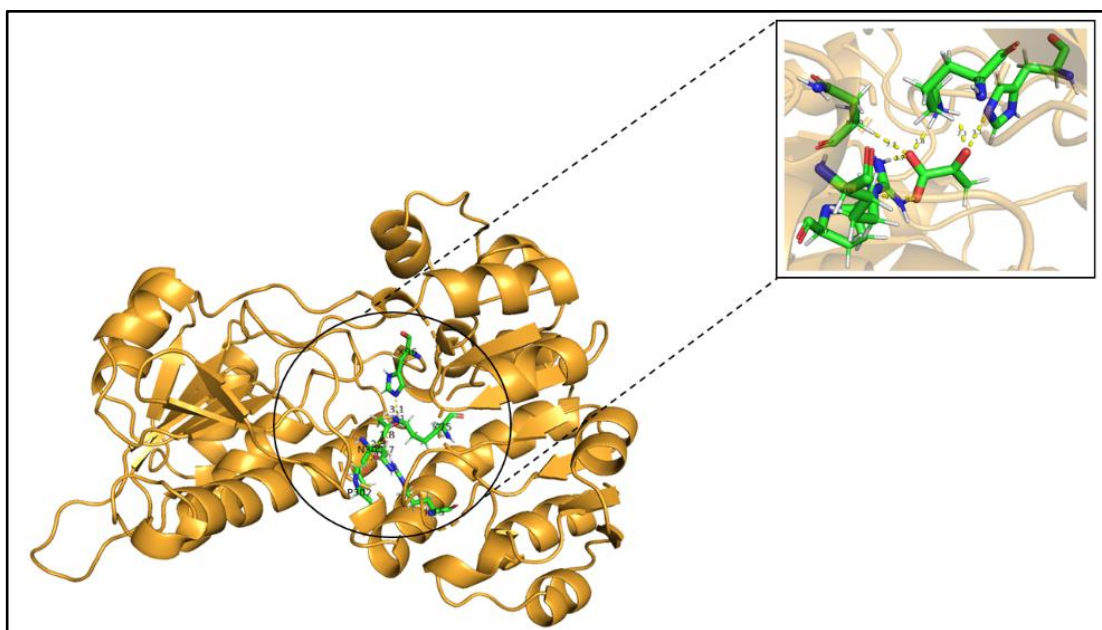


Figure 1.5: Representation of the active site of the 3D enzyme structure.

In silico modeling of enzymes has brought new insights into understanding the structure-function relationship in enzymes as well. This method allows for the selection of mutations for specific purposes, proving to be more cost-effective, less time-consuming, and less labor-intensive compared to purely experimental approaches. Moreover, when the goal is to create industrially adaptable enzymes, *in silico* modeling requires fewer raw materials while facilitating greater product generation. Ultimately, *in silico* modeling techniques like rational enzyme design offer significant advantages over traditional methods.

2. PURPOSE OF THE STUDY

Amino acids, the fundamental building blocks of proteins, are indispensable for vital biological processes and thus extensively used in modern medicinal chemistry. Their broad significance extends across various industrial sectors, from veterinary applications to the food industry. L-Alanine, in particular, is a crucial chiral amino acid for industrial uses. The enzyme L-alanine Dehydrogenase (L-AlaDH) catalyzes the reversible conversion of pyruvate to L-alanine, utilizing NAD(H) and NAD⁺ as cofactors. Specifically, reductive amination activity of the enzyme facilitates the conversion of pyruvate to L-alanine in the presence of NAD(H).

Although numerous studies have reported on the oxidative activity of L-AlaDH, its reductive amination activity remains insufficiently explored and has, moreover, been reported as low. For the first time, this research comprehensively characterizes the reductive amination activity of L-AlaDH derived from *Amycolatopsis sulphurea* (L-*AsAlaDH*). Furthermore, we report L-*AsAlaDH* variants with enhanced catalytic activity that were identified through protein engineering.

Throughout this study, we created, expressed, and purified enzyme variants identified via rational enzyme engineering techniques. We determined the optimum pH, temperature, and kinetic parameters for both the deamination and amination activities of these enzymes. We also investigated the effects of metal ions on amination activity. Statistical analysis was performed using two-way ANOVA and Dunnett test. Furthermore, we assessed the storage stability of the reductive amination activity over a two-week period at temperatures of -80°, -20°, +4°, and +20°C. To elucidate the molecular-level effects of these mutations, active site modeling and MD simulations were performed.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Devices

Table 3.1: Laboratory devices, their brands and purpose of their use during the thesis study

Device	Brand	Purpose
Autoclave	BioTek	Sterilizing the medium, buffers and consumables
Biosafety Cabinet	N-BioTek	Cell inoculation under sterile conditions
Centrifuge	Beckman Coulter	Cell and enzyme sedimentation
	Hettich	
Deep freeze (-80 °C)	Arctico	Storing the cells and proteins
Incubator	Nüve	Cell growth for enzyme production purposes
Mini Centrifuge	Sigma	Plasmid isolation
PCR	Prime	Mutant creation
Block Heater	Stuart	Denaturing DNA during visualization processs
Magnetic Stirrer	WiseStir	Homogenization of buffers, mediums and solutions
Micropipettes	Axygen	Precise measurement of liquids
Plate Reader	BioTek	Enzyme activity and concentration measurement
pH Meter	Hanna	pH arrangement
Refrigerator	Vestel	Buffer and pure enzyme storing
Precision Scale	Shimadzu	Chemical scaling
Shaking Incubator	N-BioTek	Optimum temperature providing for cell growth
Vortex	BioTek	Dissolution of cell pellets and homogenization of solutions
Water Bath	Stuart	Enzyme incubation for activity measurement
Sonicator	BANDELIN	Mechanical cell disruption
Ice maker	Brema	Enzyme protection the at room temperature
Microwave Oven	Açelik	Liquefaction of solid medium
Ultra-Pure Water Device	Milli-Q	Distilled and double distilled water supplier
Horizontal mini gel system	BioRad	Energy supplier for electrophoresis studies
Electrophoresis	Biorad Powerpac	Visualization of the purified enzymes

3.1.2. Enzymes and Strains

Several enzymes were used in experimental procedures, including lysozyme (Sigma), *DpnI* (BioLabs) and Phusion HF DNA Polymerase (BioLabs). *E. coli* DH5 α and *E. coli* BL21 (DE3) strains were supplied from New England Biolabs (NEB). L-AsAlaDH was used during the thesis study. *Amycolatopsis sulphurea* recombinant plasmid was obtained from BERC (Binay Enzyme Research Center, Kocaeli Turkey) Lab.

3.1.3. Kits and Purification Equipments

Kits and purification equipments utilized during the thesis studies were given in the Table 3.2.

Table 3.2: Purification equipments and kits used in the thesis studies.

	Kit/Purification Equipment	Brand
Kits	ZR Plasmid Miniprep- Classic	Zymo Research
	Pierce™ BCA Protein Assay Kit	Thermo Scientific
	Phusion™ Site-Directed Mutagenesis Kit	Thermo Scientific
Purification Equipments	Ni-NTA	GE Healthcare
	PD10	Cytiva
	Concentrators	Merck
	Syringe Filters	Sartorius

3.1.4. Chemicals

Table 3.3: Chemicals used in the experimental process.

Chemicals	Brand
Sodium Pyruvate	Fisher Scientific
L-Alanine	Fisher Scientific
NAD(H)	PROZOMIX
NAD ⁺	Sigma-Aldrich
Amonium chloride	Merck
Tris	Roche
Hydrochloric Acid	Sigma-Aldrich
Yeast Extract	Lab M
Peptone	Lab M
Agarose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Auto Induction Medium	Formedium™
Kanamycin	Sigma-Aldrich
Ethanol	Sigma-Aldrich

Table 3.3: Continue.

Sodium Chloride	Sigma-Aldrich
Glycine	Sigma-Aldrich
Acetic Acid	Merck
Sodium Hydroxide	Sigma-Aldrich
Sodium Phosphate	Sigma-Aldrich
Calcium Chloride Dihydrate	Sigma-Aldrich
Magnesium chloride Hephthdrate	Merck
Iron (III) Chloride Hexahydrate	ISOLAB
Potassium Chloride	Merck
Lithium Chloride	Merck
Zinc Chloride	ISOLAB
Sodium Citrate Dihydrate	MULTICELL
Sodium dihydrogen phosphate monohydrate	Merck

3.1.5. Solutions, Buffers and Media

Table 3.4: Media used in the experimental process.

Medium name	Contents	Amount
Luria-Bertani (LB)	Yeast Extract	5 g
	Peptone	10 g
	Sodium chloride	10 g
Luria-Bertani Agar (LB-Agar)	Yeast Extract	5 g
	Peptone	10 g
	Sodium chloride	10 g
	Agarose	15 g
Autoinduction Medium	Ready to use powder medium	34.85 g

All media (**Table 3.4**) was dissolved in 1000mL ddH₂O and autoclaved at 121 °C, 15 minutes under 1 atm pressure to sterilize.

Table 3.5: Solutions and buffers

	Contents	Amount	Preperation
Buffer A	NaH ₂ PO ₄ ·H ₂ O	2.76 g	Adjust the pH to 7.4 using NaOH, and then bring the final volume to 1000 mL. Subsequently, filter the buffer through a 0.45 µm filter.
	NaCl	29.22 g	
	Imidazole	2.04 g	
Buffer B	NaH ₂ PO ₄ ·H ₂ O	2.76 g	Adjust the pH to 7.4 using NaOH, and then bring the final volume to 1000 mL. Subsequently, filter the buffer through a 0.45 µm filter.
	NaCl	29.22 g	
	Imidazole	34.04 g	
	NaCl	5.84 g	

	Imidazole	0.48 g	
--	-----------	--------	--

Table 3.5: Continue.

Lysis Buffer	NaH ₂ PO ₄ ·H ₂ O	2.76 g	Adjust the pH to 7.4 using NaOH, and then bring the final volume to 200 mL. Subsequently, filter the buffer through a 0.45 µm filter.
	NaCl	5.84 g	
	Imidazole	0.48 g	

Table 3.6: Solutions used to prepare competent cells.

	Component	Amount	Final volume
Solution A (0,02 M CaCl ₂ and 0,08 M MgCl ₂)	CaCl ₂	0,176	Bring the final volume to 60mL with ddH ₂ O
	MgCl ₂	0,975	
Solution B (0,1M CaCl ₂)	CaCl ₂	0,882	Bring the final volume to 60mL with ddH ₂ O

Solutions used in optimum pH determination experiments

- Citric Acid-Sodium Citrate solution:

The solution was prepared with 100mM citric acid and 100mM sodium citrate, then pH was arranged between pH 3.0-5.5 range. Double-distilled water (ddH₂O) was used for solution preparation, and the resulting solution was filtered through a 0.45 µm filter.

- Sodium phosphate solution:

The solution was prepared with 100mM sodium phosphate and pH was arranged between pH 6.0-7.5 range. Double-distilled water (ddH₂O) was used for solution preparation, and the resulting solution was filtered through a 0.45 µm filter.

- Tris-HCl solution

The solution was prepared with 100mM tris and 100mM HCl, then pH was arranged between pH 7.5-9.0 range. Double-distilled water (ddH₂O) was used for solution preparation, and the resulting solution was filtered through a 0.45 µm filter. Glycine-NaOH Solution

The solution was prepared with 100mM glycine and 100mM NaOH, then pH was arranged between pH 9.0-10.0 range. ddH₂O was used during the solution preparation and filtered with 0.45 µm filter.

- Sodium Carbonate-Sodium Bicarbonate Solution

The solution was prepared with 100mM sodium carbonate and 100mM sodium bicarbonate, then pH was arranged between pH 9.5-11.0 range. Double-distilled water (ddH₂O) was used for solution preparation, and the resulting solution was filtered through a 0.45 µm filter.

Solutions used in Metal binding Experiments

Chloride compounds of sodium, lithium, potassium, zinc, iron, magnesium and calcium were used in the reaction as 1, 3, 5, 7 and 10 µM. Double-distilled water (ddH₂O) was used for solution preparation, and the resulting solution was filtered through a 0.45 µm filter.

Table 3.7: Solutions used in SDS-PAGE gel electrophoresis

Solution name	Component	Amount	Preparation
10% APS solution	Ammonium persulfate	0.1 g	Dissolve in 1mL ddH ₂ O and use fresh.
10% SDS solution	Sodium dodecyl sulfate	5 g	Dissolve in ddH ₂ O and bring total volume to 50mL.
30% Acrylamide Solution	Acrylamide	30 g	Dissolve in ddH ₂ O and bring total volume to 50mL.
	Bis-acrylamide	0.8 g	
1.5 M Tris Buffer (pH 8.9)	Tris	36.34 g	Dissolve in ddH ₂ O and bring the total volume to 200 mL. Adjust the pH to 8.9 using HCl.
1 M Tris Buffer (pH 6.8)	Tris	12.11 g	Dissolve in ddH ₂ O and bring the total volume to 100 mL. Adjust the pH to 6.8 using HCl.
SDS Running Buffer	Tris	3.03 g	Dissolve in ddH ₂ O as the total volume is 1000 mL.
	Glycine	14.04 g	
	SDS	1 g	
4X Sample Loading Buffer	Bromophenol blue	4 mg	Homogenize all components.
	Tris buffer (1 M, pH 6.8)	0.6 mL	
	Glycerol,	2.5 mL	
	SDS (10% w/v)	3 mL	
	ddH ₂ O	1.4 mL	
SDS Gel Staining Solution:	Coomassie Brilliant Blue R250	0.25 g	Homogenize all components.
	Methanol	125mL	
	Acetic acid	25 mL	
	ddH ₂ O	100mL	

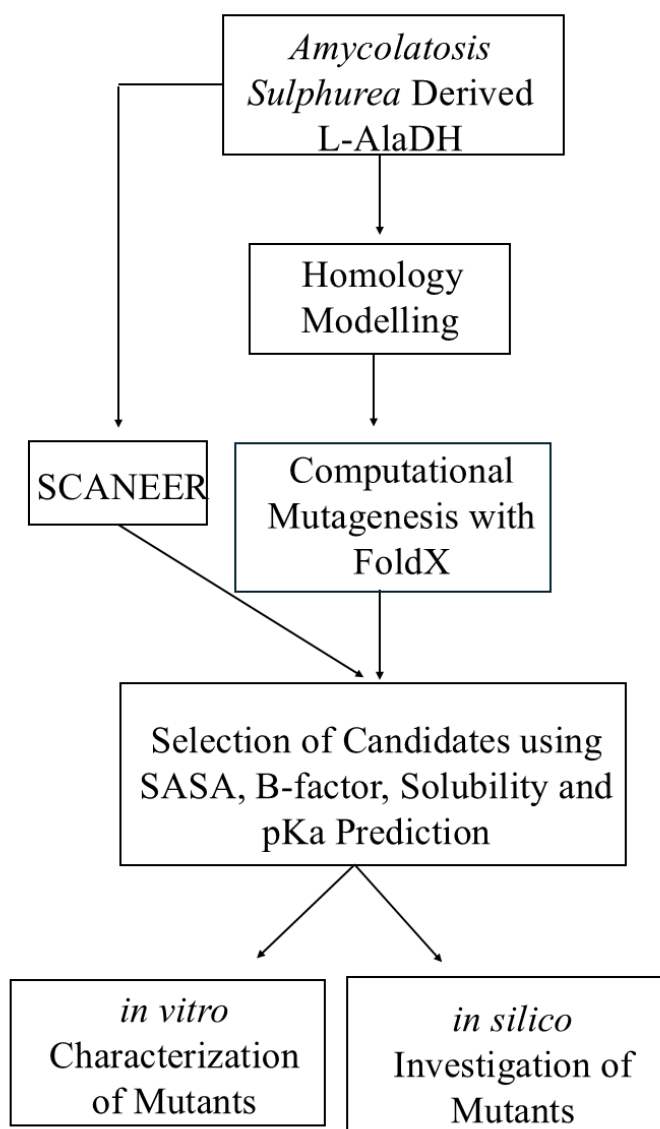
30µL sample was mixed with 6X dye and then incubated at 90°C for 10 minutes to prepare sample for the SDS-PAGE gel electrophoresis. Subsequently, the samples were centrifuged at 13,500 rpm for 10 minutes prior to the experiment.

3.1.6. Databanks, Programmes, Softwares, Servers and Hardwares

Table 3.8: Utilized softwares, servers and hardware for mutation scanning and MD simulation performing and analyzing.

Tools and Hardwares	Class	Usage
MSI workstation, NVIDIA GeForce RTX 3060 Ti	Hardware	Mutation scanning studies, MD simulations and analysis.
Clustal Omega	Web server	Alignment studies
SWISS MODEL	Web server	Homology modelling
Protein Databank	Databank	Protein sequence obtaining
Uniprot		
YASARA	Web server	Energy minimization of models
FoldX	Stand-alone	Computational mutagenesis
PyMol	Stand-alone	Visualization of the protein structures.
SODA	Web server	Solubility analysis
ProbKa	Web server	pKa analysis
SCANEER	Web server	Mutation prediction
GROMACS	Stand-alone	MD simulations.
VMD	Stand-alone	Visualization of protein structures and simulation trajectories.
gRINN	Stand-alone	Analysis of pairwise amino acid interaction energies in MD simulations
gmx_MMPBSA	Stand-alone	Binding free energy analysis between NAD and protein

3.2. Methods



3.2.1. Determination of the Mutant Candidates Based on Rational Enzyme Design

Due to limited prior research on *A. sulphurea* L-AsAlaDH, an experimentally-determined three-dimensional (3D) structure was unavailable. Consequently, the first

step involved creating a homology model of protein to facilitate virtual mutation experiments through rational design.

To create a homology model, we first performed a Multiple Sequence Alignment (MSA) using AlaDH sequences from the most-extensively studied microorganisms (**Table 3.9**). Sequence information was retrieved from UniProt (Bateman et al., 2025) and Protein Data bank (PDB) (Berman et al., 2000). Clustal Omega was used to create MSA, which yielded a percent sequence identity matrix (Madeira et al., 2024). The AlaDH sequence with the highest percentage of sequence identity with the *A. sulphurea* L-AlaDH (L-AsAlaDH) sequence and an experimentally determined structure was then chosen as template. Homology model was created using SWISS-MODEL (Waterhouse et al., 2018). Once the model was obtained, NAD(H) and pyruvate molecules were manually inserted into the generated model by taking their coordinates from the template structure. After that, the structure was energy-minimized to prepare the structure for mutagenesis studies using YASARA web server (Krieger et al., 2009).

Table 3.9: Organisms from which L-alanine dehydrogenases were characterized previously in literature

Organism	Reference
<i>Staphylococcus haemolyticus</i>	(Takeuchi et al., 2005)
<i>Bacillus sphaericus</i>	(Sakamoto et al., 2001)
<i>Bacillus subtilis</i>	(Grimshaw & Cleland, 1981)
<i>Alkalihalophilus pseudofirmus</i>	(Janto et al., 2011)
<i>Shewanella sp.</i>	(Irwin et al., 2003)
<i>Phormidium lapideum</i>	(Sawa et al., 1994)
<i>Thermomicrobium roseum</i>	(Dedeakayoğulları et al., 2023)
<i>Mycobacterium tuberculosis</i>	(Hutter & Singh, 1998)

A 7 Å distance between alpha-carbons was taken as cutoff to determine pairs of amino acids involved in non-covalent interactions (Nagarajan et al., 2016). To narrow down the search space for potentially activity-enhancing mutations in the enzyme structure, residues within 7Å distance around the substrate (pyruvate) in the generated model

were identified by using VMD (Humphrey et al., 1996). These positions then underwent saturated mutagenesis using FoldX (Schymkowitz et al., 2005). FoldX is a software that allows quick evaluation of the mutation effects on proteins and nucleic acids. It provides $\Delta\Delta G$ score for evaluation of stability, dynamics behaviour and folding profile of protein. FoldX uses own empirical force field to calculate $\Delta\Delta G$ ($\Delta G_{\text{mutant}} - \Delta G_{\text{wild-type}}$) between wild-type and mutated protein structures. According to the calculated $\Delta\Delta G$ values, a list of stabilizing mutant candidates ($\Delta\Delta G < 0$) was generated. Then, educated predictions were made to decrease the number of candidate mutations further to be tested in experimental studies (Gu et al., 2023).

In addition to FoldX, SCANEER (D. Kim et al., 2022) web server was also used to obtain mutant candidates for enhanced activity. SCANEER focuses on making predictions for increasing enzyme activity based on sequence based co-evolutionary analysis and evaluates the effect of mutations by calculating a sequence co-evolution index value. Sequence co-evolution index is determined by calculating the frequency of co-evolving amino acid pairs.

After candidate mutations were identified, they were implemented in the homology model using PyMOL. Then, further analysis was performed on all mutant candidates, which involved B-factor analysis using PyMOL, solubility detection using the SODA server (Paladin et al., 2017), Solvent Accessible Surface Area (SASA) using PyMOL, and pKa analysis using the Probka server (Søndergaard et al., 2011). Active sites of the tested mutants were visualized using Discovery Studio (BIOVIA, 2012). Finally, a final set of selected mutants were further studied in experimental studies.

3.2.2. Primer Design and Site Directed Mutagenesis

Primers were designed according to the protocol of Agilent Technologies Quickchange II Site-Directed Mutagenesis Kit (Agilent Technologies, 2023). According to the kit, primers must have the following properties:

- Primers must be between 25-45 bases,
- T_m of the primers is higher than or equal to 78 °C,
- Ratio of GC bases must not exceed 40%,
- 3' of the primer must include at least one G or C base,
- Loop formation probability of the primer must be weak

Primers were designed using the OligoEvaluator™ web tool and then commercially synthesized. Polymerase Chain Reaction (PCR) components and their amounts are presented in **Table 3.10**; additionally, the PCR process was optimized.

Table 3.10: PCR components

Component	Amount(μL)
10μM Forward Primer	2,5 μL
10μM Reverse Primer	2,5 μL
Template DNA	1 μL
dNTPs	1 μL
High Fidelity Buffer	10 μL
Nuclease Free water	32,5 μL
Taq Polymerase	0,5 μL
Total	50 μL

After the process was completed, 40 μL of PCR products were treated with 4 μL *DpnI* enzyme and 5 μL tango buffer (1X). Mixture was incubated overnight at 37 °C. *DpnI* treated and non-treated plasmids were run on the agarose gel electrophoresis. 1%(w/v) agarose was used in Tris-Borate-EDTA (TBE) buffer (X) and samples were treated with loading dye (X). Finally, samples were run on the gel at 90 Volt. Success of both *DpnI* enzyme application and PCR process was evaluated and then mutant plasmids were transformed into competent cells.

3.2.3. Transformation of L-AsAlaDH Plasmids to Competent Cells

All plasmids were transformed into E. coli DH5α competent cells using the heat shock method. During the process, New England Biolabs High Efficiency Transformation protocol was followed. According to the protocol, the following steps were performed:

- E. coli DH5α competent cells were removed from -80 deep freezer and allowed to adapt to their environment.
- According to the agarose gel electrophoresis results, the amount of the PCR products was used in transformation were determined as 3 – 5 μL.

- Then, the competent cell and plasmid products were mixed gently and incubated on ice for 30 minutes.
- After that mixtures were shocked at 42 °C for 30 seconds in a water bath.
- Finally, heat shock was completed via 5 minutes incubation on ice of the mixtures.

After the process was completed, 500 µL LB medium was added and incubated at 37 °C and 180 rpm for 1 hour. Then the 80-150µL cell were spread on kanamycin including LB-Agar medium and incubated overnight at 37 °C. After transformation was completed, three growing colonies were selected and growth on 5mL kanamycin including LB medium overnight at 37 °C and 180 rpm. Obtained cells were used in plasmid extraction studies. Plasmids were extracted according to ZR Plazmid Miniprep™- Classic kit and then 15 µL of the plasmids were sent to Eurofins for sequencing with the purpose of controlling the mutations. After the all plasmid extraction process was completed, purity (260/280 ratio) and concentration of the plasmids were controlled at 260 nm with nanodrop. After sequence verification, plasmids of the mutants were used to transform competent E. coli BL21 (DE3) cells, a strain frequently employed in high-yield protein expression studies, using the standard heat shock procedure.

3.2.4. Preparation of Glycerol Stock and Pellet Stock

Competent E. coli BL21 (DE3) cells were inoculated from solid media into 5 mL of LB medium supplemented with kanamycin (50 µg/mL) and incubated overnight at 37 °C with shaking at 200 rpm. To create glycerol stocks, 250 µL of preculture was mixed with an equal volume of 50% (v/v) glycerol and stored at -80°C.

Cells are stored as pellets at -20°C. To obtain these pellets, a preculture was inoculated into 200 mL of auto-induction Studier medium containing kanamycin (50 µg/mL) and incubated overnight at 30 °C with shaking at 200 rpm. Subsequently, cells for both the wild-type and mutant strains were centrifuged at 5000 rpm and +4°C for 20 minutes, and the resulting cell pellet was stored at -20°C.

3.2.5. Expression and Purification of L-AsAlaDH Enzymes

The precipitated cells were resuspended in 20 mL of lysis buffer, which contained Buffer A (20 mM NaPi, 500 mM NaCl, 30 mM imidazole, pH 7.4), 1 mg/mL lysozyme, and 1 mM phenylmethylsulfonyl fluoride at final concentrations. The resuspended cells were then placed in a continuously shaking incubator on ice for 20 minutes. The cell suspension was sonicated and subsequently centrifuged at 10,000 rpm, 4 °C for 40 minutes. Following this process, the supernatant was filtered using a 0.45 µm pore filter.

The supernatant was loaded onto a 5 mL HisTrap column (GE), which had been equilibrated with Buffer A and activated with nickel nitrilotriacetic acid (Ni-NTA) resin. The column was then re-washed with Buffer A. Subsequently, fractions were eluted by applying a gradient of imidazole concentrations (100, 200, 400, and 500 mM), prepared using Buffer A and Buffer B (20 mM NaPi, 500 mM NaCl, 500 mM imidazole, pH 7.4). Both mutant and wild-type L-AsAlaDH enzyme eluted throughout this imidazole gradient.

Proteins were concentrated using ultrafiltration (MM Amicon Ultracel-30), and their imidazole and salts were exchanged using a PD-10 (GE) column. The concentration of the purified enzymes was then measured with a bicinchoninic acid (BCA) protein kit at 562 nm, following its protocol.

Expression of the pure samples were investigated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). First, proteins were denatured in loading dye with ratio 1:3 at 95 °C for 10 minutes. Then mixtures were centrifuged at 10.000 rpm for 5 minutes and loaded on 8% stacking gel which was found above the 12% separating gel.

3.2.6. Optimization of Activity Parameters for L-AsAlaDHs

During these experiments, we investigated and optimized both the oxidative deamination and reductive amination processes of the L-AsAlaDH enzyme. For the reductive amination, we optimized the process by preparing 5 mM pyruvate, 0.5 M ammonium chloride, and 0.5 mM NAD(H) in its specific buffer, along with the required amount of enzyme. The reaction was kinetically analyzed for 10 minutes at 340 nm, and NAD(H) consumption was monitored spectrophotometrically.

For the oxidative deamination reaction, 5 mM L-alanine was mixed with the appropriate buffer, the required amount of enzyme, and 1 mM NAD⁺. The reaction was kinetically analyzed for 10 minutes at 340 nm, and NAD(H) formation was monitored spectrophotometrically.

The enzyme unit (U) of L-AlaDH was defined as the amount of enzyme required to produce (in oxidative deamination) or consume (in reductive amination) 1 μ M of NAD(H) per minute.

3.2.7. Determination of Optimum Working Conditions

We determined the optimum working temperature and pH conditions for both the oxidative deamination and reductive amination reactions of wild-type and mutant L-*AsAlaDH* enzymes. The pH buffer optimization process utilized a range of 100 mM solutions: citric acid for pH 3.0-5.5, sodium phosphate for pH 6.0-7.5, Tris-HCl for pH 7.5-9.0, glycine for pH 9.0-10.0, and carbonate for pH 9.5-11.0. Depending on the specific reaction, buffers were combined with the substrate, cofactor, enzyme, or other necessary components, and NAD(H) consumption or formation was monitored at 340 nm for 10 minutes.

After determining the optimum pH, the optimum temperature for both reductive amination and oxidative deamination reactions was determined, ranging from 25-55°C. The optimized buffer was then combined with the optimized amount of NAD(H)/NAD⁺ (depending on the specific reaction), substrate (also dependent on the reaction), ammonium chloride, and enzyme. Prior to spectrophotometric measurement, samples were incubated for 10 minutes at the optimized working temperature. Subsequently, NAD(H) consumption or formation was monitored at 340 nm for 10 minutes at the same working temperature.

3.2.8. Determination of Kinetic Parameters of L-*AsAlaDH* Enzymes

The kinetic characteristics of both the reductive amination and oxidative deamination reactions for wild-type and mutant L-*AsAlaDH* enzymes were determined. During kinetic parameter determination, substrates were incubated at their optimum temperature for 10 minutes. Their concentrations were tested across a range from 0.1 mM to 50 mM. For reductive amination, the reaction mixture included a suitable amount of enzyme, 5 mM pyruvate, 0.5 M ammonium chloride, and 0.5 mM NAD(H).

For oxidative deamination, the mixture contained a suitable amount of enzyme, 5 mM L-alanine, and 1 mM NAD⁺.

Specific activity was determined tracing NAD(H) consumption for reductive amination and NAD(H) formation for oxidative deamination during 10 minutes at 340nm absorbance. Specific activity was calculated by terms of U/mg and extinction coefficient (ϵ) was used as 6220 M⁻¹ cm⁻¹. After that k_{cat} , K_M and k_{cat}/K_M values were calculated via Michaelis-Menten graph which were drawn by GraphPad Prism 8.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com.

3.2.9. Storage Stability and Effect of Metal on L-AsAlaDH Enzymes for Reductive Amination Activity

In these experiments, we investigated the effects of metal ions on the reductive amination reaction of L-AlaDH enzymes. We used five different concentrations (1, 3, 5, 7, and 10 μ M) of the chloride salts of six metal ions: Zn (II), Fe (II), K⁺, Na⁺, Li⁺, Mg⁺² and Ca⁺².

We used optimized reaction components in the optimum buffer. NAD(H) consumption was measured at 340 nm for 10 minutes at room temperature. The significance of our results were assessed, compared to control using Two-way ANOVA and Dunnett test (for multiple comparisons), with a confidence level of 0.001. All statistical analyses were performed using GraphPad Prism.

Storage stability of L-AlaDH enzymes for amination activity were investigated at -80, -20, 4 and 25°C in their optimum buffers for 14 days. Absorbance measurement of reactions were performed at 25 °C for 10 minutes at 340nm absorbance.

3.2.10. Molecular Dynamics (MD) Simulations

Molecular Dynamics (MD) simulations were employed to investigate the effects of mutations on the L-AsAlaDH enzyme at the molecular level. For this computational study, homology models generated during the *in silico* mutation design efforts were utilized in the MD simulations. L-AsAlaDH is a homo-hexameric enzyme (AKTAŞ, 2021). However, simulating the homo-hexameric structure requires excessive computing power that severely limits the number of systems and replicas to be simulated, in addition to the time scales of simulations. Moreover, it is also common practice in the literature that a single chain of enzymes is used for homology modelling

and molecular simulation (Ling et al., 2012). Simulations were prepared with proper amount of Na⁺ ions to neutralize the system in TIP3 water model. The systems were replaced in a box with at least 10 Å padding distance to box boundaries to provide required periodic boundary conditions (PBC) for simulations. Simulations were conducted for 100 ns by using GROMACS 2023.3 (Abraham et al., 2015). AMBER99SB force field was used to parametrize amino acids, ions, and water molecules in the system (Case et al., 2023). Parametrization of ligand molecules was performed using the aceptype server (Silva & Vranken, 2012) with GAFF2 atom type selection. Routine energy minimization and equilibration steps were applied prior to production simulations. Minimization was performed using the gradient-descent algorithm for up to 5000 steps until forces anywhere in the system converged below 500 kJ. Equilibration was conducted in two steps dedicated to temperature and pressure control in the NVT and NPT ensembles, respectively. In these steps, the protein and ligand complex was subjected to position restraints. Position restraints were applied to heavy atoms with 1000 kJ mol⁻¹ nm⁻². Temperature control was performed for 10ps (5000 steps), followed by pressure control for 10ps (5000 steps). All systems were simulated at 310 K temperature and 1 bar pressure. A 2-femtosecond time step was used. Total simulation time for 12 systems was 1.2 microseconds. After the simulation process, trajectories of simulations were visualized using Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) program.

3.2.11. Analysis of MD Simulations

Once simulations were completed, trajectories were wrapped to retain only protein, NAD and/or pyruvate and to remove periodic boundary artefacts. Then, a visual check has been performed to confirm the overall stability of protein-ligand interactions. Only those trajectories that were deemed to yield stable protein-ligand interactions were included in analysis.

3.2.11.1. RMSD and RMSF Profiles of MD Simulations

Dynamic behavior of the protein with respect to its initial coordinates was investigated by computing Root Mean Square Deviation (RMSD) time profiles. For this purpose, trajectories were first superposed onto the first frame as reference based on alpha-

carbon coordinates. RMSD of a given conformation was then calculated using the following equation:

$$RMSD = \sqrt{\frac{1}{S} \sum_{i=1}^S (r_i - r_i^{ref})^2}$$

where S is the number of the atoms, r_i is the initial position of atom i, r_i^{ref} is the position of atom i in the reference frame.

Root Mean Square Fluctuations (RMSF) were also computed by superposing conformations onto an average conformation based on alpha-carbon coordinates to analyze per-residue fluctuations. Only the portion of trajectories that were deemed to represent equilibrated conformations was included in the analysis (20-100 ns). RMSF was calculated using the following equation:

$$RMSF_i = \sqrt{\frac{1}{N} \sum_{i=1}^N (x_{i(t)} - \bar{x}_i)^2}$$

N is the number of frames in the simulation, $x_{i(t)}$ is the coordinate of atom i at time t, $\bar{x}_i$ is the average position of atom i at simulation. ProDy (Bakan et al., 2011) was used for both RMSD and RMSF calculation.

3.2.11.2. Molecular Mechanics Poisson–Boltzmann Surface Area (MMPBSA) Analysis

Molecular Mechanics Poisson–Boltzmann Surface Area (MMPBSA) method was used to calculate Binding Free Energy (FEB) of NAD to AlaDH (Miller et al., 2012). A total of 100 frames from the equilibrated portion of the simulations were included in this analysis (selection was made with an appropriate stride value). Analysis was conducted using gmx_MMPBSA (Valdés-Tresanco et al., 2021). In the MM/PBSA method, the difference in Gibbs free energy between complex and free forms of protein and ligand are computed as follows:

$$\Delta G_{bind} = G_{complex} - (G_{protein} + \Delta G_{ligand})$$

3.2.11.3. Calculation of Non-bonded Pairwise Amino Acid Interaction Energies and Construction of Protein Energy Networks

Residue-residue non-bonded interaction energies throughout simulations were calculated using gRINN (get Residue Interaction eNergies and Networks) (Serçinoğlu & Ozbek, 2018). gRINN is a software that uses atomistic MD simulation trajectories as input, and calculates interaction energies between residue pairs. A modified version of the original tool, which accepts heteroatoms in the input trajectory, was employed (<https://www.github.com/osercinoglu/grinn>). Trajectories were processed to yield only protein and ligand in the output. While the tool can compute both van der Waals and electrostatic interactions, only van der Waals interactions were included in our analysis since electrostatic interactions may fail to yield accurate values in the absence of water molecules. Only those pairs whose centers-of-mass are within 10 Å of each other in the initial frame were included in the analysis. For each simulation covering 100 ns of simulation time, a total of 100 evenly-spaced frames were included in this analysis.

After interaction energies were calculated, protein energy networks (PENs) were constructed for each simulation. Here, amino acids were taken to represent nodes, and the existence of van der Waals interactions were used to add edges between pairs of amino acids. Only those interactions with van der Waals interaction energies higher than 1 kcal/mol in magnitude, and covalently-bound residues were considered to add edges to the network. Once a PEN was constructed, betweenness centralities of each residue were computed using Brandes' algorithm (Brandes, 2001), which is based on Dijkstra's shortest path finding algorithm (E.W. Dijkstra, 1959).

4. RESULTS

4.1. Rational Design of L-AsAlaDH Enzymes

Mutation studies using rational enzyme design require the protein's three-dimensional (3D) structure, which can be either experimentally-resolved or computationally-predicted. Databases such as UniProt and PDB serve as key repositories for protein structural information. Since the L-AsAlaDH enzyme lacks an experimentally-resolved structure in these databases, a homology model was created within the scope of this thesis. To identify the best template candidate, we focused on eight commonly-studied AlaDH enzymes from the literature. To determine best template, first a multiple sequence alignment (MSA) (**Figure 4.1**) was constructed with the following AlaDH sequences from *A. sulphurea* (UniProt Accession Number: A0A2A9FGJ5), *Staphylococcus haemolyticus* (UniProt Accession Number: Q4L750), *Bacillus sphaericus* (UniProt Accession Number: Q9KWR0), *Bacillus subtilis* (UniProt Accession Number: Q08352), *Alkalihalophilus pseudofirmus* (UniProt Accession Number: D3FTT5), *Shewanella sp.* (UniProt Accession Number: Q8RLH6), *Phormidium lapideum* (UniProt Accession Number: O52942), *Thermomicrobium roseum* (UniProt Accession Number: B9L0I6), *Mycobacterium tuberculosis* (UniProt Accession Number: P9WQB1). MSA results revealed that active site residues, including critical residues for ligand binding (Arg15, Lys75) and proton transfer (His96, Asp270) are conserved in all sequences (Ågren et al., 2008; Dave & Kadeppagari, 2019b; Tripathi & Ramachandran, 2008).

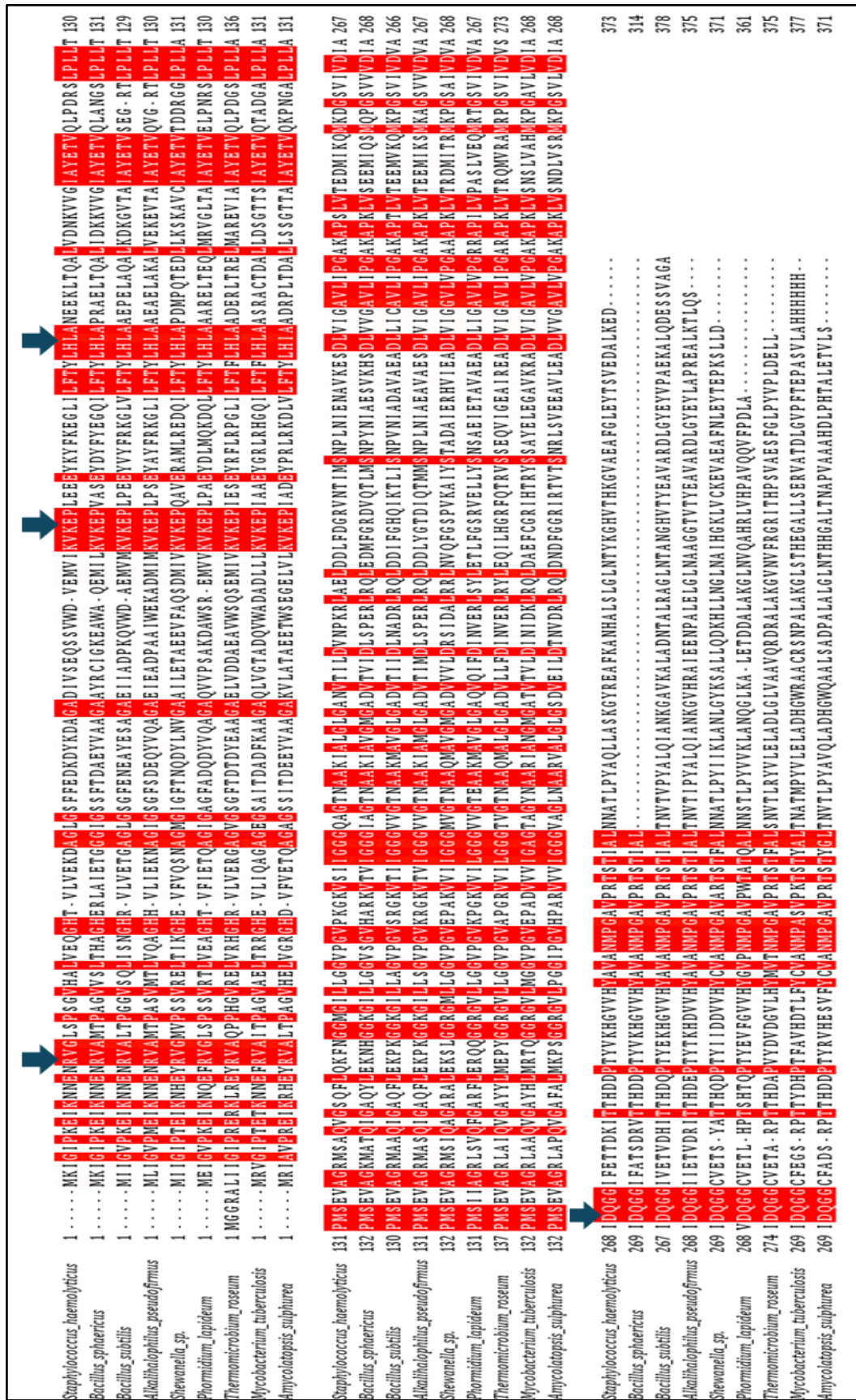


Figure 4.1: Multiple Sequence Alignment Result. Conserved residues are shown in red, and active site residues are shown with arrows. residues respectively (Arg15, Lys75, His96 and Asn270).

A sequence percent identity matrix was also created (**Figure 4.2**). Accordingly, *Amycolatopsis sulphurea* L-AsAlaDH was found to show the highest sequence identity (%63,34) with AlaDH of *Mycobacterium tuberculosis*.

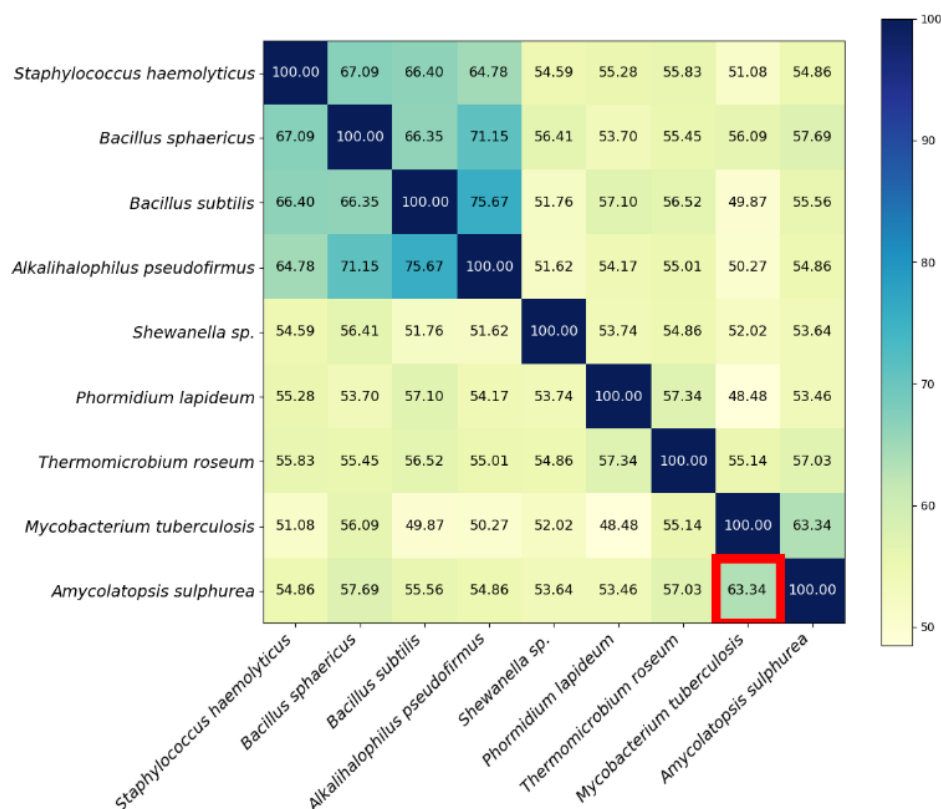


Figure 4.1: Heatmap of percent sequence identities matrix.

Mycobacterium tuberculosis AlaDH is a well-studied and characterized enzyme in the literature (Ågren et al., 2008; Giffin et al., 2016; He et al., 2018). Its 3D structure has also been resolved previously. Moreover, the structure features the substrate pyruvate, and the cofactor NAD(H) in its structure (**Figure 4.3.**), with a resolution below 2.5 Å (Ågren et al., 2008). With a sequence identity percentage higher than 50%, it was considered to be well-suited as a template for the L-AsAlaDH enzyme.

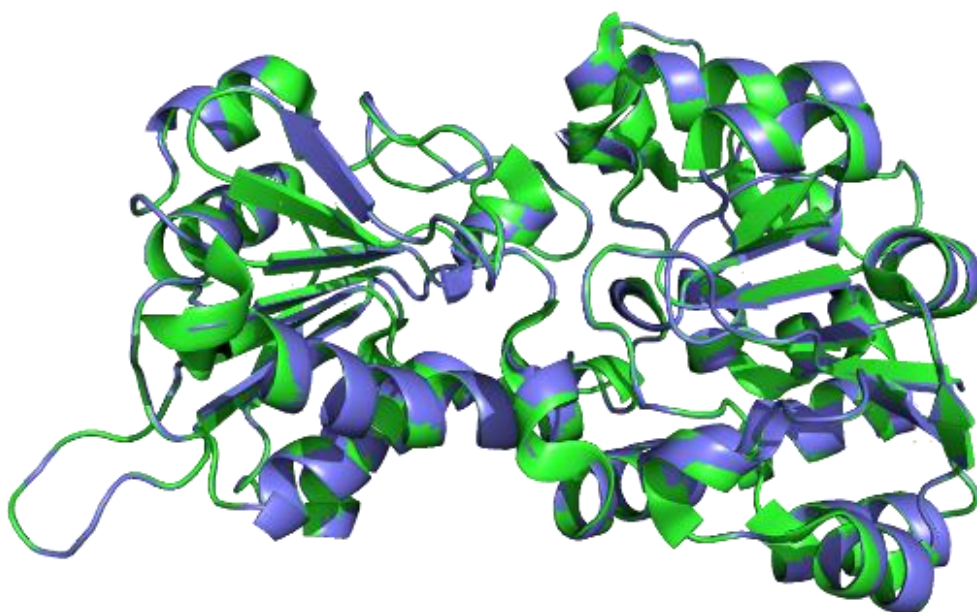


Figure 4.2: Superposition of *Mycobacterium tuberculosis* L-alanine dehydrogenase (PDB id: 2VHX, green) and homology model of the *Amylocaptosis sulphurea* L-alanine dehydrogenase (Purple).

Hence, *Mycobacterium tuberculosis* AlaDH was used as template for homology modelling. The homology model was created using SWISS-MODEL (**Figure 4.4b**). A physically-valid model was obtained, as evidenced by the torsional angles falling within the allowed regions in the Ramachandran plot being higher than 95%, with no outliers (**Figure 4.4a**). Once a homology model was constructed, pyruvate and NAD(H) were inserted manually into the active site, by taking their structures from the template (the template included both pyruvate and NAD(H)).

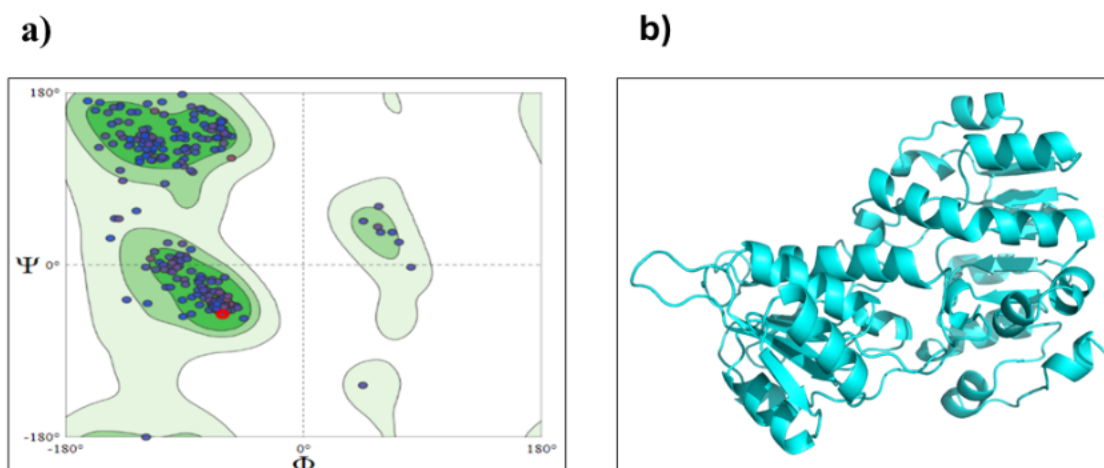


Figure 4.3: Ramachandran plot result **(a)** and model structure **(b)** of the homology modelling study.

We next identified residues whose alpha-carbons were within a 7 Å distance from those of the active site residues (Arg15, Lys75, His96, and Asp270) using VMD. The purpose of this analysis was to establish a region within the protein structure with high potential to increase local stability around the active site, and thus enhance catalytic activity. A total of 54 residues/positions were identified (**Figure 4.5**).

Next, a computational saturation mutagenesis procedure was then applied to these residues (**Figure 4.6**) by mutating each to the 19 other standard amino acids using the FoldX program. This process generated 1026 virtual mutants, along with their $\Delta\Delta G$ (kcal/mol, $\Delta G_{\text{mutant}} - \Delta G_{\text{wild-type}}$) scores. Mutants with a $\Delta\Delta G < 0$ score were considered as stabilizing mutants (Christensen & Kepp, 2012). These were then evaluated further to narrow down the list of mutations to be studied experimentally.

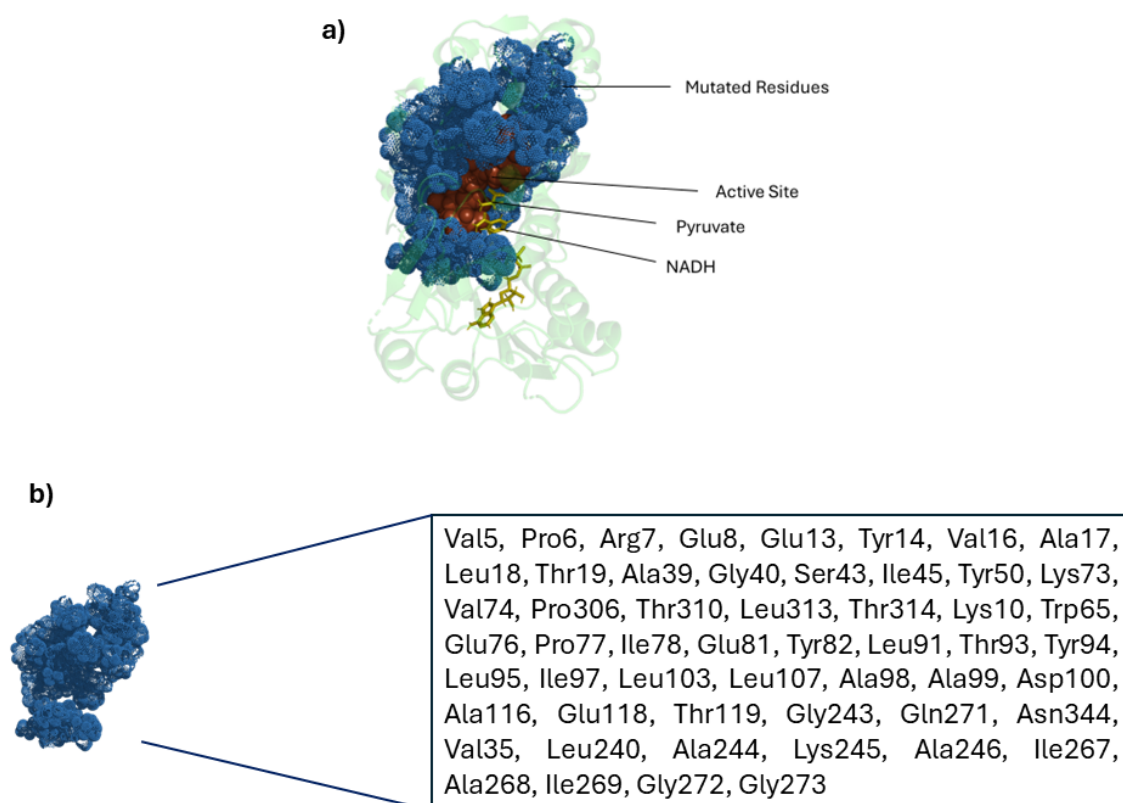


Figure 4.4: Active site, ligands and location of computationally mutated residues **(a)**, the list of computationally mutated residues in *A. sulphurea* alanine dehydrogenase **(b)**.

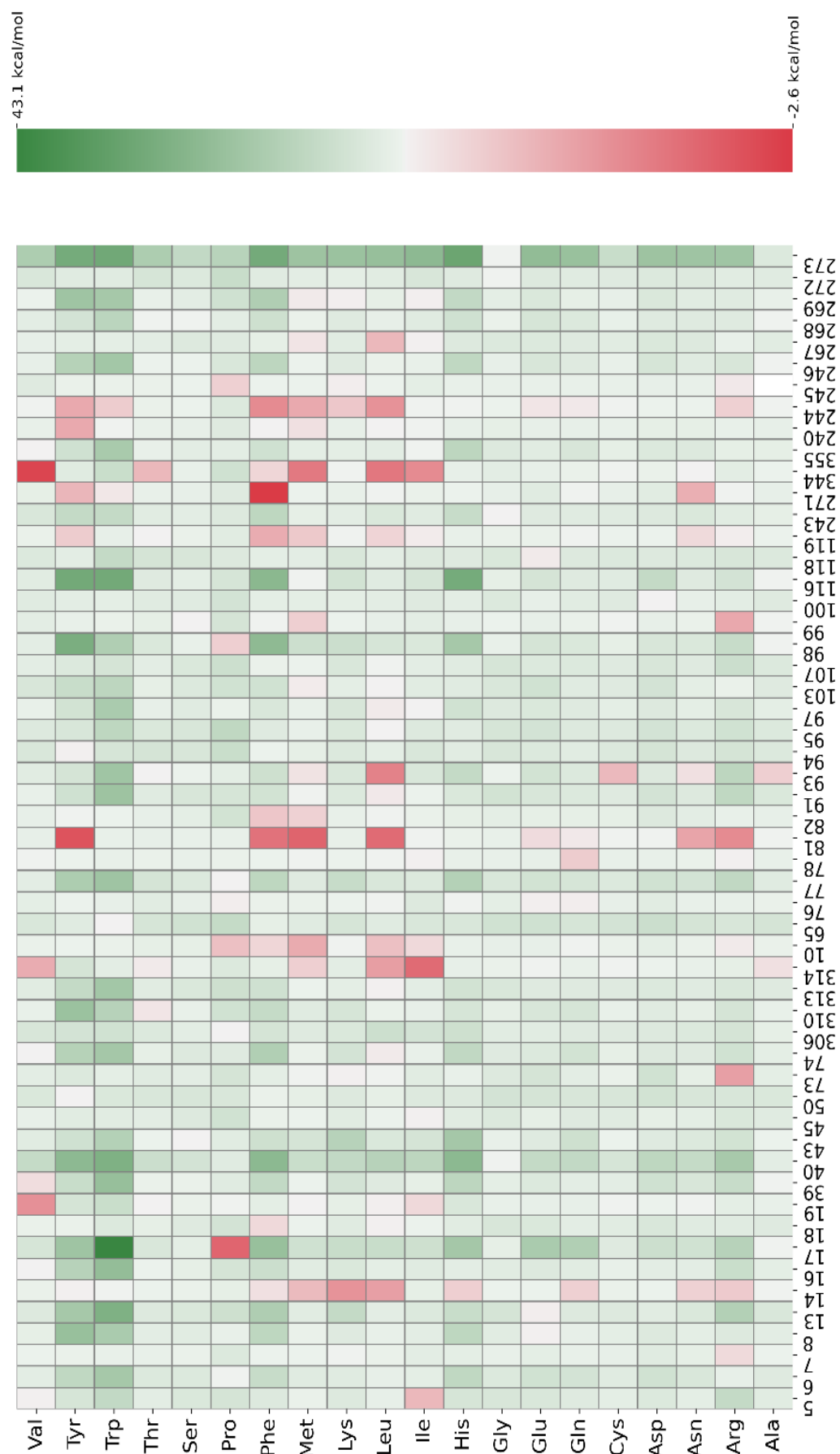


Figure 4.6: Heatmap of $\Delta\Delta G$ – mutated positions in 1026 mutants. X -axis shows the mutated position, Y-axis shows amino acids in the mutated structure. Each cell shows $\Delta\Delta G$ scores of the mutants in kcal/ mol. Stabilizing mutants (negative values) are shown in green, destabilizing mutants (negative values) are shown in red.

Gu et al. (2023) performed a study using *Bacillus subtilis* 168, *Bacillus cereus*, *Mycobacterium smegmatis* MC2 155 and *Geobacillus stearothermophilus* organisms derived AlaDH enzymes under same conditions, and evaluated their kinetic parameters (Gu et al., 2023a). Using a MSA of the respective AlaDH sequences from *Bacillus subtilis* 168 (UniProt Accession Number: Q08352), *Bacillus cereus* (UniProt Accession Number: A0A2C3GCW7), *Mycobacterium smegmatis* MC2 155 (UniProt Accession Number: A0QVQ8) *Geobacillus stearothermophilus* (UniProt Accession Number: P17557)), and that of the L-AsAlaDH enzyme sequence (Figure 4.6.), an assessment of experimental activity values were made, by considering those mutants that yielded $\Delta\Delta G < 0$ from saturation mutagenesis. The purpose of this analysis was to establish a potential link between amino acid differences and experimental enzyme activities, and to further identify stabilizing mutants of *A. sulphurea* AlaDH with potentially-enhanced activity. Highly-conserved positions within or near the active site were excluded since mutating catalytically-important positions often results in inactive variants (Fernandes et al., 2015). As a result, 12 different positions were identified (Table 4.1). Figure 4.7. shows that there are only two positions that differ between four other enzymes and L-AsAlaDH, which are Tyr14 and Ile97. FoldX-based analysis yielded Tyr14Asn and Ile97Leu mutants as stabilizing. Thus, these were then prioritized for experimental studies.

In addition, SCANEER server was also used to identify potentially catalytic activity enhancing mutants. Here, SCANEER predicted Ser192Ala mutation with the highest SCI value. This mutant was also selected for further analysis.

As a result, the final list of mutants included (Tyr14Asn, Ile97Leu, and Ser192Ala). These were then subjected to solubility analysis, B-factor analysis, pKa analysis of active site positions and SASA analysis to further confirm their suitability for experimental studies.

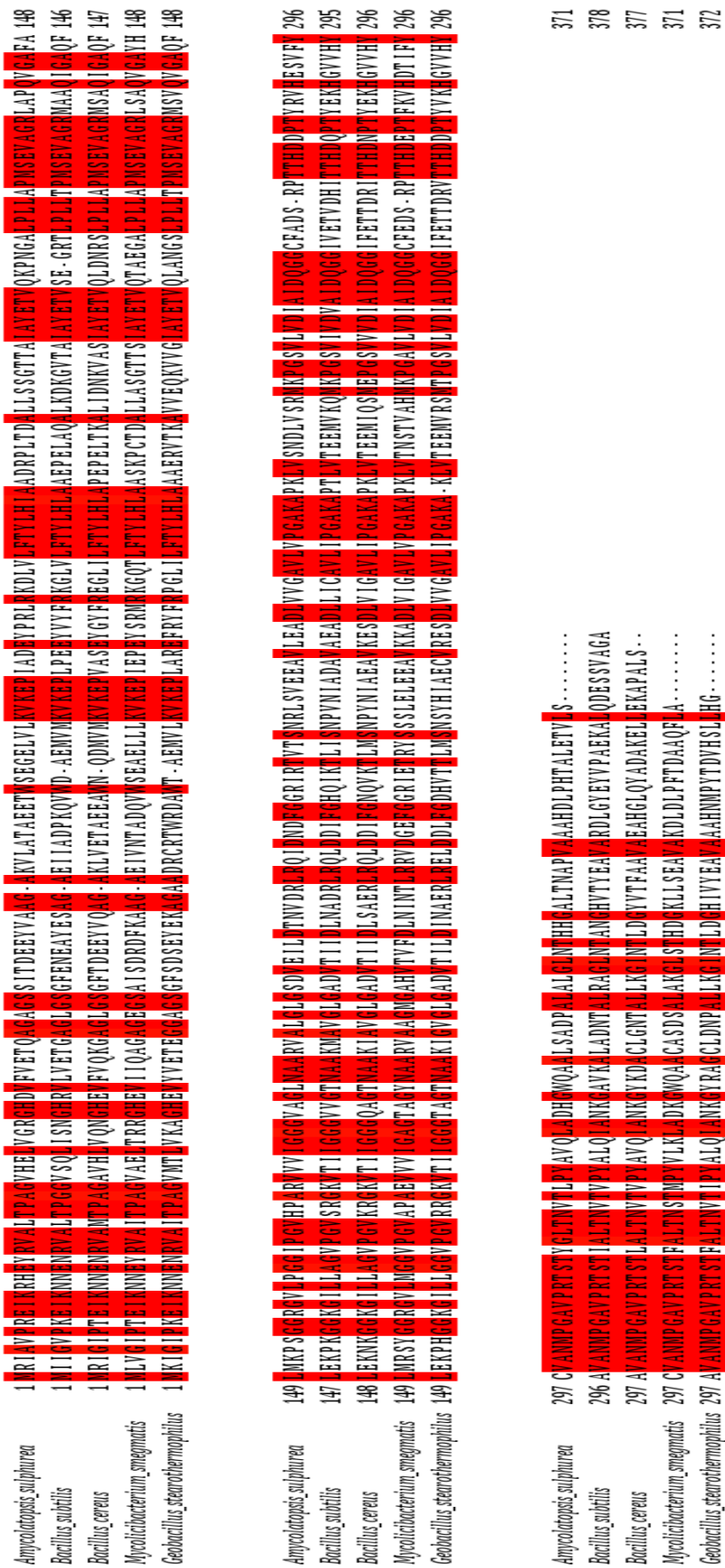


Figure 4.5: MSA result of *Bacillus subtilis* 168, *Bacillus cereus*, *Mycobacterium smegmatis* MC2 155 and *Geobacillus stearothermophilus* organisms derived L-AlaDH enzyme sequences.

Table 4.1: Experimental activity results of AlaDH from *Bacillus subtilis* 168, *Bacillus cereus*, *Mycobacterium smegmatis* MC2 155 and *Geobacillus stearothermophilus*, and amino acids found in positions identified from MSA and computational saturation mutagenesis results.

	Specific Activity (U/mg)	K _M (mM)	V _{max} (μmol/min)	K _{cat} (s ⁻¹)	k _{cat} /K _M (s ⁻¹ mM ⁻¹)	5	7	14	18	39	78	82	97	103	99	344	267
<i>Bs</i> AlaDH	5.22	2.25	64.70	36.34	16.68	V	K	N	L	A	L	Y	L	L*	A	N	V
<i>Bc</i> AlaDH	5.64	1.10	66.91	40.43	37.92	I*	S	N	M	A	V	Y	L	L	P	N	I
<i>Gs</i> AlaDH	2.00	0.81	10.70	11.94	16.29	I	K	N	I	G	L	F*	L	V	A	N	I
<i>Ms</i> AlaDH	4.48	1.69	23.69	28.28	17.02	I	T	Y	I	A	I*	Y	L	C	A	S	I
<i>As</i> AlaDH	-	-	-	-	-	V	R	Y	L	A	I	Y	I	L	A	N	I
FoldX-generated ΔΔG (Kcal/mol)						-0,82	ND	-0,45	ND	ND	-0,02	-0,62	-0,11	-0,01	ND	ND	ND

*Represents mutants detected by FoldX

ND: There is no detected FoldX-predicted ΔΔG values for any of variants

According to the B-factor analysis results (Figure 4.7), the average B-factor value was 0.84 Å². Specifically, Tyr14, Ile97 and Ser192 exhibited values of 0.80 Å², 0.86 Å², and 0.83 Å² respectively. As shown in **Figure 4.8**, the B-factor values for these selected positions were very close to the overall average. Both candidate positions are near the active site, and their rigidity/flexibility profile falls approximately within the middle range.

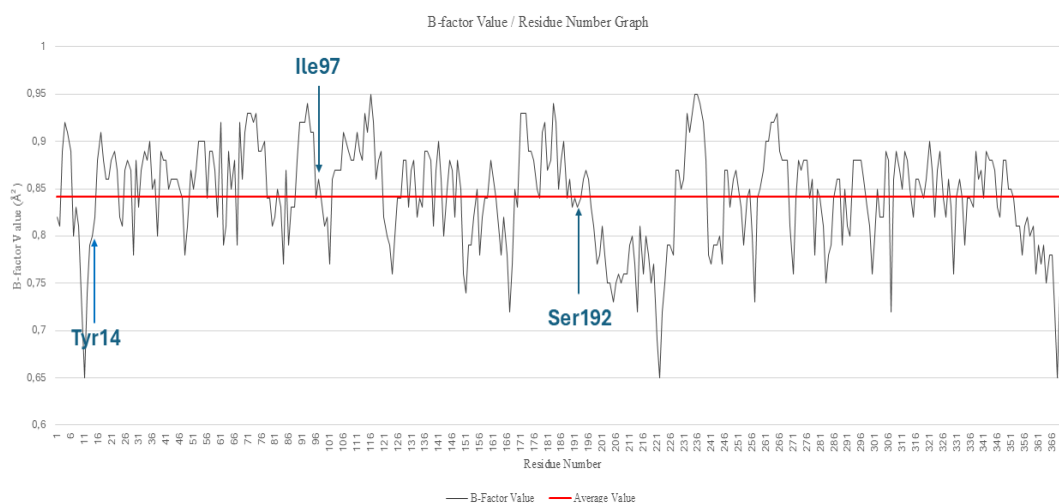


Figure 4.6: B-factor values of each aminoacids in the wild-type protein. Black line shows B-factor values, red line shows the average B-factor value.

Mutants generated with FoldX were used to calculate their Solvent Accessible Surface Area (SASA) values. In this SASA analysis, only the mutated positions were investigated and compared with the wild-type (**Table 4.2**). According to the results, the Tyr14Asn mutation decreased the SASA value at position 14. Similarly, the Ile97Leu and Ser192Ala mutation also led to a decrease in the SASA value at position 97 and 192, respectively. Furthermore, the results indicated that position 14 exhibited a higher SASA value than position 97 and 192. This observation may be attributed to the characteristics of the amino acids involved. Tyrosine, located at position 14, contains a hydroxyl group and may be situated somewhat exposed on the protein's surface. Conversely, isoleucine at position 97, possessing methyl groups in its structure, might adopt a more internal position within the protein due to its hydrophobic nature (Moret & Zebende, 2007; Pandey et al., 2023). Similarly, serine amino acid include hydrophobic side chain, this likely leads to more interactions with

water than Ile97. Decreasing SASA can lead to increased interaction with the ligand rather than water. However, a reduction in SASA might also result in decreased solubility.

Table 4.2: SASA values of position 14, 97 and 192 detected for mutants and wild-type.

Position	Wild-type	Tyr14Asn	Ile97Leu	Ser192Ala
14	104,117	49,042	-	-
97	1,713	-	1,17	-
192	6,156	-	-	5.713

Solubility of an enzyme is important for enzymatic activity (Paladin et al., 2017). We investigated changes in the solubility properties of selected mutants using the SODA server. Mutant sequences were provided to the server to measure their solubility profiles compared to the wild-type. Results indicated that both the Tyr14Asn and Ile97Leu mutations led to increased solubility compared to the wild-type. However, the Ser192Ala mutation did not result in higher solubility than the wild-type.

pKa analysis was performed on wild-type, Tyr14Asn, Ile97Leu and Ser192Ala. L-*AsAla*DHs to investigate changes in the pKa values of active site residues using the PropKa server. According to **Table 4.3**, the pKa values of His97 and Asp270 increased in the Ile97Leu mutant compared to the wild-type, but decreased in the Tyr14Asn mutant. Conversely, the pKa values of Arg15 decreased in all mutants, while those of Lys75 increased across all mutants.

Table 4.3: Changing the pKa values of active site residues on Tyr14Asn, Ile97Leu and Ser192Ala compared to wild-type *AsAla*DH.

Active Site Residue	Wild-type pKa Value	Tyr14Asn pKa Value	Ile97Leu pKa Value	Ser192Ala pKa Value
Arg15	11,7	10,78	11,61	10.74
Lys75	9,99	11,86	10,82	8.16
His96	0,17	0,09	0,82	0
Asp270	4,28	3,86	4,59	6.42

4.2. Mutant Production of L-AsAlaDH

Primers were prepared using OligoEvaluator™ web tool showed in the **Figure 4.9**, **Figure 4.10**, **Figure 4.11**.

The designed primer for Tyr14Asn mutant and its properties (**Figure 4.9**) that evaluated by Sigma OligoEvaluator as follows.

Forward: 5' GCGTGAAATTAAACGTCATGAAAATCGTGTGGCAC 3'

Backward: 3' GTGCCACACGATTTTCATGACGTTTAATTTACGC 5'

1. Analysis Results												
Sequence: 5' GCGTGAAATTAAACGTCATGAAAATCGTGTGGCAC 3'												
Base Count	Molecular Weight	Extinction Coefficient	Oligo Type	µg/OD at 260 nm	Length (bp)	Tm (°C)	GC%	GC Clamp	Run Length (bp)	Secondary Structure	Primer Dimer	BLAST
A = 12, U = 0, G = 9, C = 6, T = 8, I = 0, Total = 35	10828.2	351.4	No Mod	30.8	35	79.1	42.9	3	4	Weak	No	Sequence

Figure 4.7: Analysed properties of primer designed for Tyr1 Asn mutant.

The designed primer for Ile97Leu mutant and its properties (**Figure 4.10**) that evaluated by Sigma OligoEvaluator as follows.

Forward: 5' GTTCTGTTTACCTATCTGCATCTTGCGGCCGATCG 3

Backward: 3' CGATCGGCCGCAAGATGCAGATAGGTAAACAGAAC 5'

1. Analysis Results												
Sequence: 5' GTTCTGTTTACCTATCTGCATCTTGCGGCCGATCG 3'												
Base Count	Molecular Weight	Extinction Coefficient	Oligo Type	µg/OD at 260 nm	Length (bp)	Tm (°C)	GC%	GC Clamp	Run Length (bp)	Secondary Structure	Primer Dimer	BLAST
A = 4, U = 0, G = 8, C = 10, T = 13, I = 0, Total = 35	10671.0	312.5	No Mod	34.1	35	80.4	51.4	2	3	Weak	No	Sequence

Figure 4.8: Analysed properties of primer designed for Ile97Leu mutant

The designed primer for Ser192Ala mutant and its properties (**Figure 4.11**) that evaluated by Sigma OligoEvaluator as follows.

Forward: 5' GCATTAGGTCTGGGTGCCGATGTTGAAATTCTGG 3'

Backward: 3' CCAGAATTTCAACATCGGCACCCAGACCTAATGC 5'

1. Analysis Results												
Sequence: 5' GCATTAGGTCTGGGTGCCGATGTTGAAATTCTGG 3'												
Base Count	Molecular Weight	Extinction Coefficient	Oligo Type	µg/OD at 260 nm	Length (bp)	Tm (°C)	GC%	GC Clamp	Run Length (bp)	Secondary Structure	Primer Dimer	BLAST
A = 6, U = 0, G = 12, C = 5, T = 11, I = 0, Total = 34	10560.0	325.5	No Mod	32.4	34	80.2	50.0	2	3	Weak	No	Sequence

Figure 4.9: Analysed properties of primer designed for Ser192Ala mutant.

We optimized the PCR process for all mutants and the wild-type in a thermocycler, using commercially synthesized primers (**Table 4.4**).

Table 4.4: Optimized PCR steps during mutat creation process.

Steps		Tyr14Asn (Temperature/Time)	Ile97Leu (Temperature/Time)	Ser192Ala (Temperature/Time)
1	Initial Denaturation	98 °C / 10 s	98 °C / 10 s	98 °C / 10 s
2	Denaturation	98 °C / 10 s	98 °C / 30 s	98 °C / 10 s
3	Denaturation	98 °C / 8 s	98 °C / 8 s	98 °C / 8 s
	Annealing	62,26/ 20s	65,7 °C / 20s	65,5 °C/ 20s
	Extension	72°C / 3 mins	72°C / 3 mins	72°C / 3 mins
4	Final Extension	72°C / 7,5 mins	72°C / 7,5 mins	72°C / 7,5 mins
	Cycle	30	30	30

After optimization, plasmid concentration was measured at 260 nm. All plasmids had concentrations between 70-130 ng/µL, and their 260/280 ratios ranged from 1.7-2.0 (**Table 4.5**.) (Lucena-Aguilar et al., 2016). Agarose gel electrophoresis was performed on the plasmid DNAs. As seen in **Figure 4.12**, all mutant samples, including those treated with DpnI enzyme, appeared at approximately 6.4 kbp on the agarose gel (Aktaş, 2021a).

Table 4.5: Concentration and 260/280 ratio of wild-type, Tyr14Asn, Ile97Leu and Ser192Ala.

	Concentration (ng/ μ L)	260/280
Wild-type	70,901	1,882
Tyr14Asn	117,200	1,841
Ile97Leu	125,798	1,836
Ser192Ala	128,065	1,732

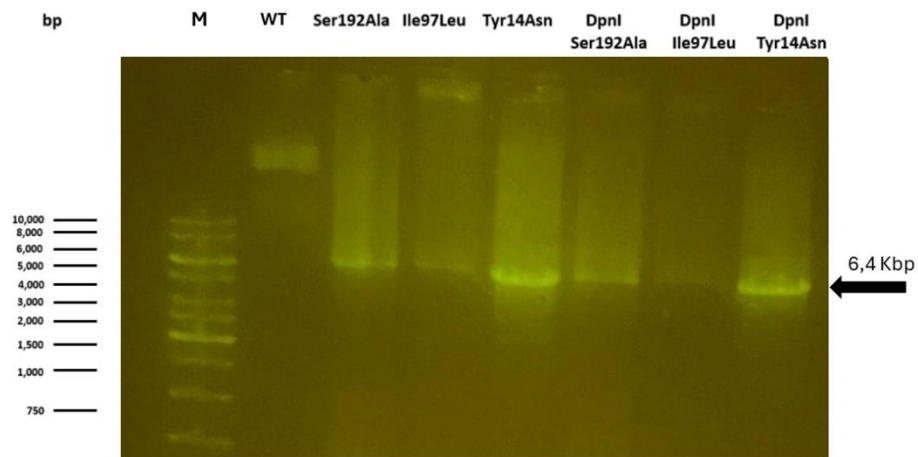


Figure 4.10: Visualization of mutants and wild-type on agarose gel electrophoresis. **M:** Molecular Weight Marker, **WT:** Wild-type plasmid, **Ser192Ala:** Ser192Ala mutant plasmid, **Ile97Leu:** Ile97Leu Mutant, **Tyr14Asn:** Tyr14Asn mutant, **DpnI Ser192Ala:** DpnI manipulated Ser192Ala mutant, **DpnI Ile97Leu:** DpnI manipulated Ile97Leu mutant, **DpnI Tyr14Asn:** DpnI manipulated Tyr14Asn

Following agarose gel verification of all plasmids, all mutants were confirmed by sequencing. The sequencing process was performed by Eurofins, and the results were aligned using ClustalOmega and visualized with Jalview.

As shown in **Figure 4.13**, the Tyr14Asn mutant was obtained by changing the TAT codon to AAT at position 14. The Ile97Leu mutant resulted from the conversion of the ATT codon to CTT; however, during plasmid design, the GCA codon was also changed to GCG to adhere to design rules, despite both codons encoding the same amino acid. The Ser192Ala mutant was designed by converting the AGC codon to GCC.

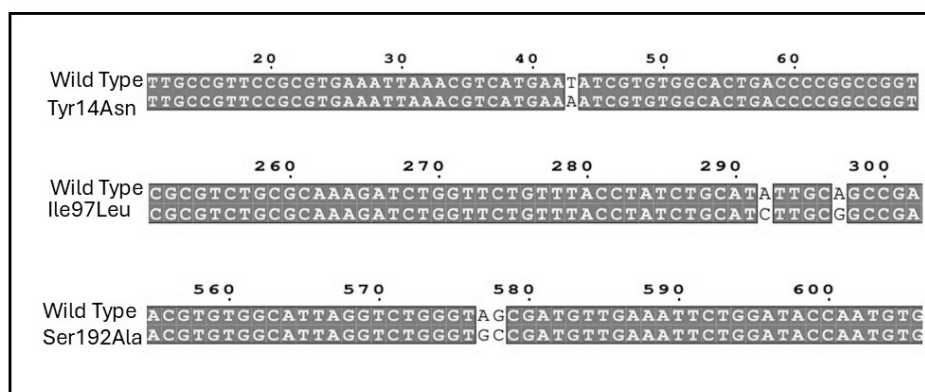


Figure 4.11: Alignment of sequencing results compared to wild-type.

After mutant verification, plasmids were transformed into *E. coli* BL21 competent cells. Based on the agarose gel electrophoresis results, the amounts of PCR products used for transformation were 3 μ L for Ser192Ala and the wild-type, and 5 μ L for Ile97Leu and Tyr14Asn mutants. The transformed cells were then stored in glycerol at -80°C . These cells were subsequently used for both preculture formation and large-scale cell production. The produced cells were stored at -20°C for future purification steps.

4.3. Purification of Wild-type and Mutant *L-AsAlaDH* Enzymes

Mutant and wild-type *L-AsAlaDH* were purified with Ni-NTA column chromatography method. During the chromatography, different imidazole concentration containing buffers (100, 200, 400 and 500mM) were eluted from the column. The purity level of the samples was visualized with SDS-PAGE gel electrophoresis. As shown in the **Figure 4.14** purity of wild-type and Ile97Leu *L*-alanine dehydrogenase enzyme was investigated. When, wild-type enzyme was investigated as pure 200/5 and 400/1-3 fractions, Ile97Leu mutant was seen as pure 200/3-5 and 400/1-2 fractions.

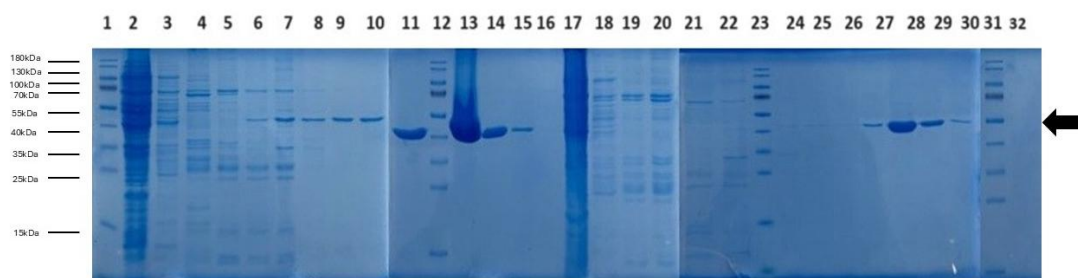


Figure 4.12: After purification, SDS page image belong to Ile97Leu and wild-type. **1, 12, 23 and 31:** Protein marker, **2:** Flowthrough elution fraction belongs to Ile97Leu, **3:** Buffer A elution fraction belongs to Ile97Leu, **4, 5, 6:** 100mM imidazol including buffer A elution fractions belongs to Ile97Leu, **7, 8, 9, 10,11:** 200mM imidazol including buffer A elution fractions belongs to Ile97Leu, **13, 14, 15:** 400mM imidazol including buffer A elution fractions belongs to Ile97Leu, **16:** Buffer B elution fraction belongs to Ile97Leu, **17:** Flowthrough elution fraction belongs to wild-type, **18:** Buffer A elution fraction belongs to wild-type, **19, 20, 21:** 100mM imidazol including buffer A elution fractions belongs to wild-type, **22, 24, 25, 26, 27:** 200mM imidazol including buffer A elution fractions belongs to wild-type, **28, 29, 30:** 400mM imidazol including buffer A elution fractions belongs to wild-type, **32:** Buffer B elution fraction belongs to wild-type.

Figure 4.15 shows the purity of the Ser192Ala and Tyr14Asn enzymes. The Ser192Ala enzyme appeared pure in fractions 200/3-5 and 400/1-2, while the Tyr14Asn enzyme was pure in fractions 200/5 and 400/1-2. In all figures, the purified enzymes from these mentioned fractions consistently showed a molecular weight of approximately 41.09 kDa (Aktaş, 2021b).

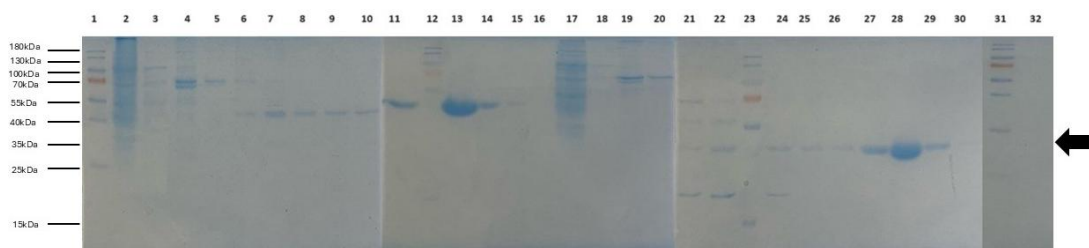


Figure 4.13: After purification, SDS page image belongs to Ser192Ala and Tyr14Asn. **1, 12, 23 and 31:** Protein marker, **2:** Flowthrough elution fraction belongs to Ser192Ala, **3:** Buffer A elution fraction belongs to Ser192Ala, **4, 5, 6:** 100mM imidazol including buffer A elution fractions belongs to Ser192Ala, **7, 8, 9, 10,11:** 200mM imidazol including buffer A elution fractions belongs to Ser192Ala, **13, 14, 15:** 400mM imidazol including buffer A elution fractions belongs to Ser192Ala, **16:** Buffer B elution

fraction belongs to Ser192Ala, **17**: Flowthrough elution fraction belongs to Tyr14Asn, **18**: Buffer A elution fraction belongs to Tyr14Asn, **19, 20, 21**: 100mM imidazol including buffer A elution fractions belongs to Tyr14Asn, **22, 24, 25, 26, 27**: 200mM imidazol including buffer A elution fractions belongs to Tyr14Asn, **28, 29, 30**: 400mM imidazol including buffer A elution fractions belongs to Tyr14Asn, **32**: Buffer B elution fraction belongs to Tyr14Asn.

Afterward, all detected fractions were combined, and impurities and salts were removed using ultrafiltration with a 30 kDa pore size filter. Subsequently, PD-10 desalting columns were applied to all purified samples to exchange the solution with their respective buffers at the optimum pH.

4.4. Determination of Optimum pH and Temperature for L-AsAlaDH Enzymes

Optimum working conditions of enzymes were determined to obtain maximum kinetics values of enzymes. During the optimum pH detection process, effect of 20 pH values (pH 3.0-11.0) from 5 different buffers (Citric Acid-Sodium Citrate, Sodium Phosphate, Tris-HCl, Glycine-NaOH, Sodium Carbonate-Sodium Bicarbonate) was investigated.

Figure 4.16 illustrates the effect of pH on reductive amination reaction catalysis. The optimum pH for both the wild-type and Tyr14Asn enzyme was determined to be pH 9.0 in Tris-HCl buffer. For Ile97Leu and Ser192Ala, the optimal buffers were pH 9.5 Sodium carbonate-sodium bicarbonate and pH 10.0 sodium carbonate-sodium bicarbonate, respectively.

As depicted in **Figure 4.16**, the Tyr14Asn mutant (**Figure 4.16b**) shifted the enzyme's optimal working pH to a more basic condition than the wild-type. Conversely, while Ser192Ala (**Figure 4.16d**) allowed for a broader working range, Ile97Leu (**Figure 4.16c**) decreased this range.

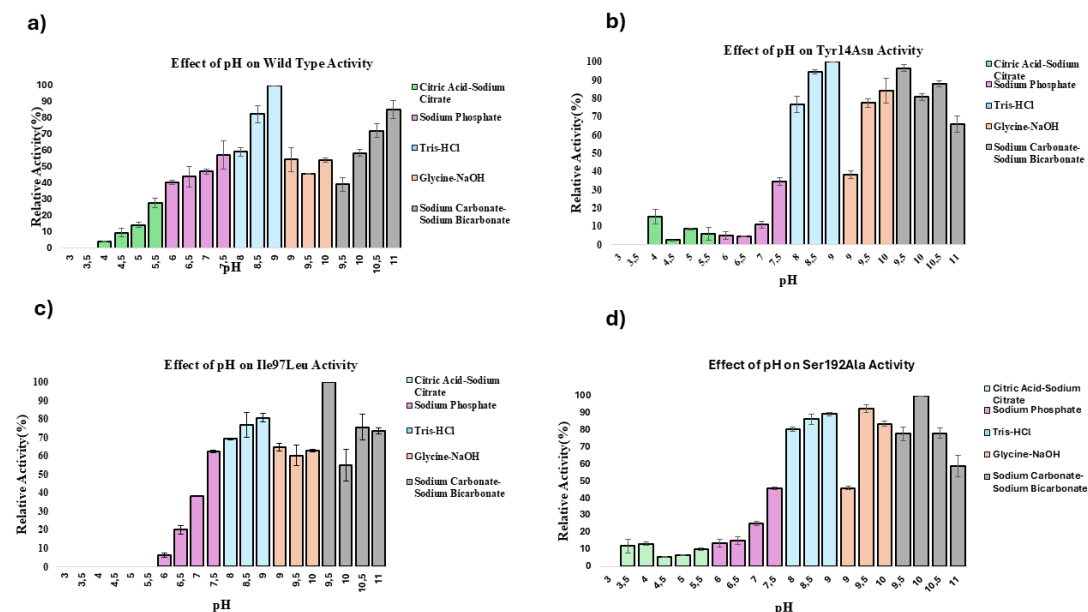


Figure 4.14: Effects of different pH (Citric acid-sodium citrate: pH 3.0-5.5, Sodium phosphate: pH 6.0-7.5, Tris-HCl: pH 8.0-9.0, Glycine-NaOH: pH 9.0-10.0, Sodium carbonate-sodium bicarbonate: pH 9.5-11.0) on reductive amination catalysed by L-AsAlaDH: **(a)** wild-type **(b)** Tyr14Asn **(c)** Ile97Leu and, **(d)** Ser192Ala.

Figure 4.17 illustrates the effect of pH on oxidative deamination reaction catalysis. The optimum pH for both the wild-type (**Figure 4.17a**) and Ile97Leu (**Figure 4.17c**) enzymes were determined to be pH 10.5 in sodium carbonate-sodium bicarbonate buffer. Conversely, pH 10.0 sodium carbonate-sodium bicarbonate buffer was identified as optimal for both Tyr14Asn (**Figure 4.17b**) and Ser192Ala (**Figure 4.17d**). Notably, the optimum pH for wild-type oxidative deamination aligns with previously reported literature (Aktaş, 2021). As depicted in **Figure 4.17**, all mutants showed a decreased working range compared to the wild-type enzyme.

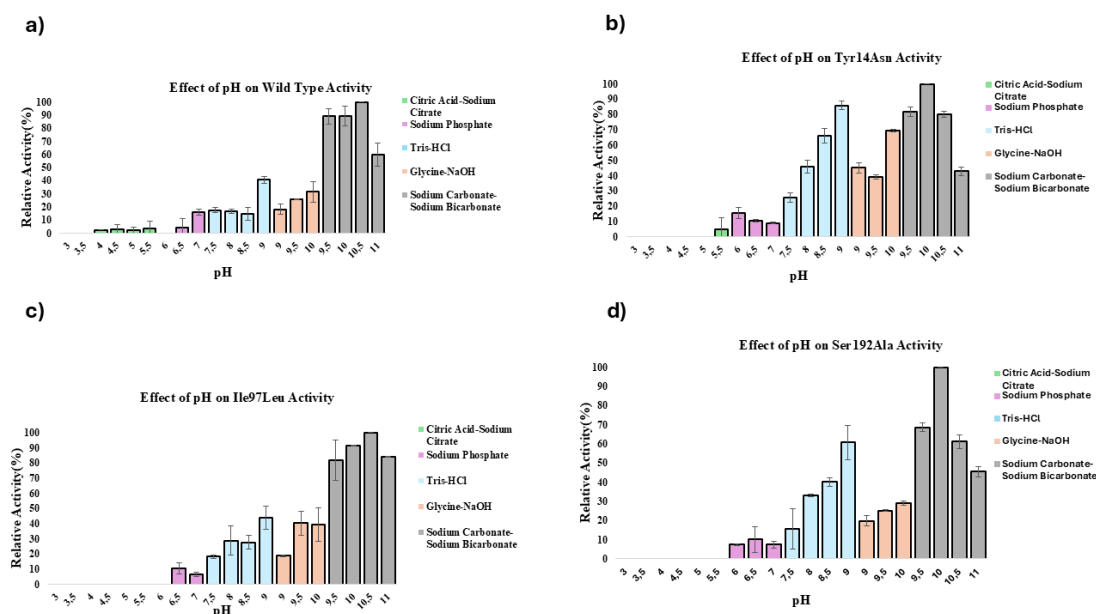


Figure 4.15: Effects of different pH (Citric acid-sodium citrate: pH 3.0-5.5, Sodium phosphate: pH6.0-7.5, Tris-HCl: pH8.0-9.0, Glycine-NaOH: pH 9.0-10.0, Sodium carbonate-sodium bicarbonate: pH9.5-11.0) on oxydative deamination catalysed by L-AsAlaDH: **(a)** wild-type **(b)** Tyr14Asn **(c)** Ile97Leu and, **(d)** Ser192Ala.

After establishing the optimum working pH, the optimal temperatures were determined for both reductive amination and oxidative deamination reactions across a range of 25-55°C. **Figure 4.18** illustrates the effect of temperature on the reductive amination reaction for the wild-type enzyme and each mutant. The optimum temperatures were determined to be 30°C for the wild-type, 35°C for Tyr14Asn, 45°C for Ile97Leu, and 40°C for Ser192Ala. While all enzymes in this study exhibited a distinct decrease in activity above their optimum temperature, the Ile97Leu mutant showed minimal change between 35-45°C. Furthermore, Ile97Leu maintained activity at 55°C, in contrast to the wild-type and other mutants.

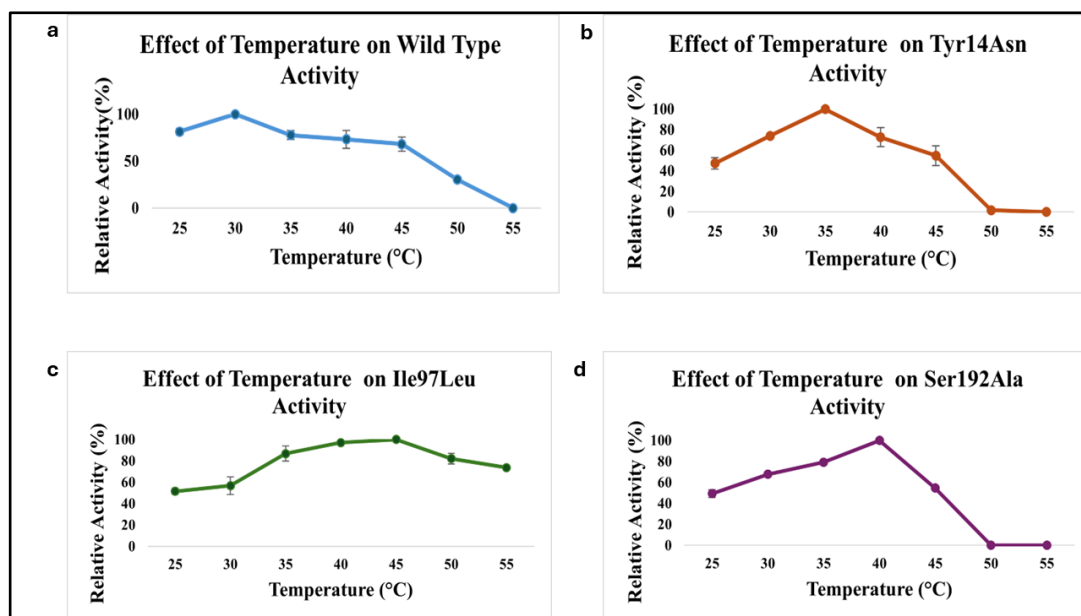


Figure 4.16: Effects of different temperature (25-55°C) on reductive amination catalysed by L-AsAlaDH: **(a)** wild-type **(b)** Tyr14Asn **(c)** Ile97Leu and, **(d)** Ser192Ala.

Figure 4.19 illustrates the effect of temperature on the oxidative deamination reaction for the wild-type enzyme and each mutant. The optimum temperatures were determined to be 25°C for the wild-type, 30°C for Tyr14Asn, 40°C for Ile97Leu, and 35°C for Ser192Ala. Notably, the optimum temperature for wild-type oxidative deamination aligns with previously reported literature (Aktaş, 2021). None of the enzymes exhibited any activity at 55°C. Furthermore, Tyr14Asn and Ser192Ala showed no activity at 50°C.

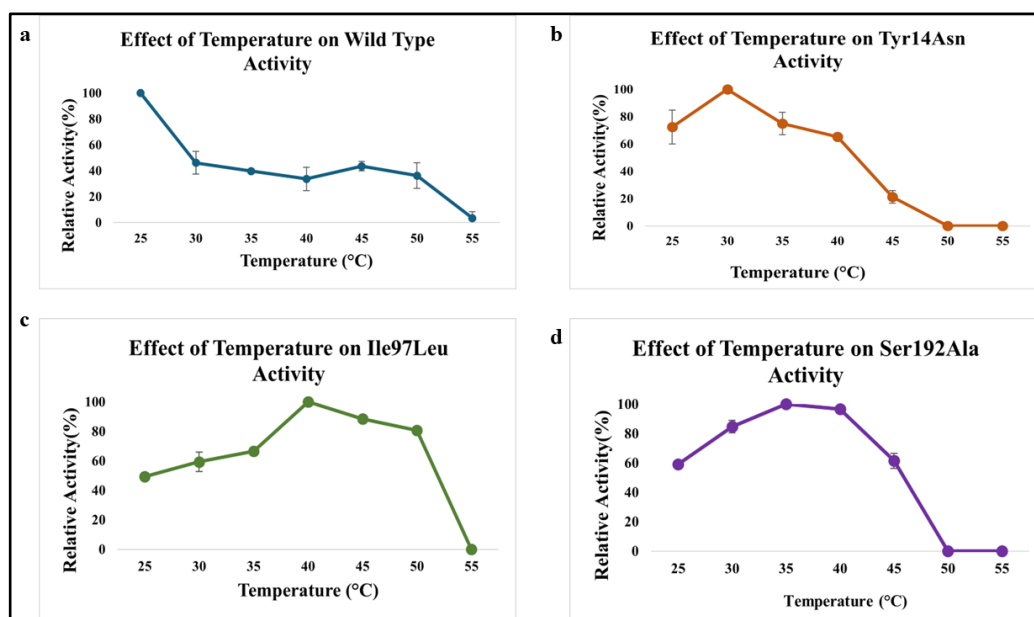


Figure 4.17: Effects of different temperature (25-55°C) on oxydative deamination catalysed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala.

4.5. Determination of Kinetics Parameters of L-AsAlaDH Enzymes

In this thesis, we determined the kinetic parameters for both the oxidative deamination and reductive amination reactions. We used varying substrate concentrations (0.1-50 mM) of L-alanine and pyruvate, and for each enzyme and reaction pathway, we utilized the optimum pH and temperature. Pure enzymes, prepared in their respective optimum buffers, were used for all reactions. The reductive amination reaction was performed with 0.5 mM NAD(H) and 0.5 M ammonium chloride as the NH_4^+ source.

Oxidative deamination reaction was performed with 1mM NAD^+ as cofactor. NAD(H) depletion/formation was investigated in the reaction at 340nm for 10 minutes. Specific activity values were calculated using $\epsilon=6220 \text{ M}^{-1} \text{ cm}^{-1}$ value. Michaelis-Menten graphs were drawn and kinetic parameters which are K_M and $V_{\max}$ (maximum rate) values were calculated with GraphPad Prism 8. In **Figure 4.20** and **Figure 4.21**, Michaelis-Menten graphs of wild-type and mutants belong to reductive amination and oxidative deamination reaction, respectively, can be investigated. All the graphs fit to Michaelis-Menten model.

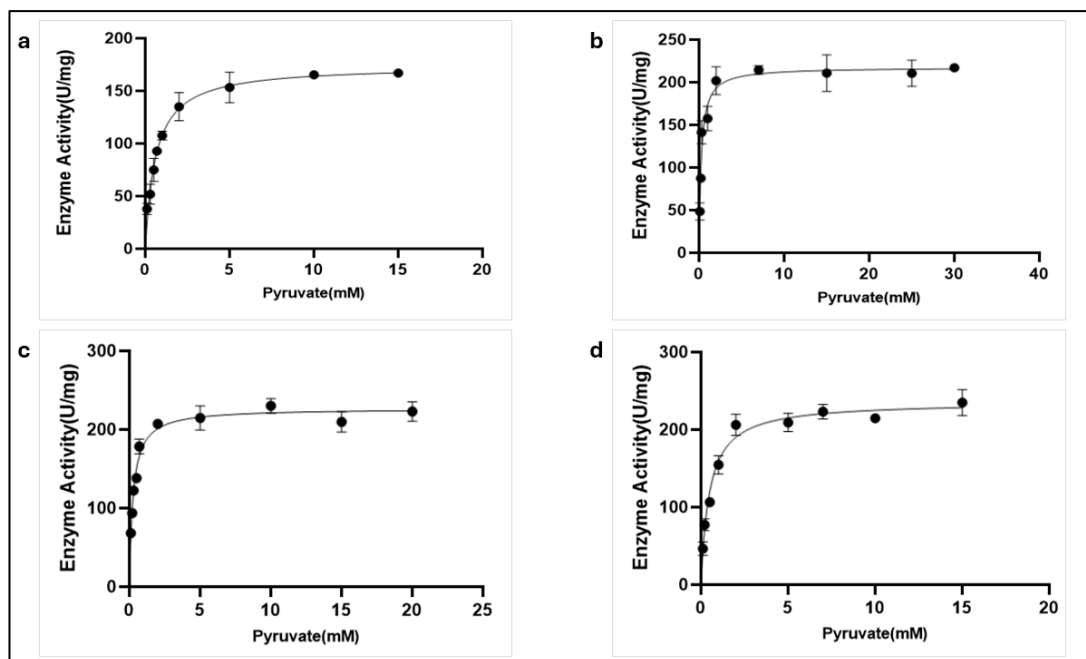


Figure 4.18: Michaelis-Menten graphs of reductive amination catalyzed by L-AsAlaDH: (a) wild-type (b) Tyr14Asn (c) Ile97Leu and, (d) Ser192Ala.

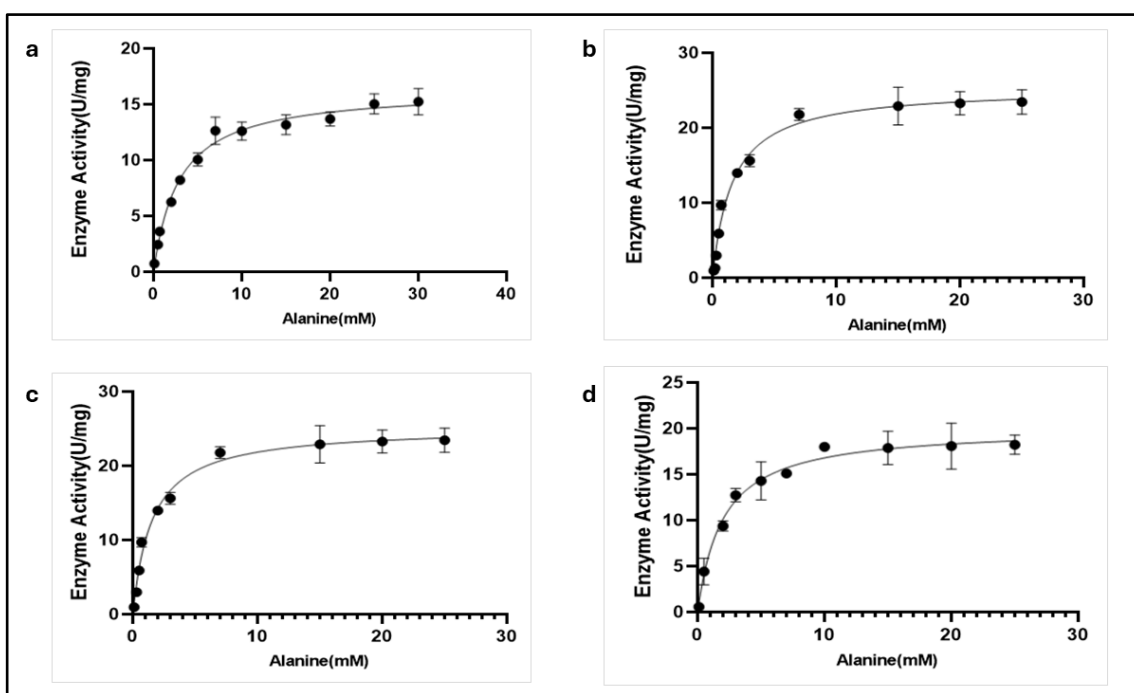


Figure 4.19: Michaelis-Menten graphs of oxidative deamination catalyzed by L-AsAlaDH: (a) wild-type, (b) Tyr14Asn, (c) Ile97Leu and, (d) Ser192Ala

Table 4.6 presents the k_{cat} , K_M , and k_{cat}/K_M values for both reductive amination and oxidative deamination reactions. For the reductive amination reaction, all mutants

exhibited an increase in both k_{cat} and K_M values, consequently leading to an enhanced k_{cat}/K_M ratio, which signifies improved catalytic efficiency. Specifically, the Tyr14Asn mutant demonstrated a 3-fold increase in catalytic efficiency, despite an accompanying 2.27-fold increase in its K_M value. The Ile97Leu mutant showed a 3.13-fold enhancement in catalytic efficiency, coupled with a 2.36-fold increase in K_M . Similarly, Ser192Ala exhibited an improved activity, increasing 1.73-fold, alongside a 1.28-fold increase in K_M .

In the oxidative deamination reaction, an inverse relationship was observed between k_{cat} and K_M values across all mutants: as k_{cat} increased, K_M decreased, consequently leading to an enhanced k_{cat}/K_M ratio. Specifically, the Tyr14Asn mutant exhibited a 2.79-fold increase in catalytic efficiency, accompanied by a 1.80-fold decrease in its K_M value. Similarly, Ile97Leu demonstrated a 3.04-fold increase in catalytic efficiency and a 1.92-fold reduction in K_M . The Ser192Ala mutant also showed improved activity, increasing 1.80-fold, alongside a 1.46-fold decrease in K_M .

Table 4.6: Kinetics values belong to reductive amination and oxidative deamination catalysis of wild-type and mutant *L-AsAlaDH*.

Reductive Amination			
	$k_{\text{cat}} (\text{s}^{-1})$	$K_M (\text{mM})$	$k_{\text{cat}}/K_M (\text{s}^{-1}\text{mM}^{-1})$
Wild-type	119,30±8,62	0,615±0,17	193,98±40,0
Tyr14Asn	149,02±10,99	0,27±0,10	581,65±70,98
Ile97Leu	155,39±7,89	0,26±0,03	607,22±38,31
Ser192Ala	161,55±10,84	0,478±0,09	336,99±29,07
Oxidative Deamination			
	$k_{\text{cat}} (\text{s}^{-1})$	$K_M (\text{mM})$	$k_{\text{cat}}/K_M (\text{s}^{-1}\text{mM}^{-1})$
Wild-type	11,20±0,79	2,89±0,80	3,88±0,80
Tyr14Asn	17,36±1,29	1,60±0,48	10,85±2,41
Ile97Leu	17,76±1,86	1,50±0,63	11,80±3,67
Ser192Ala	13,79±1,31	1,97±0,82	6,99±2,27

4.6. Effects of Metals on L-AlaDH Enzymes and Statistical Analysis

Metal ions can either inhibit or enhance enzyme activity, a phenomenon that has been extensively investigated for various L-AlaDH enzymes in the literature (Dedeakayoğulları et al., 2023; He et al., 2018). Based on this, effects of 7 different metals (Zn (II), Fe (III), K⁺, Na⁺, Li⁺, Mg⁺² and Ca⁺²) with 5 different concentrations (1, 3, 5, 7 and 10 µM) in reductive amination activity were investigated. Pure enzymes in its optimum buffer were combined with 0,5mM NAD(H) and 0,5M NH₄Cl. NAD(H) depletion was investigated at 340nm for 10 minutes. Experiments were performed with three replicates. We compared the obtained results to a control (without metal) by calculating the relative values, and then generated heatmap graphics for each mutant and the wild-type. The significance level of the results was analyzed using Two-way ANOVA and Dunnett Test (for multiple comparisons), with a confidence level of 0.001.

Figure 4.22 presents heatmap graphics for the mutants and wild-type. In these heatmaps, red indicates increased activity while green signifies decreased activity compared to the control. For statistical analysis, a significance level of $p < 0.001$ was accepted for the sensitivity of the test, and these statistically significant values are underlined in the heatmaps.

Figure 4.22a displays the relative (%) activity values, showing the effect of metal ions on the reductive amination reaction of wild-type and mutant enzymes. Most metals, in particular, increased the activity of the wild-type. However, 10 µM of K⁺ is calculated as the highest increased enzyme activity with ~80%, also Fe⁺³ lead to inhibition on the enzyme activity ~ 25%. In the **Figure 4.22b** most of the metal concentrations lead to increasing activity on Tyr14Asn enzyme. 10 µM of Ca^{+ 2} ion including reaction showed ~ 60% increased enzyme activity. However, Zn^{+ 2} ion showed inhibitory effect ~ 30%.

In **Figure 4.22c** most of the metal concentrations lead to inhibitory effect on Ile97Ile enzyme. 3 µM of Zn^{+ 2} ion including reaction showed ~ 15% increased enzyme activity. However, Fe⁺³ ion showed inhibitory effect ~ 60%. In **Figure 4.22d** Ser192Ala enzyme was investigated. 3 µM of Fe⁺³ ion including reaction showed ~

75% increased enzyme activity. However, 10 μM Fe^{+3} ion showed inhibitory effect $\sim 40\%$.

a						b					
	1 μM	3 μM	5 μM	7 μM	10 μM		1 μM	3 μM	5 μM	7 μM	10 μM
Control	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	Control	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
Na ⁺	93,11 $\pm$ 1,95	111,23 $\pm$ 1,43	135,44 $\pm$ 0,97	109,2 $\pm$ 2,07	95,36 $\pm$ 2,55	Na ⁺	110,22 $\pm$ 5,43	117,78 $\pm$ 1,28	119,56 $\pm$ 0,08	137,69 $\pm$ 6,54	150,02 $\pm$ 3,03
Fe ²⁺	140,33 $\pm$ 2,45	150,13 $\pm$ 0,94	145,26 $\pm$ 3,06	93,08 $\pm$ 2,34	76,47 $\pm$ 2,61	Fe ²⁺	144,29 $\pm$ 0,5	133,31 $\pm$ 9,94	95,52 $\pm$ 8,93	102,73 $\pm$ 7,52	97,41 $\pm$ 1,62
Zn ²⁺	83,27 $\pm$ 5,44	118,56 $\pm$ 1,48	122 $\pm$ 4,27	184,71 $\pm$ 4,78	138,18 $\pm$ 1,07	Zn ²⁺	68,33 $\pm$ 4,48	90,32 $\pm$ 7,17	113,56 $\pm$ 1,54	94,07 $\pm$ 1,86	87,46 $\pm$ 4,24
K ⁺	99,68 $\pm$ 6,18	122,69 $\pm$ 3,93	146,74 $\pm$ 2,72	157,16 $\pm$ 2,26	186,76 $\pm$ 5,72	K ⁺	127,77 $\pm$ 4,42	132,93 $\pm$ 4,07	147,58 $\pm$ 6,91	132,12 $\pm$ 3,58	109,05 $\pm$ 0,35
Li ⁺	123,27 $\pm$ 1,51	137,52 $\pm$ 3,22	151,24 $\pm$ 1,76	141,57 $\pm$ 0,58	133,04 $\pm$ 5,75	Li ⁺	129,37 $\pm$ 4,19	126,32 $\pm$ 8,86	121,57 $\pm$ 6,69	151,42 $\pm$ 6,53	109,64 $\pm$ 4,7
Mg ²⁺	110,34 $\pm$ 3,1	141,54 $\pm$ 2,81	147,76 $\pm$ 3,24	128,7 $\pm$ 3,16	98,52 $\pm$ 1,68	Mg ²⁺	116,42 $\pm$ 4,06	130,42 $\pm$ 6,45	151,16 $\pm$ 4,44	114,79 $\pm$ 6,67	100,85 $\pm$ 9,07
Ca ²⁺	93,4 $\pm$ 0,09	101,12 $\pm$ 0,17	104,29 $\pm$ 2,73	131,66 $\pm$ 9,07	164,29 $\pm$ 3,96	Ca ²⁺	84,16 $\pm$ 4,74	97,26 $\pm$ 6,37	135,98 $\pm$ 0,53	133,51 $\pm$ 5,54	158,65 $\pm$ 0,16

c						d					
	1 μM	3 μM	5 μM	7 μM	10 μM		1 μM	3 μM	5 μM	7 μM	10 μM
Control	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	Control	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
Na ⁺	68,79 $\pm$ 7,85	109,01 $\pm$ 2,07	98,82 $\pm$ 8,66	86,96 $\pm$ 1,89	60,34 $\pm$ 2,57	Na ⁺	85,05 $\pm$ 1,84	107,36 $\pm$ 3,19	155,33 $\pm$ 1,6	91,05 $\pm$ 6,93	81,11 $\pm$ 2,81
Fe ²⁺	44,74 $\pm$ 0,19	71,08 $\pm$ 1,17	100,72 $\pm$ 8,37	80,31 $\pm$ 3,26	62,13 $\pm$ 9,43	Fe ²⁺	76,93 $\pm$ 3,52	81,58 $\pm$ 6,05	175,71 $\pm$ 1,26	142,12 $\pm$ 5,15	58,88 $\pm$ 3,41
Zn ²⁺	65,98 $\pm$ 5,22	113,7 $\pm$ 14,6	71,18 $\pm$ 8,35	69,13 $\pm$ 3,76	82,67 $\pm$ 0,78	Zn ²⁺	76,17 $\pm$ 5,07	114,41 $\pm$ 6,72	124,31 $\pm$ 1,71	104,17 $\pm$ 4,58	68,87 $\pm$ 1,42
K ⁺	59,86 $\pm$ 4,26	74,99 $\pm$ 1,01	101,59 $\pm$ 4,92	82,18 $\pm$ 1,52	69,31 $\pm$ 2,65	K ⁺	80,03 $\pm$ 8,43	103,92 $\pm$ 2,44	103,99 $\pm$ 9,06	92,76 $\pm$ 7,78	81,89 $\pm$ 5,07
Li ⁺	75,25 $\pm$ 0,5	92,83 $\pm$ 6,46	79,86 $\pm$ 6,48	81,06 $\pm$ 2,91	60,7 $\pm$ 4,14	Li ⁺	74,17 $\pm$ 1,9	114,37 $\pm$ 1,35	125,58 $\pm$ 1,86	136,26 $\pm$ 5,88	79,94 $\pm$ 5,1
Mg ²⁺	61,39 $\pm$ 8,14	72,9 $\pm$ 4,46	84,63 $\pm$ 5,77	79,64 $\pm$ 7,97	73,98 $\pm$ 4,14	Mg ²⁺	84,14 $\pm$ 2,31	118,91 $\pm$ 2,05	77,28 $\pm$ 9,69	74,62 $\pm$ 0,07	71,46 $\pm$ 5,08
Ca ²⁺	86,68 $\pm$ 5,38	83,71 $\pm$ 6,11	99,53 $\pm$ 2,55	88,86 $\pm$ 3,18	80,59 $\pm$ 1,95	Ca ²⁺	104,25 $\pm$ 3,86	94,28 $\pm$ 4,41	79,08 $\pm$ 7,24	75,45 $\pm$ 3,68	61,53 $\pm$ 0,87

Figure 4.20: Metal binding statistics of reductive amination catalyzed by L-AsAlaDH: **a)** Wild-type **b)** Tyr14Asn **c)** Ile97Leu **d)** Ser192Ala.

4.7. Storage Stability

During storage, enzyme activity of wild-type was largely conserved at -80°C , -20°C , and $+4^{\circ}\text{C}$, while the activity of the mutants decreased at these same temperatures. The wild-type, Tyr14Asn, and Ile97Leu mutants lost activity at $+25^{\circ}\text{C}$ after 8, 6, and 3 days, respectively (**Figure 4.23**).

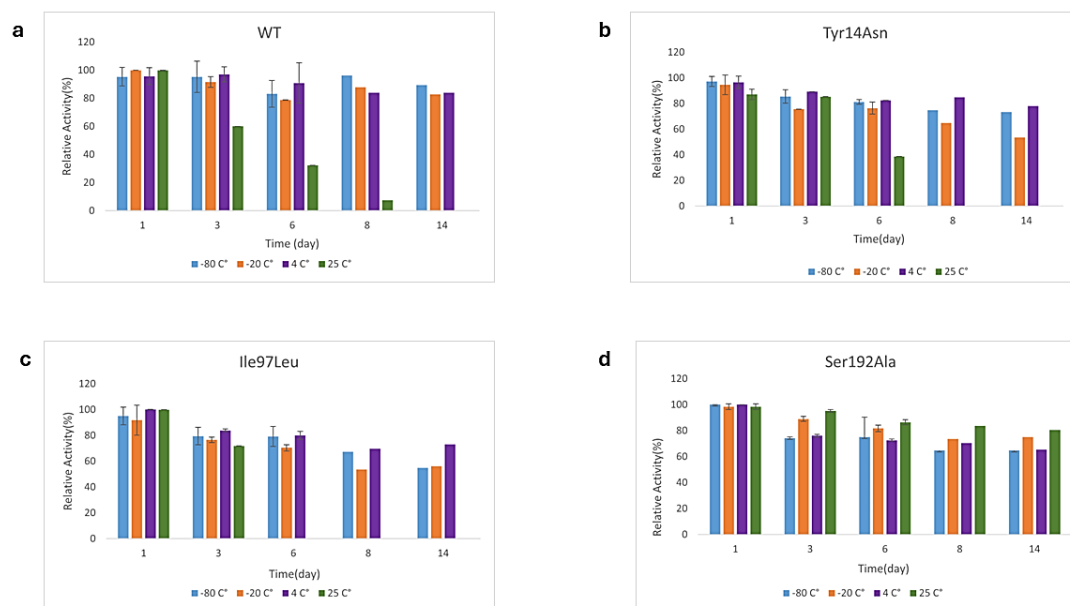


Figure 4.21: Storage stability of reductive amination catalyzed by L-AsAlaDH: **a)** wild-type **b)** Tyr14Asn **c)** Ile97Leu and, **d)** Ser192Ala.

4.8. Active Site Investigation

Potential effects of generated mutants on the active site of L-AsAlaDH were also investigated. First, mutations were applied to the homology model (wild-type) using PyMOL. The mutants were then energy-minimized using the YASARA web server. Finally, pyruvate-protein interactions within the active site were investigated using Discovery Studio and PyMOL.

The wild-type enzyme's active site (**Figure 4.24**) shows several interactions with the pyruvate molecule. His96 and Lys75 form conventional hydrogen bonds with pyruvate's O3 atom (3.1 Å and 2.1 Å, respectively). Lys75 (1.8 Å) and Arg15 (1.7 Å) establish salt bridges with the O1 atom. Additionally, Asn300 forms a carbon-hydrogen bond (2.3 Å) with the O1 atom, while Arg15 (1.7 Å) also creates conventional hydrogen bonds with the O2 atom. Pro302 is involved in a carbon-hydrogen bond (2.6 Å) with the O2 atom. Furthermore, Ala299, Glu118, Tyr94, Asp270, Leu130, and Met133 residues engage in van der Waals interactions with pyruvate

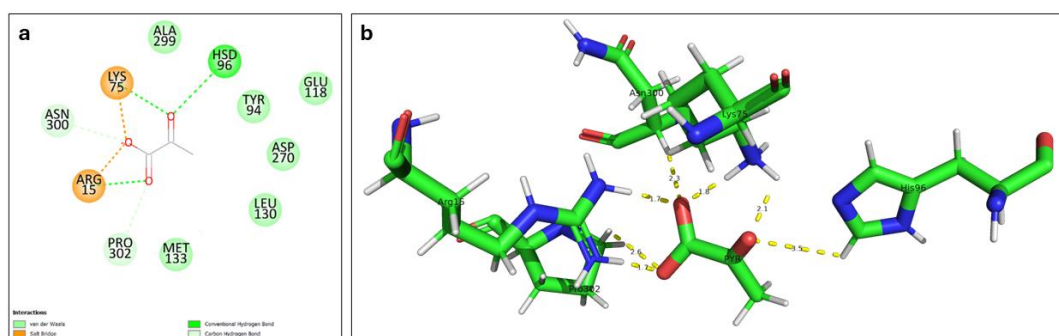


Figure 4.22: (a) 2D and (b) 3D active site representations of the wild-type enzyme in the context reductive amination activity (yellow dashes represent the bonds).

Next, the active site of Tyr14Asn mutant was investigated (**Figure 4.25**). It was detected that this mutant created the same type of bonds as those observed for the wild-type. However, some of the bonds lengths were found to differ. Conventional hydrogen bond length with His96 increased from 3.1 Å to 3.5 Å. Also the length of the pyruvate-Asn300 carbon hydrogen bond increased from 2.3 Å to 3.5 Å.

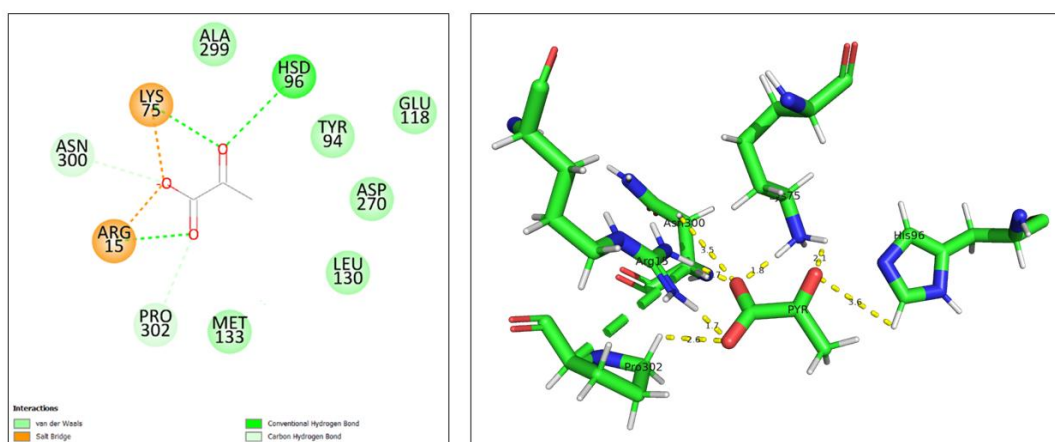


Figure 4.23: (a) 2D and (b) 3D active site representation of Tyr14Asn enzyme in the context of reductive amination activity (yellow dashes represent the bonds).

The Ile97Leu mutant (Figure 4.26) largely maintains bonding patterns similar to the wild-type, though with some key differences in bond type and length. The conventional hydrogen bond between His96 and pyruvate's O3 atom increases from 3.1 Å to 3.6 Å. Conversely, the conventional hydrogen bond between Lys75 and pyruvate's O3 atom shortens from 2.1 Å to 2.0 Å. The carbon-hydrogen bond

between Asn300 and pyruvate's O1 atom lengthens from 2.3 Å to 2.4 Å. Notably, the salt bridge between Lys75 and pyruvate's O1 atom converts to a conventional hydrogen bond (1.8 Å), and Lys75 also forms an attractive charge bond (3.7 Å) with pyruvate's O2 atom. All other bonds and their lengths remain consistent with the wild-type enzyme.

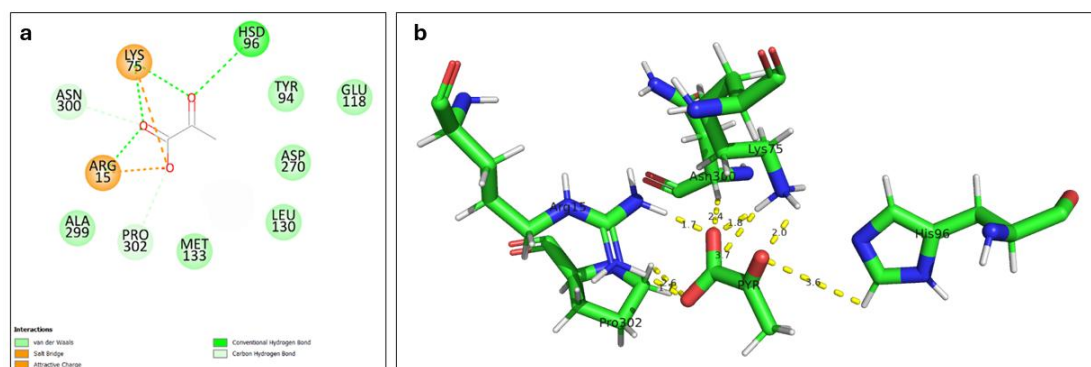


Figure 4.24: (a) 2D and (b) 3D active site representation of Ile97Leu enzyme in the context of reductive amination (yellow dashes represent the bonds).

The Ser192Ala mutant's active site (**Figure 4.27**) generally forms bonds similar to the wild-type, but with some notable differences in length and type. The conventional hydrogen bond between His96 and pyruvate's O3 atom lengthens from 3.1 Å to 3.5 Å. The carbon-hydrogen bond formed by Asn300 with pyruvate's O1 atom also increases in length from 2.3 Å to 2.4 Å. The salt bridge that Lys75 formed with pyruvate's O1 atom in the wild-type is now a conventional hydrogen bond (1.8 Å). Additionally, Lys75 creates an attractive charge bond (3.7 Å) with pyruvate's O2 atom. All other bonds and their lengths remain the same as the wild-type enzyme.

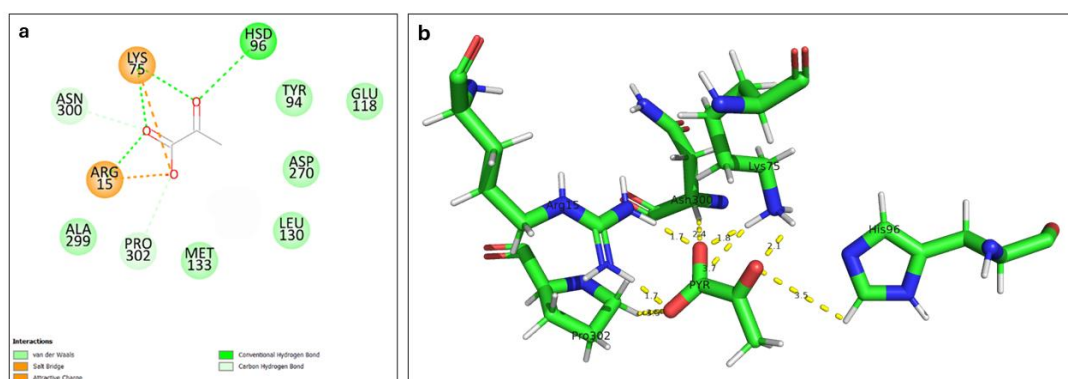


Figure 4.25: (a) 2D and (b) 3D active site representation of Ser192Ala enzyme during reductive amination activity (yellow dashes represent the bonds).

In the context of oxidative deamination, in all mutants, Tyr94, Lys75, Asn300, Arg15, Pro302, Ala299, Met133, Asp270 and Leu130 formed bonds with the alanine molecule. Bonds detected within the active site of the wild-type active site (**Figure 4.28**) are as follows; His94 (4.9 Å) and Leu130 (3.8 Å) formed pi-alkyl and alkyl bonds with C1 atom, NH1 and NH2 of alanine formed conventional hydrogen bonds with Asp270 (2.5 Å) and Ala299 (2.5 Å) residues, O1 of alanine is bound to Arg15 (3.3 Å) with a conventional hydrogen bond, Asn300 (4.0 Å) and Pro302 (3.7 Å) formed carbon hydrogen bonds with O1 atom and OH of alanine molecule formed unfavourable donor-donor interaction with Arg15 aminoacid with 1.8 Å bond length. Met133 and Lys75 create Van der Waals interaction with alanine molecule.

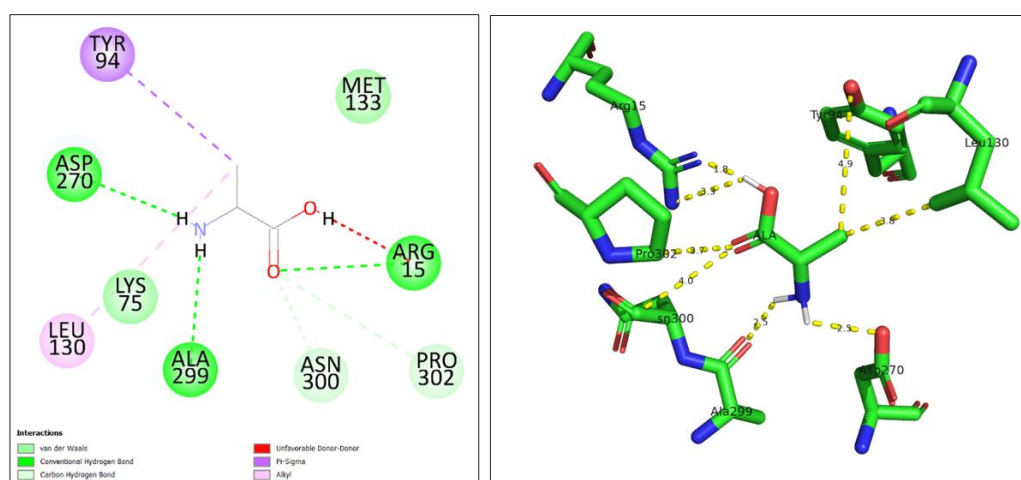


Figure 4.26: (a) 2D and (b) 3D active site representation of wild-type enzyme during oxidative deamination activity (yellow dashes represents the bonds).

Tyr14Asn and alanine interactions (**Figure 4.29**) are similar with wild-type, however some bond lengths were found to change, in addition to the presence of an additional bond. Lys75 formed a conventional hydrogen bond with O1 of alanine molecule with 3.4 Å length. All other bonds remained same with wild-type, however lengths of conventional hydrogen bonds with Asp270 and Arg15, and the unfavourable donor-donor interaction with Arg15 were found to increase from 2.5 Å to 2.6 Å, 1.8 Å to 1.9 Å and 2.9 Å to 3.0 Å, respectively. Pro302-alanine bond length decreased from 3.7 Å to 3.6 Å.

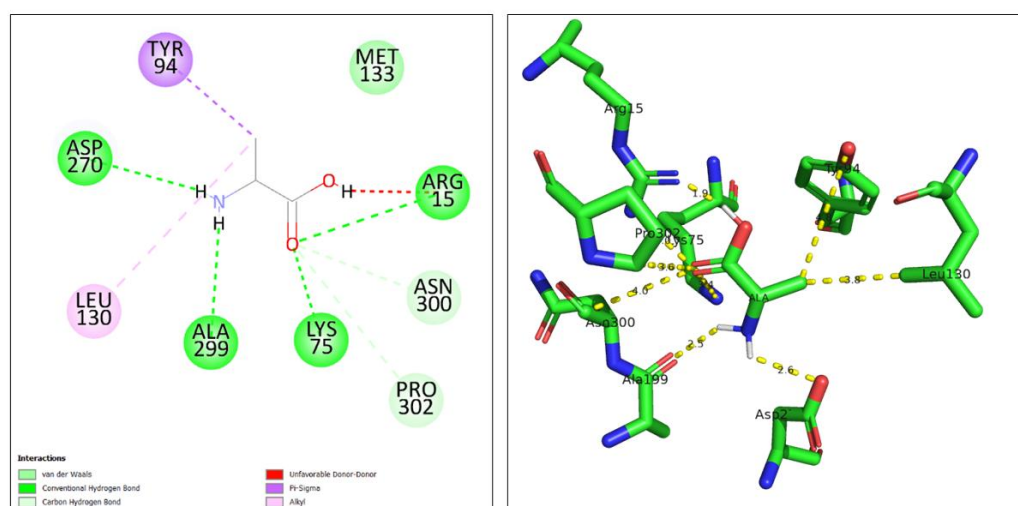


Figure 4.27: (a) 2D and (b) 3D active site representation of Tyr14Asn enzyme in the context of oxidative deamination (yellow dashes represents the bonds).

Ile97Leu mutant (**Figure 4.30**) formed the same bonds as those found in the wild-type, however some bond lengths were found to differ. Lengths of conventional hydrogen bonds of Asp270 and Arg15 were found to increased from 2.5 Å to 2.7 Å and 1.8 Å to 1.9 Å, respectively. However, pi-alkyl of His94, conventional hydrogen bond of Ala299 and carbon hydrogen bond of Pro302 were found to decrease from 4.9 Å to 4.8 Å, 2.5 Å to 2.4 Å and 3.7 Å to 3.6 Å, respectively.

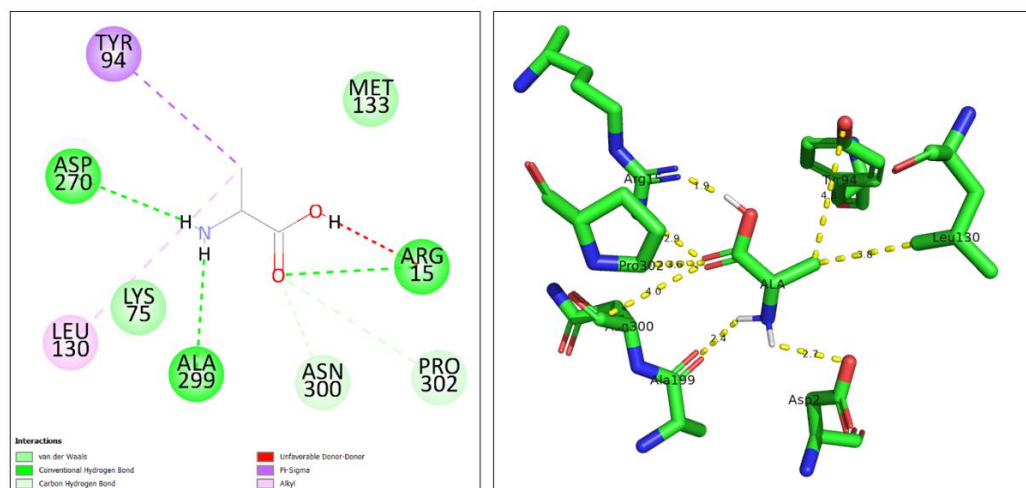


Figure 4.28: (a) 2D and (b) 3D active site representation of Ile97Leu enzyme in the context of oxidative deamination (yellow dashes represents the bonds).

Ser192Ala mutant (**Figure 4.31**) formed the same bonds as those found in the wild-type, however a length difference was also detected in one bond: Conventional hydrogen bond of Arg15 increased 1.8 Å to 1.9 Å in length.

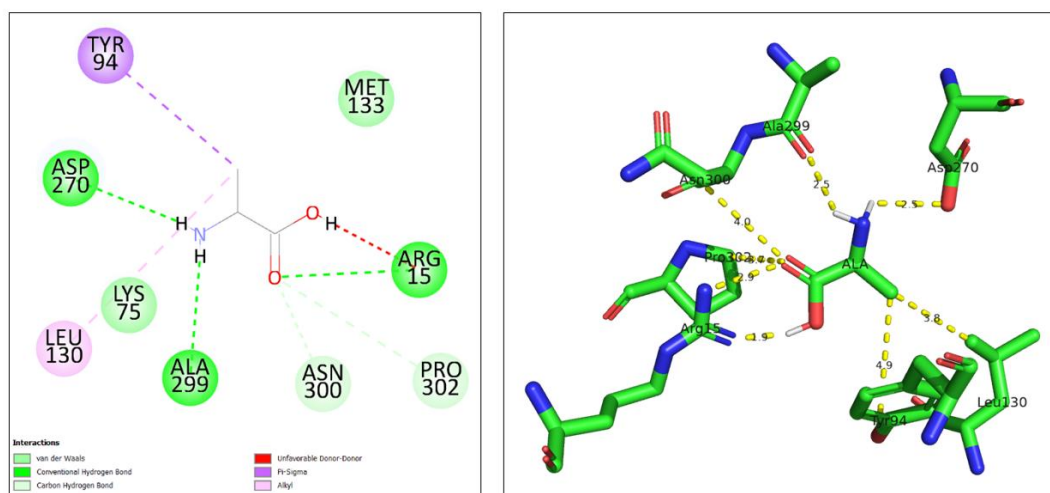


Figure 4.29: (a) 2D and (b) 3D active site representation of Ser192Ala enzyme during oxidative deamination activity (yellow dashes represents the bonds).

4.9. Analysis of Molecular Dynamics Simulations

4.9.1. RMSD, RMSF and MM-PBSA Analysis

Molecular Dynamics (MD) simulations were first performed for protein systems including the AlaDH structure and NAD(H) cofactor for each mutant and the wild-type system. Simulations were conducted in two sets, with (*L-AsAlaDH-NAD(H)-Pyr*) and without (*L-AsAlaDH-NAD(H)*) pyruvate in the active site. Each simulation ran for 100 ns, and each system was simulated three times. The conformational stabilities of the complexes were determined through these simulations at an atomistic level.

First, RMSD (Å)-time profiles were computed for *L-AsAlaDH-NAD(H)* simulations (**Figure 4.32**). In **Figure 4.32**, bold lines show the average of the RMSD values from all replica simulations, whereas lighter background areas surrounding the bolder-darker lines indicate standard deviation ranges. Accordingly, the RMSD levels of wild-type fluctuated around 1.5-3 Å. In contrast, mutant Tyr14Asn fluctuated around 1.5Å with a much smaller standard deviation than wild-type, which hints at higher conformational stability compared to that of the wild-type. Ile97Leu fluctuated around 2 Å, whereas Ser192Ala mutant fluctuated around 1.5-3.5 Å, with a slightly larger standard deviation. Ile97Leu, similar to Tyr14Asn, showed a lower level of variation in fluctuations compared to the wild-type.

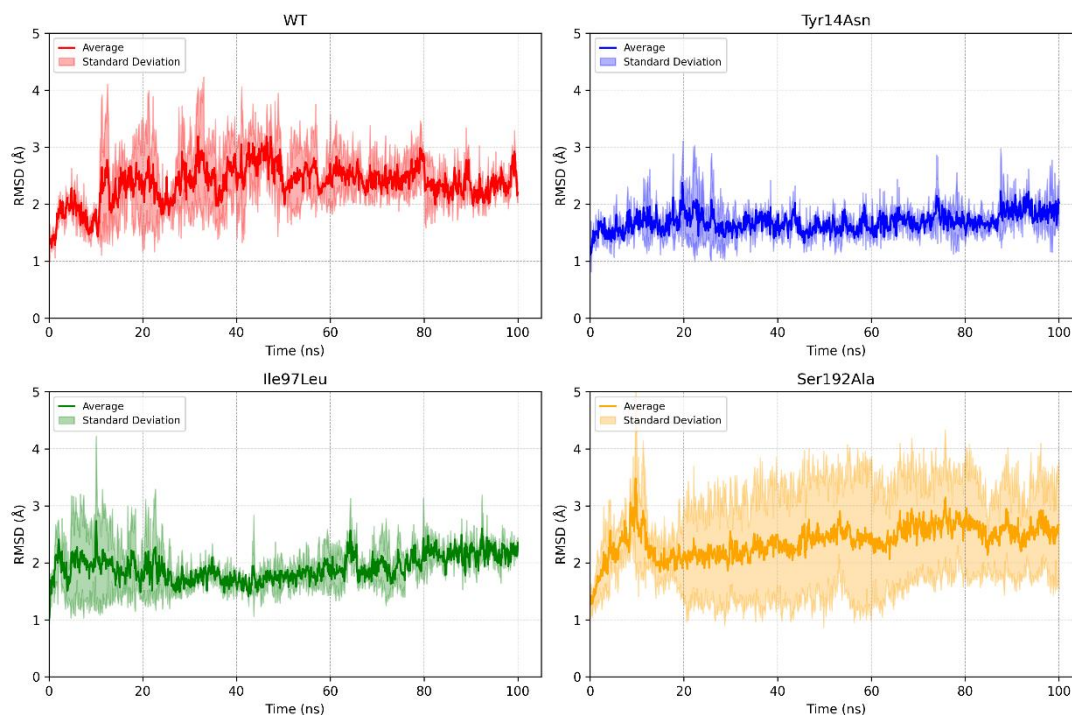


Figure 4.30: RMSD (Å)-time profiles of wild-type and mutant L-AsAlaDH-NAD(H) complex.

Per-residue Root Mean Square Fluctuation (RMSF) levels in this set of simulations revealed that regardless of the system, all residues fluctuated around similar levels. However, when RMSF levels of the mutants are compared with those of the wild-type (**Figure 4.33**), local differences are noted for only one mutant. Specifically, a local dampening of residue fluctuations around the mutated position is observed for the Ile97Leu mutant, confirming the presence of a local stabilization effect.

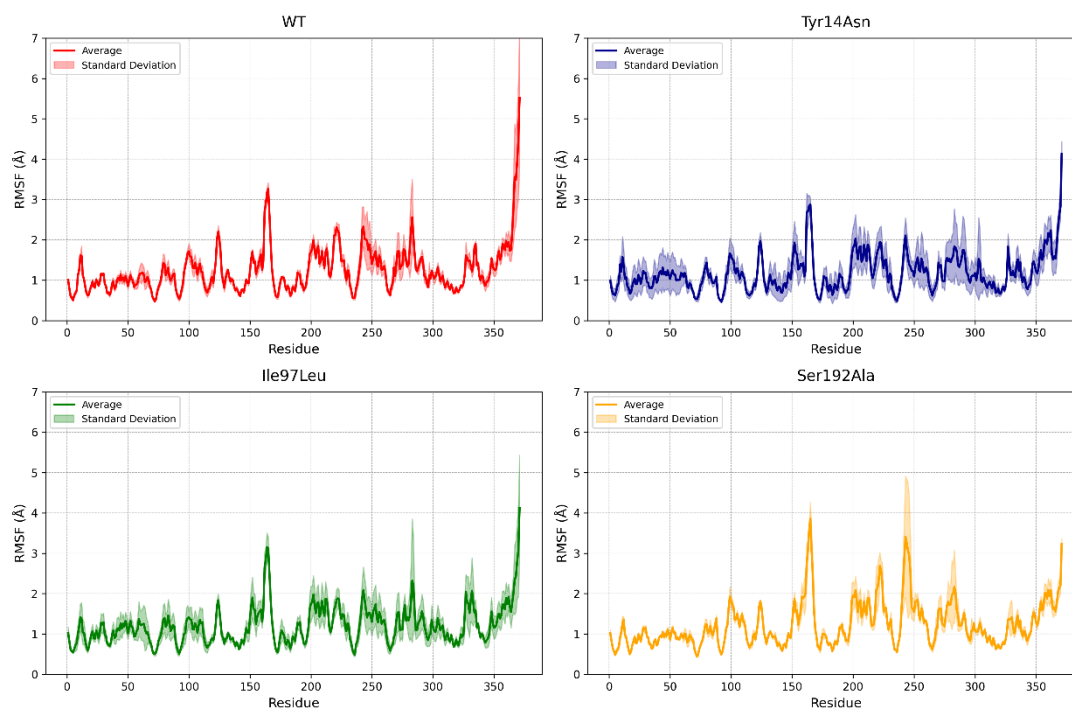


Figure 4.31: RMSF (Å)-Residue profiles of wild-type and mutant *L-AsAlaDH*-NAD(H) simulations.

Free energies of binding (FEB) (kcal/mol)-time profiles were also determined using the MM-PBSA method (**Figure 4.34**). Here, wild-type showed slightly decreasing free energy of binding from -80 kcal/mol to around -60 kcal/mol. Tyr14Asn showed a similar slightly decreasing FEB profile down to around -60 or -45 kcal/mol up until 60 ns, from which time point onwards a more stable binding free energy of around -50 kcal/mol was observed. FEB of Ile97Leu mutant yielded a fluctuation of around -65 kcal/mol. FEB fluctuates around -65 kcal/mol for Ser192Ala mutant, however after 20ns FEB drastically decreased to -55 kcal/mol. Accordingly, MM-PBSA results revealed that Ile97leu mutant yielded the most stable *L-AsAlaDH*-NAD(H) interactions.

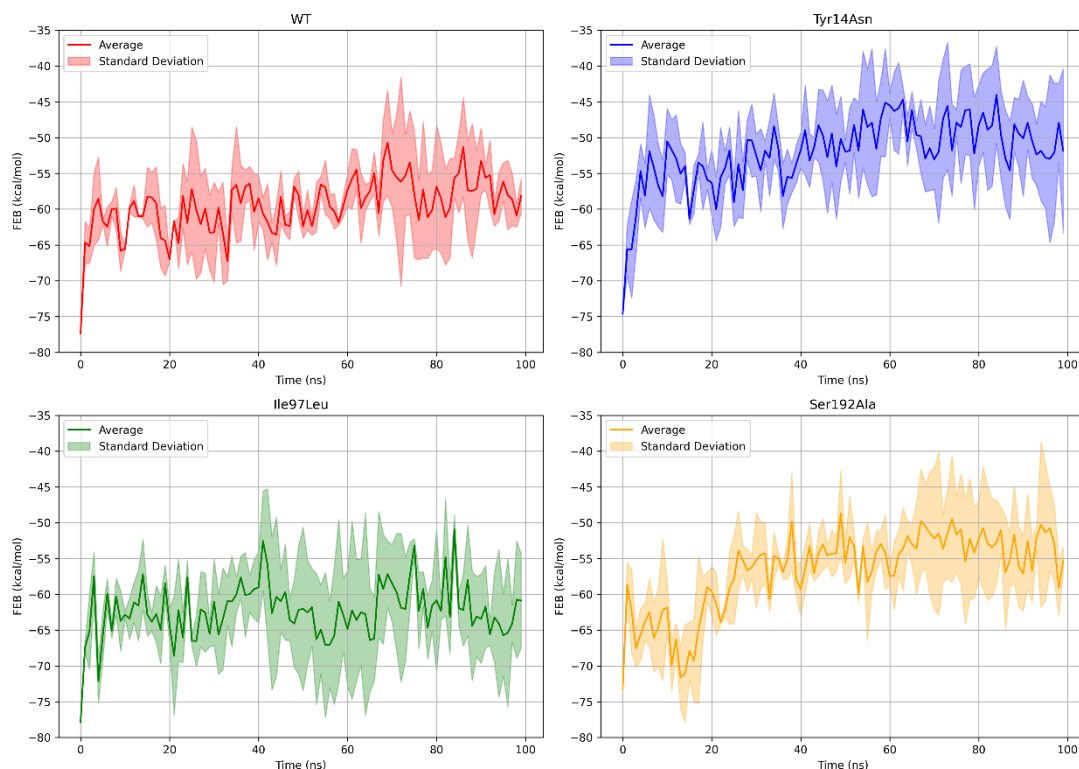


Figure 4.32: FEB (kcal/mol)-time profile of L-*AsAlaDH*-NAD(H) simulations.

MD simulations were also performed for system including NAD(H) cofactor and pyruvate and the enzyme (L-*AsALADH*-NAD(H)-Pyr). In RMSD(Å)-time profiles (**Figure 4.35**) it was observed that that wild-type fluctuated around 2.0-3.5 Å throughout the simulations. Mutant Tyr14Asn enzyme fluctuates around 2.0 Å with a much smaller standard deviation than that obtained from the wild-type simulations. Ile97Leu fluctuated around 3Å and Ser192Ala mutant fluctuated around 1.5Å. In general, all simulations were found to have slightly higher standard deviation ranges than those obtained from L-*AsAlaDH*-NAD(H) systems, indicating a lower level of conformational stability. This finding was attributed to movements of pyruvate within the active site of protein, which likely caused a perturbation near the center of the protein, leading to higher levels of conformational variation in these systems.

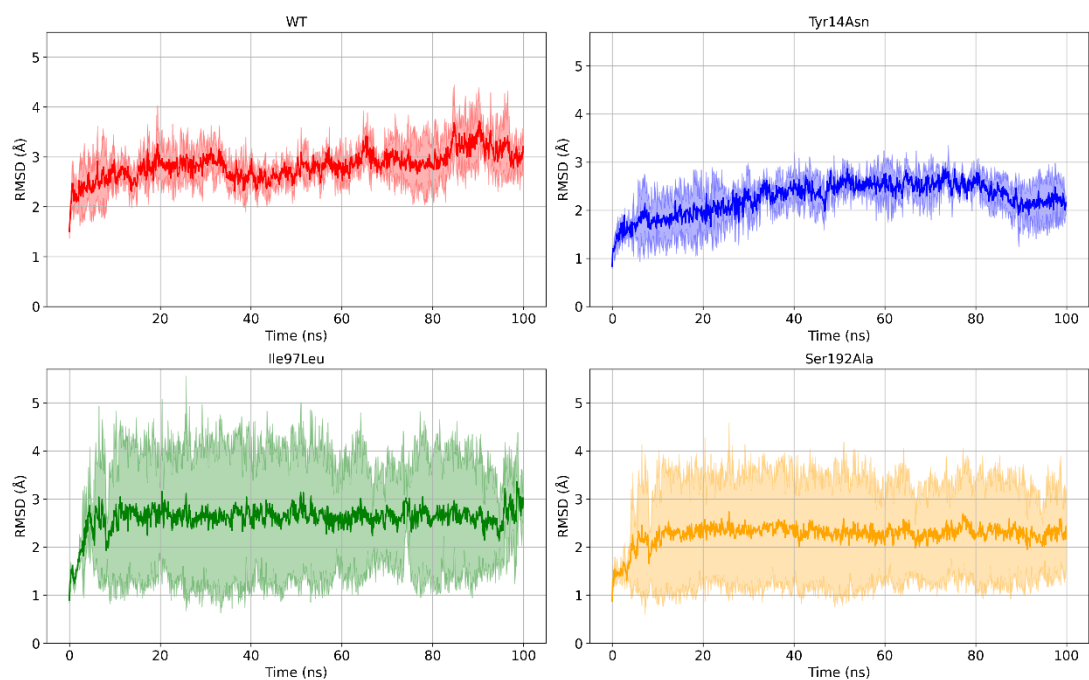


Figure 4.33: RMSD (Å)-Time profiles of L-*As*AlaDH-NAD(H)-Pyr simulations.

RMSF profiles of L-*As*ALADH-NAD(H)-Pyr systems are shown in **Figure 4.36**. Residues in all proteins deviated around a similar range which is approximately 1 Å. Significant differences in terms of per-residue fluctuations were not immediately clear.

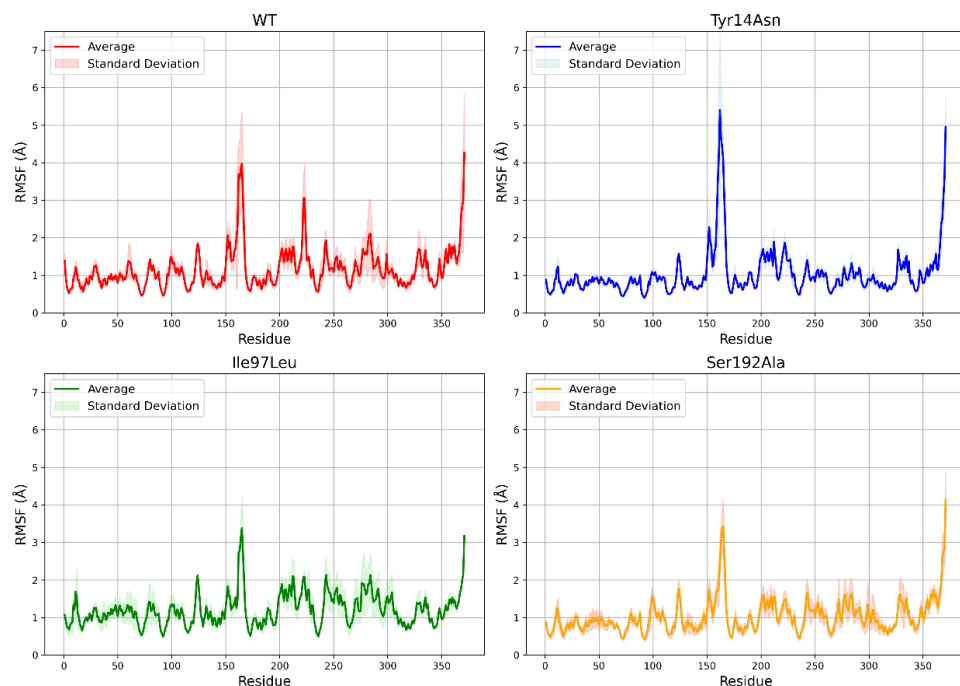


Figure 4.34: RMSF (Å)-Time profiles of L-*As*AlaDH-NAD(H)-Pyr simulations.

FEB (kcal/mol)-time profiles of L-*As*AlaDH-NAD(H)-Pyr systems were also computed (**Figure 4.37**). In contrast to systems without pyruvate, all systems showed higher standard deviation ranges. This was again attributed to pyruvate movements within the active site simulations. All systems showed a decreasing FEB trend in a certain time period, between 35-60 ns for wild-type and Tyr14Asn, 15-45 ns for Ile97Leu, 15- 60ns for Ser192Ala. However, after these initial stages, FEBs of all simulations stabilized, albeit at higher FEB levels compared to those obtained from systems without pyruvate. This may highlight that the presence of pyruvate also reinforced interactions of L-*As*AlaDH with the NAD(H).

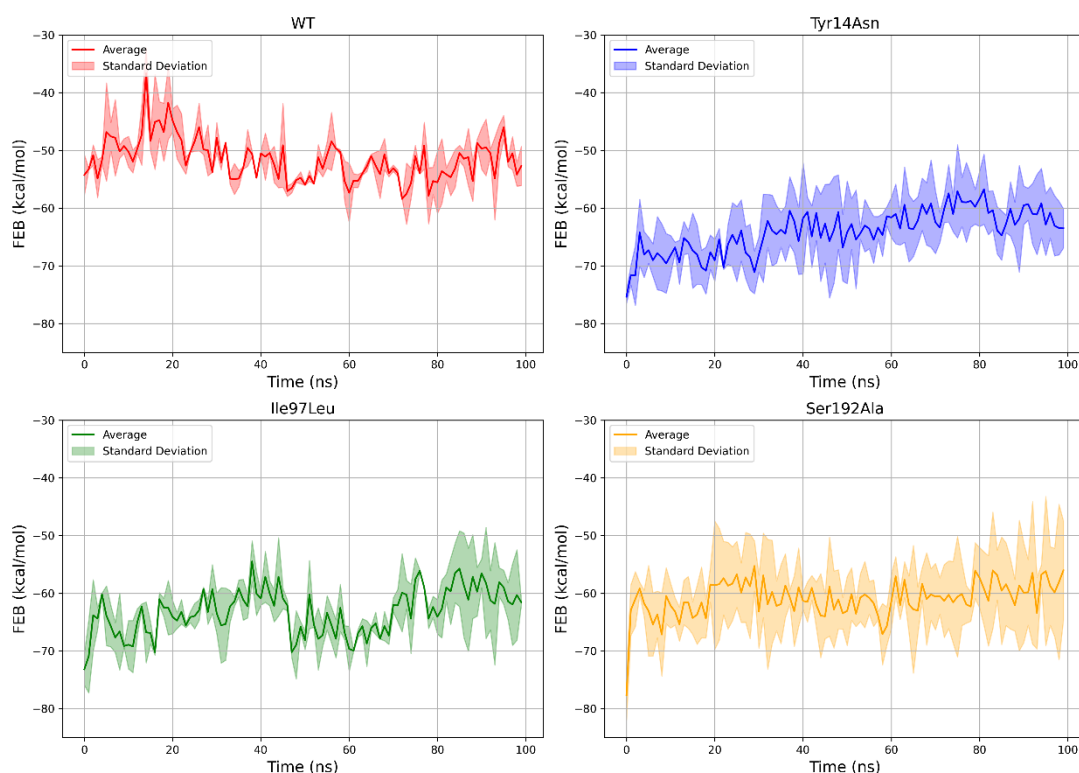


Figure 4.35: FEB (kcal/mol)-time profile NAD(H)molecule on L-*As*AlaDH-NAD(H)-Pyr simulations.

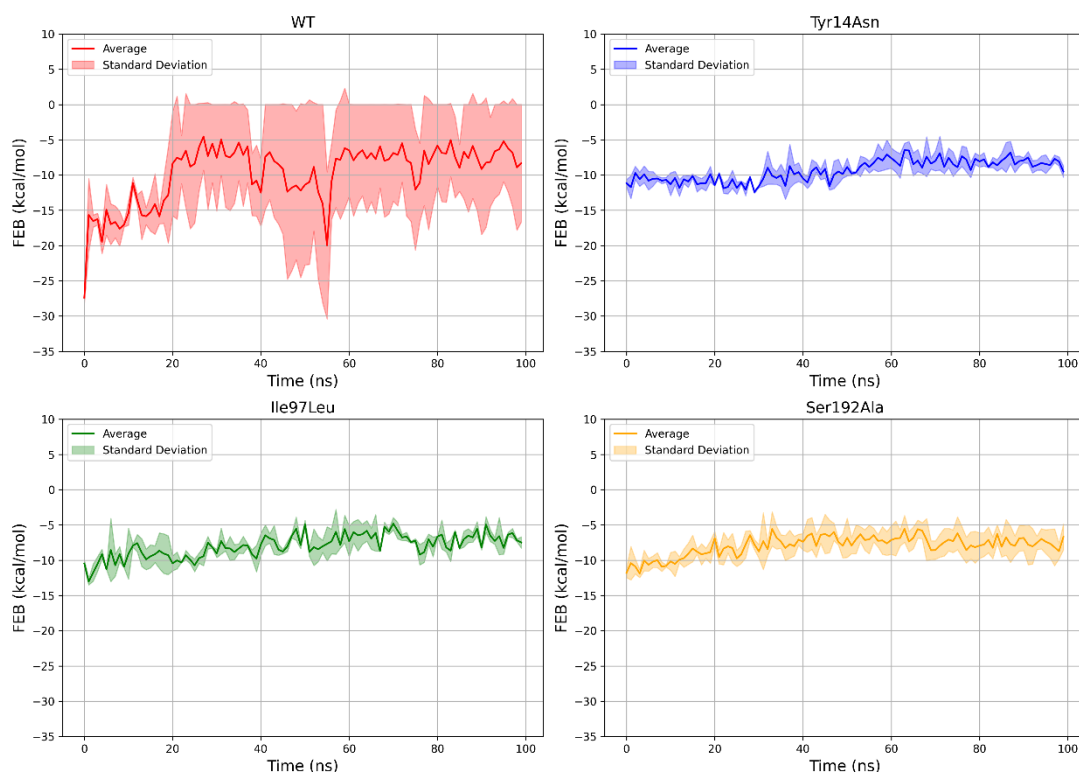


Figure 4.36: FEB (kcal/mol)-time profile pyruvate molecule on w L-*AsAlaDH*-NAD(H)-Pyr simulations.

4.9.2. Protein Energy Network (PEN) Analysis

In order to conduct a more comprehensive and in-depth analysis of the effects of mutations in the L-*AsAlaDH* structure, protein energy networks (PENs) were also constructed by using pairwise amino acid interaction energy data obtained using gRINN. PEN is a variant of residue/amino-acid interaction networks that are commonly used in structural bioinformatics to identify roles of residues in the overall conformational dynamics/stability of a protein structure. The primary feature of PENs is the use of pairwise non-bonded interaction energies as edge weights in the network. In contrast to simple distance or cutoff based protein structure network, this type of analysis enables a better representation of interaction “intensity” between residue pairs, leading to a better appreciation of physicochemical characteristics of amino acid side chains, and ultimately to better predictions of changes in protein conformational stability. A PEN analysis was deemed particularly suitable in the context of this study, as two of the studied mutants were identified through the usage of FoldX, with a the specific purpose of identifying stabilizing mutations in L-*AsAlaDH* structure. The ultimate aim of this analysis was thus to explain the mechanisms underlying the effects

of mutations on the protein conformational stability. The procedure for constructing a single PEN is explained in Methods section 3.2.11.3.

Here, the overall PEN-based analysis was conducted in a total of five stages for each set of simulations (with and without pyruvate):

- (i) calculation of non-bonded van der Waals interaction energies in each frame in each simulation
- (ii) identification of statistically-consistent pairs of interacting residues in each system
- (iii) identification of consistent pairs of interactions in mutants whose pairwise non-bonded interactions have been significantly (statistically) affected compared to the wild-type,
- (iv) construction of PENs based on interaction energies computed in step (i), for each frame in each simulation, and finally
- (v) identification of individual residues whose betweenness-centralities (BC) in PENs were significantly (statistically) affected compared to the respective values in the wild-type simulations.

Step (i) was completed by following the procedure outlined in Section 3.2.11.3. Then, in step (ii), distributions of interaction energies for each pair of interacting residues were compared between *replica simulations of the same system* by performing a one-way ANOVA. Then, only those pairs for which a p-value of *higher than* 0.05 was obtained were included in the list of *consistent pairs*. A p-value higher than 0.05 here was taken to indicate the *lack of* significantly different interaction energies between each residue pair, and hence the presence of a *consistent* interaction between the respective pair of residues. For these residues, interaction energy arrays from each simulation were concatenated into one for subsequent analyses. Then, in step (iii), distributions of interaction energies for each pair of consistent pairs were compared between *each mutant and wild-type system* by performing a Student's t-test. Here, only those pairs for which a p-value of *lower than* 0.05 were identified as those residue pairs whose interactions have been significantly affected upon mutations. Next, in step (iv), individual PENs were constructed by taking non-bonded van der Waals interaction energy levels as edge weights in each frame in each simulation as described in Section 3.2.11.3. Thus, 100 PENs were obtained from 100 frames analyzed in each simulation. Finally, in the last step (v), first betweenness-centralities (BCs) of

individual residues (nodes) were computed, yielding arrays of BCs for each residue in each system. Then, distributions of residue BCs were again compared between *each mutant and wild-type* system by performing a Student's t-test. As a result, residues whose BC levels were affected upon mutation in a statistically-significant manner were identified.

This analysis was first performed for L-*As*AlaDH-NAD(H)-Pyr systems. To perform a more focused analysis, the residue pairs list obtained at the end of step (ii) of this analysis were further filtered to include only those pairs whose absolute interaction energies were higher than 2 kcal/mol. **Figure 4.39, Figure 4.40, and Figure 4.41** highlights these residues for the Tyr14Asn, Ile97Leu, and Ser192Ala mutants, respectively, in two groups: those whose interactions became more attractive and repulsive (compared to the wild-type), respectively. The final list included residue pairs whose non-bonded van der Waals became more attractive and repulsive, respectively (**Table 4.7, Table 4.8, Table 4.9**).

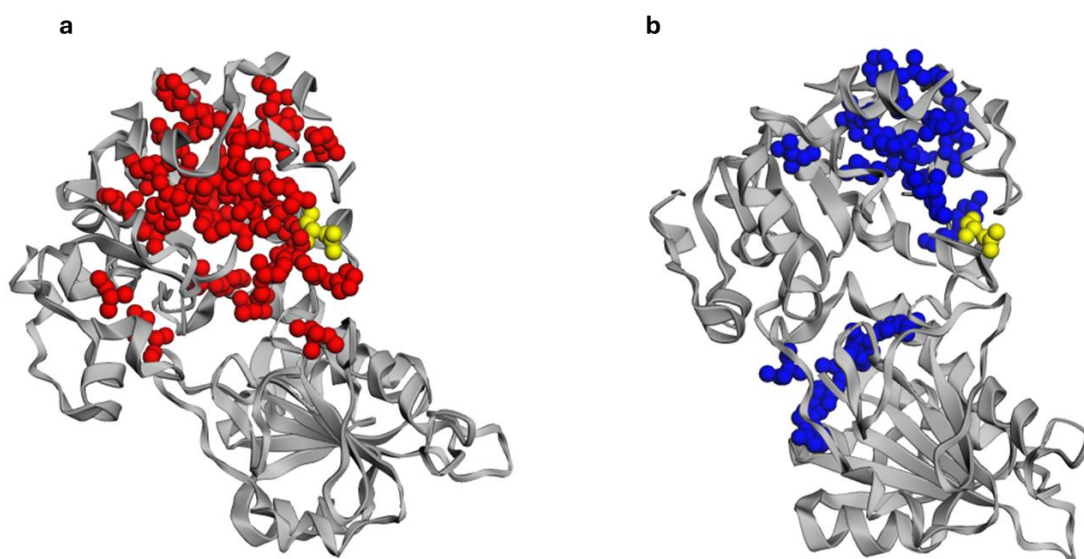


Figure 4.37: Residue pairs whose non-bonded van der Waals interactions became more **(a)** attractive or **(b)** repulsive upon introduction of the Tyr14Asn mutation into the wild-type L-*As*AlaDH enzyme in L-*As*AlaDH-NAD(H)-Pyr simulations.

Table 4.7: List of residue pairs whose non-bonded van der Waals interactions became more attractive or) repulsive upon introduction of the Tyr14Asn mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H)-Pyr simulations.

More attractive		More repulsive
Leu26-Leu70	Arg7-Lys73	Ala21-Thr311
Pro6-Trp65	Pro20-Ile45	Ile3-Val90
Leu18-Thr318	Leu18-Leu72	Val241-NAD(H)
Glu8-Glu13	Ala17-Val317	Val5-Leu18
Pro6-Val16	Leu18-Val317	Ile9-Glu36
Pro20-Tyr50	Pro6-Glu36	Val5-Tyr50
Ala4-Val90	His12-Thr282	Val5-Leu72
Pro6-Leu91	Ala17-Thr318	Pro6-Leu58
Ile3-Phe34	Arg15-Tyr94	Ile3-Leu18
Ala4-Leu18	Ile97-Ala106	Ala4-Phe34
Arg15-Pyruvate	Lys10-Lys73	
Pro6-Leu72	Arg7-Val35	

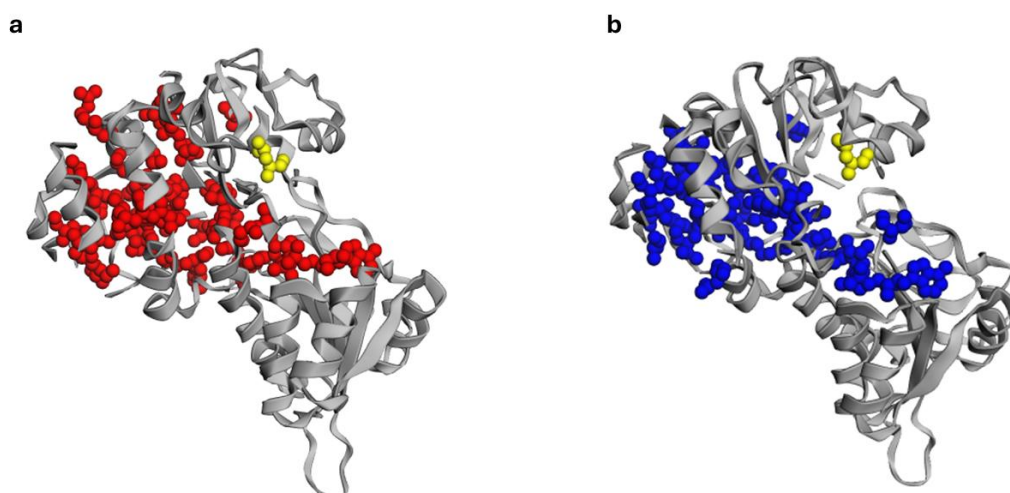


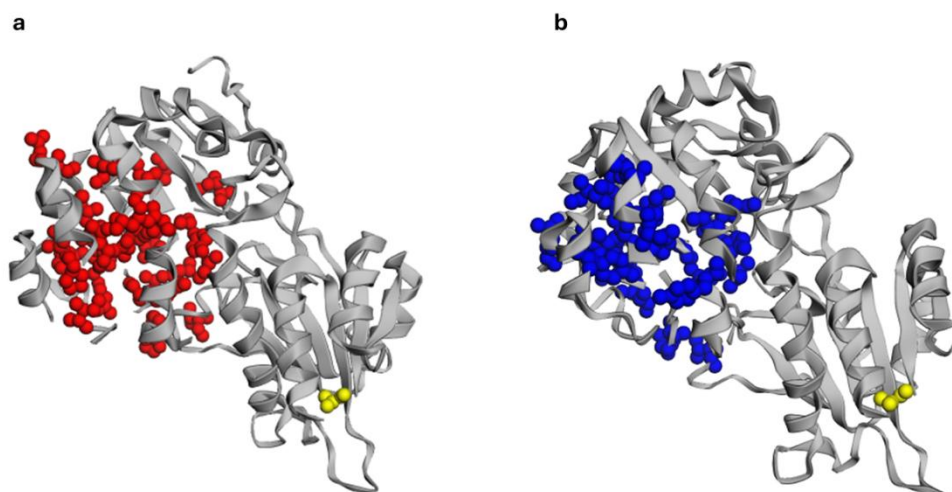
Figure 4.38: Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ile97Leu mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H)-Pyr simulations.

Table 4.8: List of residue pairs whose non-bonded van der Waals interactions became more attractive or) repulsive upon introduction of the Ile97Leu mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H)-Pyr simulations.

More Attractive		More Repulsive	
Arg2-Val33	Ala4-Val71	Ala21-Leu26	Ala4-Val33
Lys10-Glu76	Pro20-Ile45	Thr19-Thr315	Ile9-Asp80
Pro6-Val35	Ala17-Val317	Val5-Val23	Ala17-Val35
Ala4-Leu89	Pro6-Glu36	Val5-Val23	Val5-Leu18

Table 4.8: Continue.

Glu8-Val16	His12-Thr282	Ala17-Val74	Ile9-Glu36
Pro20-Tyr50	Ala17-Thr318	Ala4-Lys73	Pro6-Leu58
Ala4-Val90	Ala4-Leu18	Arg15-Asn300	Ile3-Leu18
Pro6-Leu91	Arg15-NAD(H)	Val16-Tyr50	Val5-Lys73
Ala17-Tyr94	Leu97-106A	Arg7-Thr37	Ala17-Tyr117
Ala4-Leu91	Lys10-Ala73	Tyr94-NAD(H)	Ile9-Lys75
Leu127-Pyruvate	Arg7-Val35		

**Figure 4.39:** Residue pairs whose non-bonded van der Waals interactions became more (a) attractive or (b) repulsive upon introduction of the Ser192Ala mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H)-Pyr simulations.**Table 4.9:** List of residue pairs whose non-bonded van der Waals interactions became more attractive or) repulsive upon introduction of the Ser192Ala mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H)-Pyr simulations.

More Attractive		More Repulsive	
Pro6-Val35	His12-Thr282	Ala21-Thr311	Arg11-Thr282
Arg2-Leu70	Ala17-Thr318	Ala21-Leu26	Ala17-Val35
Lys10-Ile78	Ala4-Val35	Ala17-Leu72	Val5-Tyr50
Val5-Leu70	Ala4-Leu18	Tyr94- Pyruvate	Pro6-Leu58
Pro6-Leu91	Arg15-Pyruvate	Ala4-Val33	Ile3-Leu18
Ala17-Tyr94	Arg15-Tyr94	Leu18-Val35	Ala17-Tyr117
Arg7-Lys73	Ile97-Ala106	Ile9-Asp80	Ile9-Lys75
Pro20-Ile45	Lys10-Lys73	Arg11-Thr282	
Ala17-Val317	Pro6-Glu36		

The same analysis was also conducted for *L-AsAlaDH*-NAD(H) simulations, with results shown in **Figures 4.42, 4.43, 4.44** and **Tables 4.10, 4.11, 4.12**.

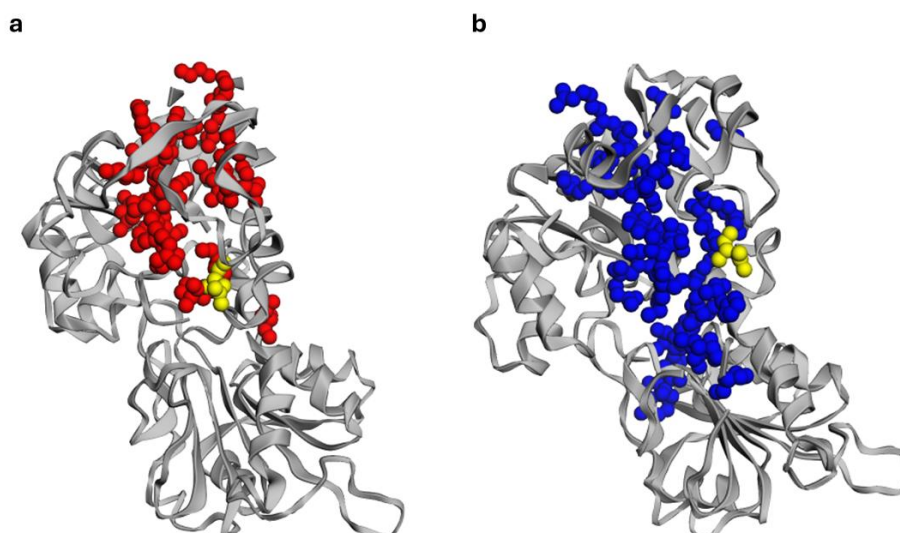


Figure 4.40: Residue pairs whose non-bonded van der Waals interactions became more **(a)** attractive or **(b)** repulsive upon introduction of the Tyr14Asn mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

Table 4.10: List of residue pairs whose non-bonded van der Waals interactions became more attractive or) repulsive upon introduction of the Tyr14Asn mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

More Attractive		More Repulsive	
Ile3-Val27	Ala4-Val33	Ala21-Leu26	Glu8-Asn300
Arg15-Val136	Arg7-Tyr14	Val5-Val71	Pro6-Glu36
Glu8-Glu13	Ala4-Leu70	Arg15-Ser310	Lys10-Val298
Ile3-Gly30	Val5-Leu72	Ile3-Val90	His12-Thr282
Ala4-Lys73	Ile3-Phe34	Pro6-Val35	Glu8-Lys75
Pro6-Leu91	Val5-Thr64	Val5-Val16	Ala4-Val35
Arg7-Lys73	Ala4-Leu18	Arg2-Leu70	Lys10-Val74
Leu18-Leu26	Val5-Tyr50	Lys10-Lys73	Val239-Pyruvate
Val5-Thr19		Val5-Leu70	Ile9-Glu76
		Glu8-Val16	His12-Pro286
		Glu8-Asn300	Glu8-Val74
		Arg15-Lys73	

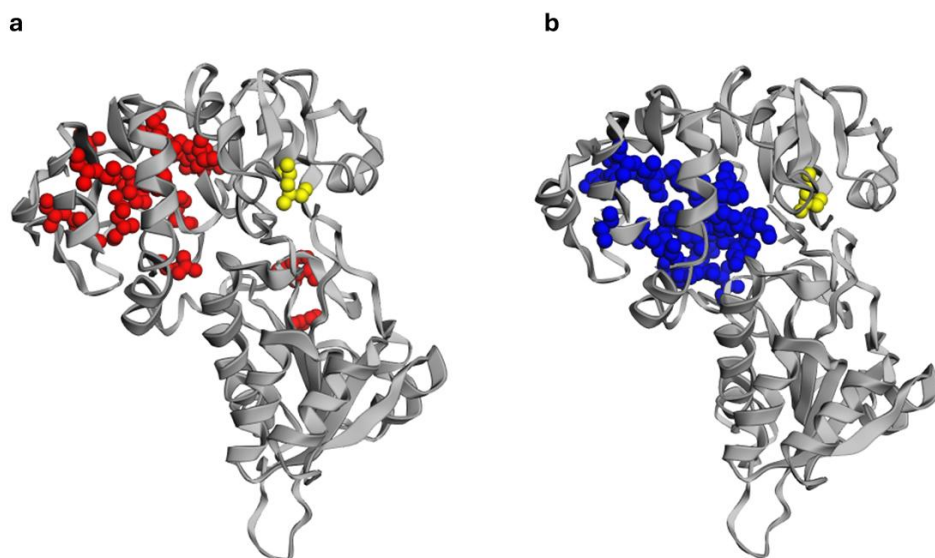


Figure 4.41: Residue pairs whose non-bonded van der Waals interactions became more **(a)** attractive or **(b)** repulsive upon introduction of the Ile97Leu mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

Table 4.11: List of residue pairs whose non-bonded van der Waals interactions became more attractive or) repulsive upon introduction of the Ile97Leu mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

More Attractive		More Repulsive	
Leu26-Leu70	Ile9-Asp80	Ala21-Leu26	Lys10-Arg15
Arg279-Tyr288	Val5-Leu72	Ala17-Leu314	Ala17-Phe92
Pro6-Trp65	Val5-Leu18	Pro6-Val16	Arg11-Thr282
Ile9-Glu13	Pro6-Leu58	Lys10-Lys73	Ala17-Val35
Pro6-Leu91	Val5-Tyr50	Ala17-Leu72	His12-Thr282
Leu18-Leu26	Val5-Trp65	Val5-Glu36	Pro6-Thr37
		Ala17-Val74	Glu8-Lys75
		Ala17-Tyr94	Ala17-Tyr117

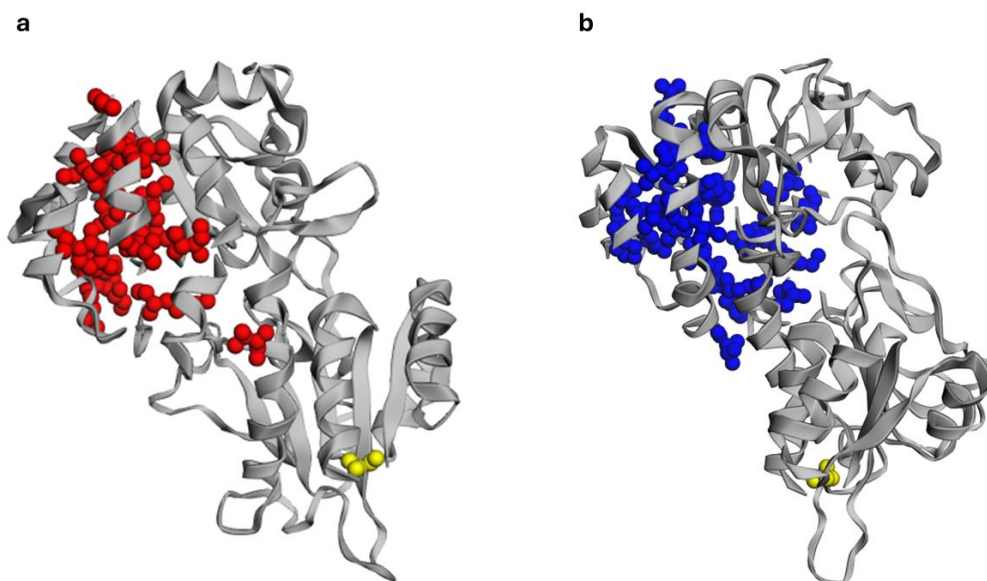


Figure 4.42: Residue pairs whose non-bonded van der Waals interactions became more **(a)** attractive or **(b)** repulsive upon introduction of the Ser192Ala mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

Table 4.12: List of residue pairs whose non-bonded van der Waals interactions became more attractive or repulsive upon introduction of the Ser192Ala mutation into the wild-type *L-AsAlaDH* enzyme in *L-AsAlaDH*-NAD(H) simulations.

More Attractive		More Repulsive	
Leu26-Leu70	Ala4-Val33	Leu18-Thr318	Glu8-Asn300
Arg15-Val136	Ile9-Asp80	Pro6-Val16	Arg15-Ala305
Thr19-Leu314	Ala4-Leu70	Arg2-Leu70	Pro20-Val35
Ile3-Gly30	Val5-Leu72	Lys10-Ile78	Leu18-Leu72
Ala4-Lys73	Val5-Tyr50	Val5-Leu70	Val23-Ala55
Arg7-Lys73	Val5-Thr19	Glu8-Val16	Leu18-Val317
Leu18-Leu26		Pro20-Tyr50	Ile9-Glu81
		Glu8-Tyr14	Ile3-Ala322

To understand the effects of mutations on single residue level rather than inter-residue interactions, we also constructed PENs and calculated betweenness centralities for each residue (step iv), which represent the average number of shortest paths passing through that residue. Residues with optimized, predominantly attractive interactions with their surroundings exhibit higher betweenness centralities. Here, only *L-AsAlaDH*-NAD(H) were considered in the analysis. *L-AsAlaDH*-NAD(H)-Pyr

simulations were considered unsuitable due to the high mobility of pyruvate within the substrate binding site, leading to inconsistent BC levels across replica simulations.

After calculating betweenness centralities for each protein conformation, we first identified residues with consistent betweenness centralities levels across all simulation replicas (with a p-value > 0.05, step v). Next, we determined a mean betweenness centralities score for each residue by averaging the median BC values from all conformations within each NAD(H) including system. Finally, we used a one-way ANOVA ($p < 0.05$) to pinpoint residues showing statistically significant differences in BC between systems. From these, we selected the top 21 residues from within this list that exhibited the most substantial increases or decreases in their median betweenness centrality levels upon NAD(H) binding in at least one mutant system.

This analysis clearly indicated that Leu130 yielded higher BC levels in Ile97Leu, and Ser192Ala, whereas the corresponding BC was lower in Tyr14Leu compared to the wild-type. Apart from this effect, mutant-dependent effects were observed for the rest of the residues in this list, which predominantly showed increased betweenness centrality and are clustered around the substrate and cofactor binding sites (**Figure 4.46**)

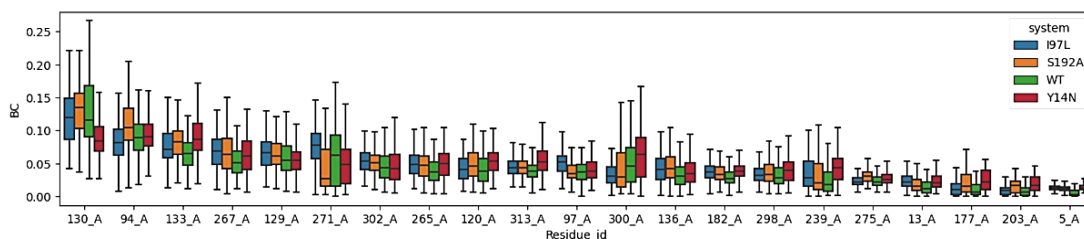


Figure 4.43: Betweenness centrality levels that changed most significantly upon mutations.

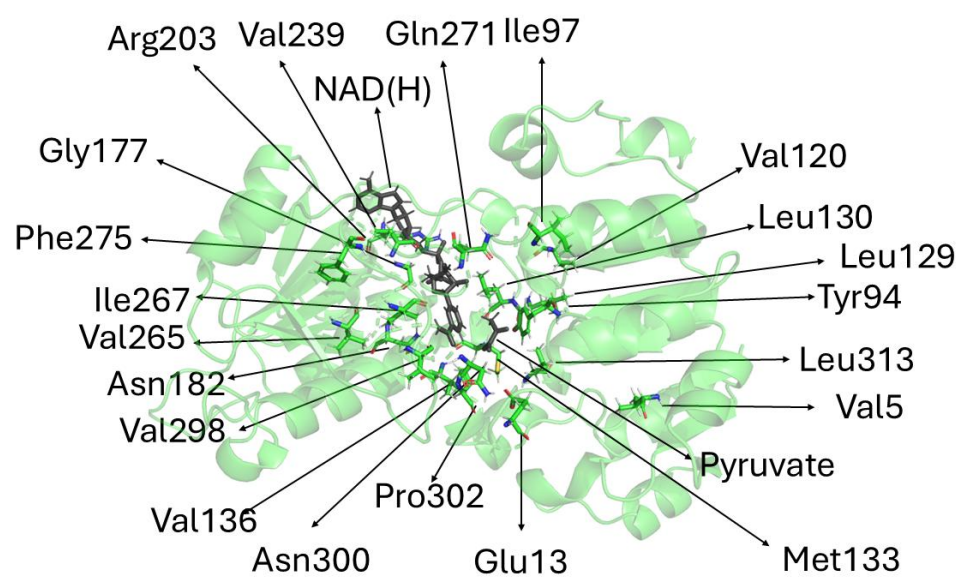


Figure 4.44: Residues with changes in their highest betweenness centrality values.

5. DISCUSSION

In this study, our primary objective was to enhance the catalytic efficiency of L-*AsAlaDH* enzyme for reductive amination through enzyme engineering. We employed a rational enzyme design approach to propose novel mutants. Following the construction of a physically valid homology model of L-*AsAlaDH*, virtual mutations were introduced into the model via computational saturation mutagenesis. Ultimately, Tyr14Asn, Ile97Leu, and Ser192Ala mutants were selected as candidates for *in vitro* validation based on their favorable B-factor, solvent accessible surface area (SASA), pKa, and solubility profiles. Specifically, the B-factor of Ile97Leu was higher than the average B-factor of all residues, while Tyr14Asn and Ser192Ala showed the opposite trend. However, none of the mutants exhibited significantly higher or lower B-factor levels compared to the average. Mutating residues with the highest or lowest B-factors was initially considered risky due to the potential disruption of the protein's overall fold and dynamics. Interestingly, some active site residues in the mutants exhibited increased pKa values, which is advantageous for interacting with negatively charged substrates. We observed an increase in active site SASA values while the SASA of the mutated residues decreased. A decrease in SASA at the active site could potentially negatively impact substrate-protein interactions. Solubility comparisons indicated that Tyr14Asn and Ile97Leu were more soluble than the wild-type, while Ser192Ala was not. However, no practical solubility issues were encountered during experimental studies.

During the experimental phase, plasmid DNAs were visualized at approximately ~6.4 kbp (Dave & Kadeppagari, 2019), and expressed and purified proteins were found to be 41.09 kDa (Aktaş, 2021). **Table 5.1** presents the optimum working conditions and kinetic parameters for both amination and deamination reactions, consistent with reported literature. Specifically, the optimal working pH for L-AlaDH typically ranges from pH 9.0-11.0 for oxidative deamination and pH 7.0-9.5 for reductive amination. Optimal working temperatures for both reactions generally fall between 25-55°C. Our experimental findings align well with these established literature values. Re-determination of deamination kinetic values and working conditions for wild-type L-*AsAlaDH* confirmed previously established optimum conditions and similar kinetic

parameters. The literature reports a diverse range of kinetic parameters for both reductive and oxidative reactions, which vary depending on optimal conditions and the source organism. To date, the reductive amination activity of these enzymes has consistently been reported as higher than their oxidative deamination activity, with only one exception (Gu et al., 2023). Overall, the kinetic parameters of both the wild-type and mutant enzymes in this study are comparable to existing literature.

Table 5.1: Reported optimum working conditions and kinetic parameters of L-AlaDH enzymes.

Oxidative Deamination						
Organism	pH	Temp (°C)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (Mm ⁻¹ s ⁻¹)	Ref
<i>Mycobacterium tuberculosis</i>	10-11	37	-	13.8	-	(Hutter & Singh, 1999)
<i>Mycobacterium tuberculosis</i>	10.2	-	7.5±0.4	5.3±0.8	1.4±0.2	(Fernandes et al., 2015)
<i>Amycolatopsis sulphurea</i>	10.5	25	13.2±0.5	2.0±0.4	6.5±1.4	(Aktaş, 2021b)
<i>Bacillus pseudofirmus</i> OF4	10.5	40	10±0.4	4±0.4	3±0.2	(He et al., 2018)
<i>Phormidium lapideum</i>	9.2	-	-	5	-	(Sawa et al., 1994) 1994)
<i>Bacillusphaericus</i>	10.5	25	-	18.9	-	(Sakamoto et al., 2001)
<i>Thermomicrobium roseum</i>	9	55	4.7±0.1	3.2±0.3	1.4±0.2	(Dedeakayogulları et al., 2023)
<i>Bacillus subtilis</i> 168	10	37	103.9±1.7	0.07±0.03	1819.4±10.2	(Gu et al., 2023b)
<i>Bacillus cereus</i>	10	37	122.3±2.1	1.1±0.08	111.8±9.6	
<i>Mycobacterium smegmatis</i> MC2 155	10	37	136.7±1.9	0.47±0.03	292.09±2.06	
<i>Geobacillus stearothermophilus</i>	10	37	108.6± 2.1	0.2±0.03	480.6±7.2	
<i>Phormidium lapideum</i>	-	45	96	0.04	-	(Ashida et al., 2004)
<i>Shewanella sp.</i>	-	30	35	0.04	-	
Wild-type L-AsAlaDH	10.5	25	11.2±0.7	2.89±0.80	3.88±0.80	This Study
Tyr14Asn L-AsAlaDH	10.0	30	17.3±1.2	1.6±0.4	10.8±2.4	
Ile97Leu L-AsAlaDH	10.5	40	17.7±1.8	1.5±0.6	11.8±3.6	

Table 5.1: Continue.

Reductive Amination						
Organism	pH	Temp (°C)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (Mm ⁻¹ s ⁻¹)	Ref
<i>Mycobacterium tuberculosis</i>	7-7.5	37	-	1.4	-	(Hutter & Singh, 1999)
<i>Phormidium lapideum</i>	8.4	-	-	0.3	-	(Sawa et al., 1994)
<i>Bacillusphaericus</i>	9	25	-	1.7	-	(Sakamoto et al., 2001))
<i>Thermomicrobium roseum</i>	9.5	55	35.0±0.50	1.9±0.20	18.4±0.1	(Dedeakayogulları et al., 2023)
<i>Bacillus subtilis 168</i>	8	37	36.3±0.1	2.2±0.4	16.6±3.4	(Gu et al., 2023b)
<i>Bacillus cereus</i>	8	37	40.4±2.9	1.1±0.1	37.9±6.7	
<i>Mycobacterium smegmatis MC2 155</i>	8	37	28.2±4.3	1.6±0.2	17±4.0	
<i>Geobacillus stearothermophilus</i>	8	37	11.9±0.06	0.8± 0.2	16.2±5.8	
<i>Melghiribacillus thermohalophilus</i>	9.5	25	139.6±19.7	1.3±0.4	108.2±12.0	
<i>Ammoniphilus sp. CFH 90114</i>	10.5	25	112.2±6.6	0.7±0.2	148.2±13.5	(Demir et al., 2024)
<i>Vagococcus lutrae</i>	7.0	25	62.5±3.4	0.3±0.0	170.0±6.7	
<i>Candidatus Bathyarchaeota archaeon</i>	8.5	25	0.5 ±0.1	0.4 ±0.0	1.3 ±0.1	
<i>Thermus thermophilus</i>	10.5	25	87.0 ±5.1	0.63 ±0.2	137.8 ±15.7	
<i>Archaeoglobus fulgidus</i>	9.5	25	15.4 ±1.0	0.2 ±0.1	66.7 ±10	
Wild-type L-AsAlaDH	9	30	119.3±8.62	0.615±0.17	193.98±40.0	This Study
Tyr14Asn L-AsAlaDH	9	35	149.02±10.99	0.27±0.10	581.65±70.98	
Ile97Leu L-AsAlaDH	9.5	45	155.39±7.89	0.26±0.03	607.22±38.31	

All mutants exhibited an increased k_{cat} value and a decreased K_M value for both amination and deamination activities, resulting in enhanced catalytic efficiency (k_{cat}/K_M) compared to the wild-type enzyme. To elucidate the underlying reasons for these changes, the active site was investigated in the presence of substrates. Previous reports indicate that pyruvate forms hydrogen bonds with Arg15, Lys75, and His96 residues in wild-type L-AsAlaDH, with non-polar interactions observed with Phe94, Leu130, Met133, and Ala299 residues (Ågren et al., 2008). These findings are consistent with our observations for the active site of L-AsAlaDH.

During reductive amination, the Tyr14Asn mutant, where a bulky aromatic tyrosine was replaced by a more polar asparagine, led to longer conventional hydrogen bonds between pyruvate and His96 compared to the wild-type. This structural alteration likely resulted in a more relaxed binding of the pyruvate molecule, contributing to the observed decrease in K_M values and a 3-fold increase in efficiency. For oxidative deamination, the Tyr14Asn mutant formed an additional hydrogen bond with Lys75, leading to increased activity and a 2.7-fold increase in stability due to enhanced alanine molecule stability.

In the Ile97Leu mutant, during reductive amination, the replacement of isoleucine with a branched-chain leucine resulted in the conversion of the salt bridge between pyruvate and Lys75 to a conventional hydrogen bond. Furthermore, the conventional hydrogen bond length between His96 and pyruvate increased compared to the wild-type. These changes, including the formation of an extra bond, likely enhanced pyruvate stability. The increased bond lengths may also have facilitated more productive binding of the pyruvate molecule, contributing to the observed catalytic improvements. This mutation led to a 3.13-fold increase in catalytic efficiency. During oxidative deamination, the Ile97Leu mutant exhibited increased conventional hydrogen bond lengths for Asp270 and Lys15, leading to enhanced activity due to alanine relaxation at the active site, resulting in a 3.04-fold efficiency increase.

Interestingly, the Ser192Ala mutant showed similar effects to the Ile97Leu mutant during reductive amination, despite a side chain change from polar to nonpolar properties and its remote location from the binding site. However, the catalytic activity enhancement was lower than that of the Ile97Leu mutant, possibly because the increase in bond length between His96 and pyruvate was less pronounced than in Ile97Leu. The greater distance of the mutation from the active site also likely contributed to a smaller activity increase compared to Ile97Leu. Nevertheless, in reductive amination, efficiency increased 1.70-fold. During oxidative deamination, the Ser192Ala mutant showed increased Lys15 and alanine bond lengths, leading to a 1.8-fold efficiency increase. This likely facilitated the relaxation of the substrate molecule, contributing to the observed decrease in the K_M value.

Beyond active site investigations, molecular dynamics (MD) simulations were performed to understand the effects of mutations on reductive amination. Two types of simulations were conducted: those including only NAD(H) and the protein, and those including pyruvate, NAD(H), and the protein. Simulations with only NAD(H)

were crucial for understanding its role in stabilizing the protein structure prior to substrate binding, as NAD(H) is known to reduce ligand-water interactions and stabilize the overall structure (Ågren et al., 2008; Ling et al., 2012; Tripathi & Ramachandran, 2008).

Protein Structure Networks (PSNs) offer a powerful framework for dissecting the intricate mechanisms underpinning protein conformational stability. By representing proteins as networks where residues are nodes and interactions (e.g., hydrogen bonds, salt bridges, van der Waals forces) are edges, PSNs allow for a quantitative analysis of structural connectivity and communication pathways within the protein (Yehorova et al., 2024). Metrics like **betweenness centrality (BC)**, which quantifies the number of shortest paths passing through a residue, are particularly insightful (Greene, 2012). Residues with high BC often act as critical hubs, facilitating information flow and allosteric communication across the protein (Foutch et al., 2021). Analyzing changes in these network properties upon mutation or ligand binding can reveal how local structural perturbations propagate through the protein, influencing global stability and function (Osuna, 2021; Rajasekaran et al., 2017). For instance, an increase in BC for a particular residue may indicate its enhanced role in mediating structural communication, potentially contributing to a more stable or efficient conformation. Conversely, a decrease could signify a loss of critical connections, potentially leading to instability. By visualizing and quantifying these network changes, we can gain deeper insights into how mutations impact the delicate balance of forces that govern protein structure and dynamics, providing a comprehensive understanding of the observed functional outcomes. This approach thus provides a systems-level view that complements traditional active site analyses. Our comparison of residue BC levels of mutants with wild-type yielded a statistically significant increase in BCs of a large group of residues surrounding the active site of the protein. This finding clearly implies a structural reorganization within the enzyme to facilitate tighter interactions with the substrate and cofactor, which ultimately leads to an increase in catalytic efficiency.

In simulations containing only NAD(H) and the protein, we observed a slight decrease in RMSD fluctuations across the mutants, although the Ser192Ala mutant exhibited more variations than others, likely due to the fluctuation of the aromatic ring on the NAD(H) molecule across replicates. Decreased RMSD indicates a stabilizing effect of the mutants on NAD(H) binding, which correlated with RMSF results. MM-PBSA

results showed that the Ile97Leu mutant had larger free energy of binding (FEB) values. Simulations involving pyruvate, NAD(H), and the protein showed a decrease in the RMSD profile, indicating a stabilizing effect, which could be further investigated in RMSF graphs. However, a binding problem was observed in all simulations performed with pyruvate, evident from unstable results in the MM-PBSA graphs. Overall, the mutants led to a slight whole-protein stabilization in their structures. At this point, increasing the simulation period could provide more insight into pyruvate's behavior.

When van der Waals interactions were investigated using gRINN, both attractive and repulsive forces within the same ligand(s)-including systems were found to be localized in similar regions. Interestingly, systems including both NAD(H) and pyruvate displayed more attractive forces than repulsive forces, primarily condensed at the pyruvate binding domain, which may indicate substrate stability. However, in simulations only including NAD(H), more repulsive forces were observed, predominantly at the pyruvate binding domain. This might suggest that the system weakens bonds at this site to facilitate pyruvate binding.

The inhibitory or enhancing effects of metal ions on enzyme activity have been widely reported (Dedeakayoğulları et al., 2023; Gu et al., 2023b; He et al., 2018; Hutter & Singh, 1999; Irwin et al., 2003). These effects vary depending on the organism and can range from highly significant to less pronounced. In this study, K^+ and Ca^{+2} increased activity 58-86% in Tyr14Asn and wild-type. However Zn is more inhibitory for Tyr14Asn enzymes 40%, increasing effect on Ile97Leu. Fe^{+3} has inhibitory effect on 5-60% on all enzymes. While metal ions generally enhanced wild-type activity, they had an inhibitory effect on the Ile97Leu mutant. Overall, metals significantly influenced the reductive activity of both wild-type and mutant enzymes. The storage stability of the wild-type and Tyr14Asn mutant enzymes was similar; also, Ile97Leu and Ser192Ala have similar profile. This can be the reason of working high optimum temperature or optimum buffer of Ile97leu and Ser192Ala. However, this can be further investigated with thermal stability experiments.

6. CONCLUSION

In this study, we successfully generated L-*As*AlaDH enzyme mutants using rational enzyme design. These mutants were expressed, purified, and characterized with the primary goal of enhancing the catalytic efficiency of L-*As*AlaDH reductive amination activity. Interestingly, the mutations also led to increased oxidative deamination. We elucidated these activity enhancements by investigating the structure-activity relationships. Specifically, we examined the stability and flexibility of the substrate at the active site, which we found to stabilize the overall enzyme structure. Furthermore, we confirmed the inhibitory or enhancing effects of various metal ions on reductive amination.

REFERENCES

- Aalbers, F. S., Fürst, M. J. L. J., Rovida, S., Trajkovic, M., Rubén Gómez Castellanos, J., Bartsch, S., Vogel, A., Mattevi, A., & Fraaije, M. W. (2020). Approaching boiling point stability of an alcohol dehydrogenase through computationally-guided enzyme engineering. *ELife*, 9. <https://doi.org/10.7554/eLife.54639>
- Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., & Lindahl, E. (2015). GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX*, 1–2, 19–25. <https://doi.org/10.1016/J.SOFTX.2015.06.001>
- Agilent Technologies. (2023). *QuikChange II Site-Directed Mutagenesis Kit Instruction Manual*. www.agilent.com/en/contact-us/page.
- Ågren, D., Stehr, M., Berthold, C. L., Kapoor, S., Oehlmann, W., Singh, M., & Schneider, G. (2008a). Three-Dimensional Structures of Apo- and Holo-l-Alanine Dehydrogenase from *Mycobacterium tuberculosis* Reveal Conformational Changes upon Coenzyme Binding. *Journal of Molecular Biology*, 377(4), 1161–1173. <https://doi.org/10.1016/j.jmb.2008.01.091>
- AKTAŞ, F. (2021). Bioinformatics and 3D homology modelling of AsAlaDH from *Amycolatopsis sulphurea*. *European Journal of Science and Technology*. <https://doi.org/10.31590/ejosat.971416>
- Aktaş, F. (2021a). Heterologous Expression and Partial Characterization of a New Alanine Dehydrogenase from *Amycolatopsis sulphurea*. *Protein Journal*, 40(3), 342–347. <https://doi.org/10.1007/s10930-021-09982-9>
- Ashida, H., Galkin, A., Kulakova, L., Sawa, Y., Nakajima, N., & Esaki, N. (2004). Conversion of cofactor specificities of alanine dehydrogenases by site-directed mutagenesis. *Journal of Molecular Catalysis B: Enzymatic*, 30(3–4), 173–176. <https://doi.org/10.1016/J.MOLCATB.2004.04.008>
- Bakan, A., Meireles, L. M., & Bahar, I. (2011). ProDy: Protein dynamics inferred from theory and experiments. *Bioinformatics*, 27(11), 1575–1577. <https://doi.org/10.1093/bioinformatics/btr168>
- Bateman, A., Martin, M.-J., Orchard, S., Magrane, M., Adesina, A., Ahmad, S., Bowler-Barnett, E. H., Bye-A-Jee, H., Carpentier, D., Denny, P., Fan, J., Garmiri, P., Gonzales, L. J. da C., Hussein, A., Ignatchenko, A., Insana, G., Ishtiaq, R., Joshi, V.,

Jyothi, D., ... Zhang, J. (2025). UniProt: the Universal Protein Knowledgebase in 2025. *Nucleic Acids Research*, 53(D1), D609–D617. <https://doi.org/10.1093/nar/gkae1010>

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., & Bourne, P. E. (2000). The Protein Data Bank. In *Nucleic Acids Research* (Vol. 28, Issue 1). <http://www.rcsb.org/pdb/status.html>

BIOVIA, D. S. (2012). *Discovery Studio Modeling Environment*.

Blanco. (2017). Medical Biochemistry. In *Medical Biochemistry*. <https://doi.org/10.1016/B978-0-12-803550-4/00003-3>

Buaki-Sogó, M., García-Carmona, L., Gil-Agustí, M., Zubizarreta, L., García-Pellicer, M., & Quijano-López, A. (2020). Enzymatic Glucose-Based Bio-batteries: Bioenergy to Fuel Next-Generation Devices. In *Topics in Current Chemistry* (Vol. 378, Issue 6). Springer Science and Business Media Deutschland GmbH. <https://doi.org/10.1007/s41061-020-00312-8>

Buß, O., Rudat, J., & Ochsenreither, K. (2018). FoldX as Protein Engineering Tool: Better Than Random Based Approaches? In *Computational and Structural Biotechnology Journal* (Vol. 16, pp. 25–33). Elsevier B.V. <https://doi.org/10.1016/j.csbj.2018.01.002>

Case, D. A., Aktulga, H. M., Belfon, K., Cerutti, D. S., Cisneros, G. A., Cruzeiro, V. W. D., Forouzes, N., Giese, T. J., Götz, A. W., Gohlke, H., Izadi, S., Kasavajhala, K., Kaymak, M. C., King, E., Kurtzman, T., Lee, T. S., Li, P., Liu, J., Luchko, T., ... Merz, K. M. (2023). AmberTools. *Journal of Chemical Information and Modeling*, 63(20), 6183–6191. <https://doi.org/10.1021/acs.jcim.3c01153>

Christensen, N. J., & Kepp, K. P. (2012a). Accurate stabilities of laccase mutants predicted with a modified FoldX protocol. *Journal of Chemical Information and Modeling*, 52(11), 3028–3042. <https://doi.org/10.1021/ci300398z>

Christensen, N. J., & Kepp, K. P. (2012b). Accurate stabilities of laccase mutants predicted with a modified FoldX protocol. *Journal of Chemical Information and Modeling*, 52(11), 3028–3042. <https://doi.org/10.1021/ci300398z>

Dave, U. C., & Kadeppagari, R. K. (2019a). Alanine dehydrogenase and its applications—A review. In *Critical Reviews in Biotechnology* (Vol. 39, Issue 5, pp. 648–664). Taylor and Francis Ltd. <https://doi.org/10.1080/07388551.2019.1594153>

Dedeakayoğulları, H., Valjakka, J., Turunen, O., Yilmazer, B., Demir, Ğ., Jänis, J., & Binay, B. (2023). Application of reductive amination by heterologously expressed *Thermomicrobium roseum* L-alanine dehydrogenase to synthesize L-alanine derivatives. *Enzyme and Microbial Technology*, 169, 110265. <https://doi.org/10.1016/J.ENZMICTEC.2023.110265>

Demir, Ğ., Valjakka, J., Turunen, O., Aktaş, F., & Binay, B. (2024). Cloning and expression heterologous alanine dehydrogenase genes: Investigation of reductive amination potential of L-alanine dehydrogenases for green synthesis of alanine derivatives. *Heliyon*, 10(5). <https://doi.org/10.1016/j.heliyon.2024.e26899>

D'Este, M., Alvarado-Morales, M., & Angelidaki, I. (2018). Amino acids production focusing on fermentation technologies – A review. In *Biotechnology Advances* (Vol. 36, Issue 1, pp. 14–25). Elsevier Inc. <https://doi.org/10.1016/j.biotechadv.2017.09.001>

Fasim, A., More, V. S., & More, S. S. (2021). Large-scale production of enzymes for biotechnology uses. In *Current Opinion in Biotechnology* (Vol. 69, pp. 68–76). Elsevier Ltd. <https://doi.org/10.1016/j.copbio.2020.12.002>

Fernandes, P., Aldeborgh, H., Carlucci, L., Walsh, L., Wasserman, J., Zhou, E., Lefurgy, S. T., & Mundorff, E. C. (2015). Alteration of substrate specificity of alanine dehydrogenase. *Protein Engineering, Design and Selection*, 28(2), 29–35. <https://doi.org/10.1093/protein/gzu053>

Giffin, M. M., Shi, L., Gennaro, M. L., & Sohaskey, C. D. (2016). Role of Alanine Dehydrogenase of *Mycobacterium tuberculosis* during Recovery from Hypoxic Nonreplicating Persistence. *PLoS ONE*, 11(5). <https://doi.org/10.1371/journal.pone.0155522>

Grimshaw, C. E., & Cleland, W. W. (1981). Kinetic Mechanism of *Bacillus subtilis* L-Alanine Dehydrogenase⁺. In *Biochemistry* (Vol. 20).

Gu, P., Ma, Q., Zhao, S., Li, Q., & Gao, J. (2023a). Alanine dehydrogenases from four different microorganisms: characterization and their application in L-alanine production. *Biotechnology for Biofuels and Bioproducts*, 16(1). <https://doi.org/10.1186/s13068-023-02373-5>

He, G., Xu, S., Wang, S., Zhang, Q., Liu, D., Chen, Y., Ju, J., & Zhao, B. (2018). A conserved residue of L-alanine dehydrogenase from *Bacillus pseudofirmus*, Lys-73, participates in the catalytic reaction through hydrogen bonding. *Enzyme and Microbial Technology*, 110, 61–68. <https://doi.org/10.1016/j.enzmictec.2017.10.001>

Humphrey, W., Dalke, A., & Schulten, K. (1996a). VMD: Visual molecular dynamics. *Journal of Molecular Graphics*, 14(1), 33–38. [https://doi.org/10.1016/0263-7855\(96\)00018-5](https://doi.org/10.1016/0263-7855(96)00018-5)

Hutter, B., & Singh, M. (1998). Host vector system for high-level expression and purification of recombinant, enzymatically active alanine dehydrogenase of *Mycobacterium tuberculosis*. *Gene*, 212(1), 21–29. [https://doi.org/10.1016/S0378-1119\(98\)00134-6](https://doi.org/10.1016/S0378-1119(98)00134-6)

Hutter, B., & Singh, M. (1999). Properties of the 40 kDa antigen of *Mycobacterium tuberculosis*, a functional L-alanine dehydrogenase. In *Biochem. J* (Vol. 343).

Industrial Enzymes Market Trend, Size, Global, Share | MRFR. (2025). https://www.marketresearchfuture.com/reports/industrial-enzymes-market-8094?utm_term=&utm_campaign=&utm_source=adwords&utm_medium=ppc&hsa_acc=2893753364&hsa_cam=19912237177&hsa_grp=148712481999&hsa_ad=659589502539&hsa_src=g&hsa_tgt=dsa-2080758265560&hsa_kw=&hsa_mt=&hsa_net=adwords&hsa_ver=3&gad_source=1

Irwin, J. A., Lynch, S. V., Coughlan, S., Baker, P. J., Gudmundsson, H. M., Alfredsson, G. A., Rice, D. W., & Engel, P. C. (2003). Alanine dehydrogenase from the psychrophilic bacterium strain PA-43: Overexpression, molecular characterization, and sequence analysis. *Extremophiles*, 7(2), 135–143. <https://doi.org/10.1007/s00792-002-0305-4>

Janto, B., Ahmed, A., Ito, M., Liu, J., Hicks, D. B., Pagni, S., Fackelmayer, O. J., Smith, T. A., Earl, J., Elbourne, L. D. H., Hassan, K., Paulsen, I. T., Kolstø, A. B., Tourasse, N. J., Ehrlich, G. D., Boissy, R., Ivey, D. M., Li, G., Xue, Y., ... Krulwich, T. A. (2011). Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. *Environmental Microbiology*, 13(12), 3289–3309. <https://doi.org/10.1111/j.1462-2920.2011.02591.x>

Kim, D., Noh, M. H., Park, M., Kim, I., Ahn, H., Ye, D. yeol, Jung, G. Y., & Kim, S. (2022). Enzyme activity engineering based on sequence co-evolution analysis. *Metabolic Engineering*, 74, 49–60. <https://doi.org/10.1016/J.YMBEN.2022.09.001>

Kim, S., Ga, S., Bae, H., Sluyter, R., Konstantinov, K., Shrestha, L. K., Kim, Y. H., Kim, J. H., & Ariga, K. (2024). Multidisciplinary approaches for enzyme biocatalysis in pharmaceuticals: protein engineering, computational biology, and nanoarchitectonics. In *EES Catalysis* (Vol. 2, Issue 1, pp. 14–48). Royal Society of Chemistry. <https://doi.org/10.1039/d3cy00239j>

Krieger, E., Joo, K., Lee, J., Lee, J., Raman, S., Thompson, J., Tyka, M., Baker, D., & Karplus, K. (2009). Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. In *Proteins: Structure, Function and Bioinformatics* (Vol. 77, Issue SUPPL. 9, pp. 114–122). <https://doi.org/10.1002/prot.22570>

Lerchner, A., Jarasch, A., & Skerra, A. (2016). Engineering of alanine dehydrogenase from *Bacillus subtilis* for novel cofactor specificity. *Biotechnology and Applied Biochemistry*, 63(5), 616–624. <https://doi.org/10.1002/bab.1414>

Ling, B., Sun, M., Bi, S., Jing, Z., & Liu, Y. (2012). Molecular dynamics simulations of the coenzyme induced conformational changes of Mycobacterium tuberculosis L-alanine dehydrogenase. *Journal of Molecular Graphics and Modelling*, 35, 1–10. <https://doi.org/10.1016/j.jmgm.2012.01.005>

Lite, N. (n.d.). *T123-TECHNICAL BULLETIN Interpretation of Nucleic Acid 260/280 Ratios*. www.thermoscientific.com/nanodrop

Madeira, F., Madhusoodanan, N., Lee, J., Eusebi, A., Niewielska, A., Tivey, A. R. N., Lopez, R., & Butcher, S. (2024). The EMBL-EBI Job Dispatcher sequence analysis tools framework in 2024. *Nucleic Acids Research*, 52(W1), W521–W525. <https://doi.org/10.1093/nar/gkae241>

Mesbah, N. M. (2022). Industrial Biotechnology Based on Enzymes From Extreme Environments. In *Frontiers in Bioengineering and Biotechnology* (Vol. 10). Frontiers Media S.A. <https://doi.org/10.3389/fbioe.2022.870083>

Miller, B. R., McGee, T. D., Swails, J. M., Homeyer, N., Gohlke, H., & Roitberg, A. E. (2012). MMPBSA.py: An efficient program for end-state free energy calculations. *Journal of Chemical Theory and Computation*, 8(9), 3314–3321. <https://doi.org/10.1021/ct300418h>

Moret, M. A., & Zebende, G. F. (2007). Amino acid hydrophobicity and accessible surface area. *Physical Review E - Statistical, Nonlinear, and Soft Matter Physics*, 75(1). <https://doi.org/10.1103/PhysRevE.75.011920>

Nagarajan, R., Archana, A., Thangakani, A. M., Jemimah, S., Velmurugan, D., & Gromiha, M. M. (2016). PDBparam: Online resource for computing structural parameters of proteins. *Bioinformatics and Biology Insights*, 10, 73–80. <https://doi.org/10.4137/BBI.S38423>

Nielsen, J. R., Weusthuis, R. A., & Huang, W. E. (2023). Growth-coupled enzyme engineering through manipulation of redox cofactor regeneration. In *Biotechnology Advances* (Vol. 63). Elsevier Inc. <https://doi.org/10.1016/j.biotechadv.2023.108102>

Okur, T., & Serçinoğlu, O. (2025). Computational Protein Engineering. *Encyclopedia of Bioinformatics and Computational Biology*, 159–171. <https://doi.org/10.1016/B978-0-323-95502-7.00261-X>

Paladin, L., Piovesan, D., & Tosatto, S. C. E. (2017a). SODA: Prediction of protein solubility from disorder and aggregation propensity. *Nucleic Acids Research*, 45(W1), W236–W240. <https://doi.org/10.1093/nar/gkx412>

Pandey, A., Liu, E., Graham, J., Chen, W., & Keten, S. (2023). B-factor prediction in proteins using a sequence-based deep learning model. *Patterns*, 4(9). <https://doi.org/10.1016/j.patter.2023.100805>

Sakamoto, S., Seki, M., Nagata, S., & Misono, H. (2001). Cloning, sequencing, and expression of the meso-diaminopimelate dehydrogenase gene from *Bacillus sphaericus*. In *Journal of Molecular Catalysis B: Enzymatic* (Vol. 12).

Sawa, Y., Tani, M., Murata, K., Shibata, H., & Ochiai, H. (1994). Purification and Characterization of Alanine Dehydrogenase from a Cyanobacterium, *Phormidium lapideum*. In *J. Biochem* (Vol. 116).

Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F., & Serrano, L. (2005). The FoldX web server: An online force field. *Nucleic Acids Research*, 33(SUPPL. 2). <https://doi.org/10.1093/nar/gki387>

Serçinoğlu, O., & Ozbek, P. (2018). GRINN: A tool for calculation of residue interaction energies and protein energy network analysis of molecular dynamics simulations. *Nucleic Acids Research*, 46(W1), W554–W562. <https://doi.org/10.1093/nar/gky381>

Sharma, A., Gupta, G., Ahmad, T., Mansoor, S., & Kaur, B. (2021a). Enzyme Engineering: Current Trends and Future Perspectives. In *Food Reviews International* (Vol. 37, Issue 2, pp. 121–154). Bellwether Publishing, Ltd. <https://doi.org/10.1080/87559129.2019.1695835>

Silva, D., & Vranken, B. F. (2012). ACPYPE-AnteChamber PYthon Parser interface. In *Research Notes* (Vol. 5). <http://www.biomedcentral.com/1756-0500/5/367>

Søndergaard, C. R., Olsson, M. H. M., Rostkowski, M., & Jensen, J. H. (2011). Improved treatment of ligands and coupling effects in empirical calculation and rationalization of p K a values. *Journal of Chemical Theory and Computation*, 7(7), 2284–2295. <https://doi.org/10.1021/ct200133y>

Takeuchi, F., Watanabe, S., Baba, T., Yuzawa, H., Ito, T., Morimoto, Y., Kuroda, M., Cui, L., Takahashi, M., Ankai, A., Baba, S. I., Fukui, S., Lee, J. C., & Hiramatsu, K. (2005). Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *Journal of Bacteriology*, 187(21), 7292–7308. <https://doi.org/10.1128/JB.187.21.7292-7308.2005>

Tripathi, S. M., & Ramachandran, R. (2008). Crystal structures of the *Mycobacterium tuberculosis* secretory antigen alanine dehydrogenase (Rv2780) in apo and ternary complex forms captures “open” and “closed” enzyme conformations. *Proteins: Structure, Function and Genetics*, 72(3), 1089–1095. <https://doi.org/10.1002/prot.22101>

Tuo, J., Nawab, S., Ma, X., & Huo, Y. X. (2023). Recent advances in screening amino acid overproducers. In *Engineering Microbiology* (Vol. 3, Issue 1). Elsevier Inc. <https://doi.org/10.1016/j.engmic.2022.100066>

Valdés-Tresanco, M. S., Valdés-Tresanco, M. E., Valiente, P. A., & Moreno, E. (2021). Gmx_MMPBSA: A New Tool to Perform End-State Free Energy Calculations with GROMACS. *Journal of Chemical Theory and Computation*, 17(10), 6281–6291. <https://doi.org/10.1021/acs.jctc.1c00645>

Victorino da Silva Amatto, I., Gonsales da Rosa-Garzon, N., Antônio de Oliveira Simões, F., Santiago, F., Pereira da Silva Leite, N., Raspante Martins, J., & Cabral, H. (2022a). Enzyme engineering and its industrial applications. In *Biotechnology and Applied Biochemistry* (Vol. 69, Issue 2, pp. 389–409). John Wiley and Sons Inc. <https://doi.org/10.1002/bab.2117>

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F. T., De Beer, T. A. P., Rempfer, C., Bordoli, L., Lepore, R., & Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, 46(W1), W296–W303. <https://doi.org/10.1093/nar/gky427>

Wendisch, V. F. (2020). Metabolic engineering advances and prospects for amino acid production. In *Metabolic Engineering* (Vol. 58, pp. 17–34). Academic Press Inc. <https://doi.org/10.1016/j.ymben.2019.03.008>

Xie, Y., An, J., Yang, G., Wu, G., Zhang, Y., Cui, L., & Feng, Y. (2014). Enhanced enzyme kinetic stability by increasing rigidity within the active site. *Journal of Biological Chemistry*, 289(11), 7994–8006. <https://doi.org/10.1074/jbc.M113.536045>

Xing, H., Zou, G., Liu, C., Chai, S., Yan, X., Li, X., Liu, R., Yang, Y., & Zhou, Z. (2021). Improving the thermostability of a GH11 xylanase by directed evolution and rational design guided by B-factor analysis. *Enzyme and Microbial Technology*, 143. <https://doi.org/10.1016/j.enzmictec.2020.109720>

Zhao, Y., Li, D., Bai, X., Luo, M., Feng, Y., Zhao, Y., Ma, F., & Yang, G.-Y. (2021). Improved thermostability of proteinase K and recognizing the synergistic effect of Rosetta and FoldX approaches. *Protein Engineering, Design and Selection*, 34, 1–7. <https://doi.org/10.1093/protein/gzab024>

BIOGRAPHY

Tuba Okur Aksu graduated from Yıldız Technical University with a Bioengineering degree in 2021. She then began her master's studies in bioengineering at Gebze Technical University in 2022. During her master's program, she gained experience in both experimental and computational enzyme engineering.

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

OKUR TUBA, SERÇİNOĞLU ONUR, AKTAŞ FATİH, BİNAY BARIŞ (2024). Rational Engineering of Amycolotopsis Sulphurea L-Alanine Dehydrogenase for Enhanced Alanine Production. V. International Enzyme and Bioprocess Days (Özet Bildiri/Poster) (Yayın No: 9159690)

OKUR TUBA, SERÇİNOĞLU ONUR, AKTAŞ FATİH, BİNAY BARIŞ (2023). Engineering of l-alanine dehydrogenase for improving catalytic activity. 4th V. International Enzyme and Bioprocess Days (Özet Bildiri/Sözlü Sunum) (Yayın No: 8490127)

Okur, T., & Serçinoğlu, O. (2025). Computational Protein Engineering. *Encyclopedia of Bioinformatics and Computational Biology*, 159–171. <https://doi.org/10.1016/B978-0-323-95502-7.00261-X>