

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**HETEROLOGOUS EXPRESSION OF NOVEL L-ALANINE
DEHYDROGENASES AND GENERATION OF MUTANTS FOR
THE SYNTHESIS OF UNNATURAL AMINO ACIDS**

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A THESIS OF DOCTORATE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ADVISOR: PROF. DR. BARIŞ BİNAY

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

YENİ L-ALANİN DEHİDROGENAZLARIN HETEROLOG
EKSPRESYONU VE DOĞAL OLMAYAN AMİNO ASİTLERİN
SENTEZİ İÇİN MUTANTLARIN ELDE EDİLMESİ

ĞARİP DEMİR

DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK
ANABİLİM DALI

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*To my family, my beloved
mother, father and brothers*

ABSTRACT

This thesis focuses on design, characterization, protein engineering, and modeling an NAD(H)-dependent L-alanine dehydrogenase (L-AlaDH) for asymmetric amino acid synthesis. It examines enzyme expression, purification, and activity, identifying key parameters for optimal catalytic efficiency. Mutagenesis studies aimed to enhance enzyme activity against specific pyruvate derivatives, including α -ketovalerate, α -ketobutyrate, and α -ketocaproate.

The results indicate that L-AlaDH enzymes are active against these ketoacids. In particular, the L-AlaDH enzyme from *Thermus thermophilus* (TtAlaDH) demonstrated high catalytic efficiency and specific activity against all tested substrates, positioning it as a promising candidate for protein engineering. Mutational analysis of the Tyr92 and Tyr115 positions showed that the Ser92 mutant notably enhanced enzyme activity and effectiveness against α -ketovalerate and α -ketocaproate, though it lost effectiveness against α -ketobutyrate. For α -ketocaproate, the k_{cat} increased 99-fold, while K_M increased 26-fold, yielding a 6-fold improvement in catalytic efficiency. For α -ketovalerate, the k_{cat} increased 2.2-fold, and K_M increased 1.8-fold, leading to a 2-fold increase in catalytic efficiency. However, for α -ketobutyrate, the k_{cat} decreased 18-fold, and K_M increased eight-fold, resulting in a 150-fold reduction in catalytic efficiency.

A second mutation at the Met115 position in the Ser92 mutant significantly boosted enzyme activity and improved its performance against α -ketocaproate, while reducing effectiveness against α -ketovalerate and showing little change against α -ketobutyrate. In terms of α -ketocaproate, catalytic activity increased substantially compared to both the wild-type enzyme and the Tyr92Ser mutant. The k_{cat} increased 155-fold over the wild type, while K_M increased 37-fold, resulting in a 4-fold improvement in catalytic efficiency. Compared to Tyr92Ser, the k_{cat} increased 1.5-fold, and the K_M increased 1.4-fold, resulting in a 1.2-fold improvement.

For α -ketovalerate, however, catalytic activity decreased significantly compared to both the wild-type and Tyr92Ser mutants. The k_{cat} decreased 4-fold, and K_M increased 5-fold compared to the wild type, leading to a 20-fold reduction in catalytic efficiency. Compared to Tyr92Ser, the k_{cat} decreased 8.2-fold, and K_M increased 4-fold, resulting in a 33.5-fold decrease. For α -ketobutyrate, a notable reduction in catalytic activity was observed. The k_{cat} decreased 6.5-fold, and K_M increased 49-fold over the wild type, leading to a 375-fold decrease in efficiency. Compared to Tyr92Ser, the k_{cat} increased 2.8-fold, but K_M increased 6.2-fold, resulting in a 2.5-fold reduction in activity.

These findings highlight the impact of specific mutations on L-AlaDH activity and substrate specificity. Understanding the structure-function relationships of these enzymes enables the design of biocatalysts with enhanced properties for industrial applications. Engineered L-AlaDH enzymes have potential in biochemistry, food processing, and pharmaceutical production, facilitating the creation of novel drugs, nutritional supplements, and other high-value products. This contributes to sustainable and innovative solutions across various sectors.

Keywords: L-alanine dehydrogenase, Unnatural amino acids (UAAs), Reductive amination mechanism, Enzyme Engineering

ÖZET

Tez çalışması L-alanin türevlerinden yapay amino asitlerin asimetrik sentezi için yeni bir NAD(H)-bağımlı L-alanin dehidrogenaz (L-AlaDH) enzim tasarımı, karakterizasyonu, protein mühendisliği ve modellemesi üzerine odaklanmaktadır. Farklı organizmalardan seçilen L-AlaDH enzimlerinin ifadesi, saflaştırılması ve aktiviteleri kapsamlı bir şekilde incelenmiştir. En yüksek katalitik verimliliğe sahip enzimleri seçmek için optimum pH, sıcaklık ve kinetik değerler gibi önemli parametreler belirlenmiştir. Pirüvat türevlerine (α -ketovalerat, α -ketobütriyat ve α -ketokaproat) karşı enzim aktivitesini artırmak amacıyla mutasyon çalışmaları yapılmıştır.

Elde edilen sonuçlar kapsamında, L-AlaDH enzimlerinin çalışma kapsamında kullanılan ketoasitlere karşı aktivite gösterdiği belirlenmiştir. Özellikle, *Thermus thermophilus* (TtAlaDH) kaynaklı L-AlaDH enzimi, tüm test edilen substratlar karşısında yüksek katalitik verimlilik ve spesifik aktivite sergileyerek, protein mühendisliği için umut verici bir adaylar olarak görülmüştür. Tyr92 ve Tyr115 pozisyonlarına yapılan mutasyonel analizlere göre, Ser92 mutantının enzim aktivitesinin α -ketovalerat ve α -ketokaproata karşı etkinliğini artırdığını, ancak α -ketobütriyata karşı etkinliğini kaybettiğini göstermiştir. α -ketokaproat için, k_{cat} 99-kat artmış, K_M 26-kat artmış ve bu da katalitik verimliliğin 6-kat artmasına yol açmıştır. α -ketovalerat için, k_{cat} 2.2-kat artmış ve K_M 1.8-kat artmış, bu da katalitik verimliliğin 2-kat artmasına yol açmıştır. Ancak, α -ketobütriyat için, k_{cat} 18-kat azalmış ve K_M 8-kat artmış, bu da katalitik verimliliğin 150-kat azalmasına neden olmuştur.

Ser92 mutantındaki ikinci bir mutasyon, Met115 pozisyonunda, enzim aktivitesini önemli ölçüde artırmış ve α -ketokaproat karşısındaki performansını iyileştirmiş, ancak α -ketovalerat karşısındaki etkinliğini azaltmış ve α -ketobütriyat karşısında önemli bir değişiklik göstermemiştir. α -ketokaproat açısından, katalitik aktivite, hem yabanıl tip enzimiyle hem de Tyr92Ser mutantıyla karşılaştırıldığında önemli ölçüde artmıştır. k_{cat} , yabanıl tipe göre 155-kat artmış, K_M 37-kat artmış ve bu da katalitik verimliliğin 4-kat artmasına yol açmıştır. Tyr92Ser ile karşılaştırıldığında, k_{cat} 1.5-kat artmış ve K_M 1.4-kat artmış, bu da 1.2-katlık bir iyileşmeye neden olmuştur.

Ancak, α -ketovalerat için, katalitik aktivite hem yabanıl tip hem de Tyr92Ser mutantları ile karşılaştırıldığında önemli ölçüde azalmıştır. k_{cat} yabanıl tipe göre 4-kat azalmış ve K_M 5-kat artmış, bu da katalitik verimliliğin 20-kat azalmasına yol açmıştır. Tyr92Ser ile karşılaştırıldığında, k_{cat} 8.2-kat azalmış ve K_M 4-kat artmış, bu da 33.5 katlık bir azalmaya yol açmıştır. α -ketobütriyat için, katalitik aktivitede belirgin bir azalma gözlemlenmiştir. k_{cat} yabanıl tipe göre 6.5-kat azalmış ve K_M 49-kat artmış, bu da verimliliğin 375-kat azalmasına neden olmuştur. Tyr92Ser ile karşılaştırıldığında, k_{cat} 2.8-kat artmış, ancak K_M 6.2-kat artmış, bu da aktivitenin toplamda 2.5-kat azalmasına yol açmıştır.

Sonuç olarak, Tyr115Met mutasyonu α -ketokaproat karşısındaki katalitik aktiviteyi artırmış, ancak α -ketovalerat ve α -ketobütriyat karşısındaki etkinliği önemli ölçüde azaltmıştır. L-AlaDH enzimlerinin yapı-fonksiyon ilişkisini anlamak, gelişmiş özelliklere sahip yeni enzimlerin geliştirilmesine olanak sağlayarak biyokimya, gıda ve ilaç endüstrilerinde çeşitli endüstriyel uygulamalar için değerli hale getirebilir.

Anahtar Kelimeler: L-alanine dehidrogenaz, Doğal olmayan amino asitler (UAAs), Redüktif aminasyon mekanizması, Enzim Mühendisliği

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LIST OF SYMBOLS AND ABBREVIATIONS

UAA	: Unnatural Amino Acids
L-AlaDH	: L-Alanine Dehydrogenase Enzyme
ald	: L-Alanine Dehydrogenase Gene
IUBMB	: International Union of Biochemistry and Molecular Biology
Met	: <i>Melghiribacillus thermohalophilus</i>
Acfh	: <i>Ammoniphilus</i> sp. CFH 90114
Vl	: <i>Vagococcus lutrae</i>
Tt	: <i>Thermus thermophilus</i>
Cb	: <i>Candidatus Bathyarchaeota archaeon</i>
Af	: <i>Archaeoglobus fulgidus</i>
MEC	: Multi-Enzymatic Cascade
Ni-NTA	: Nickel-Nitrilotriacetic Acid
Tris-HCl	: Tris(hydroxymethyl)aminomethane Hydrochloride
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PMSF	: Phenylmethylsulfonyl fluoride
EDTA	: Ethylenediaminetetraacetic Acid
NAD(H)	: Nicotinamide Adenine Dinucleotide Hydrogen
BLAST	: The Basic Local Alignment Search Tool
NAMD	: Nanoscale Molecular Dynamics
VMD	: Visual Molecular Dynamics
TIP3P	: Transferable Intermolecular Potential With 3 Points
SOC	: Super Optimal broth with Catabolite repression
CAST/ISM	: The combinatorial active site saturation test based/ site saturation mutagenesis
k_{cat}	: Turnover number of an enzyme
kDa	: kiloDalton
K_M	: Apparent Michaelis–Menten constant
mM	: Millimolar
DNA	: Deoxyribonucleic Acid
BCA	: Bicinchoninic Acid Assay

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1. INTRODUCTION

A wide range of industries utilize natural amino acids. In the field of synthetic organic chemistry, the utilization of optically active and cost-effective amino acids is very prevalent, especially in asymmetric synthesis. The field of pharmaceutical chemistry has received significant focus because to its frequent utilization in asymmetrical synthesis, mostly attributed to the fundamental biological functions exhibited by amino acid derivatives. Specifically, non-proteinaceous or unnatural amino acids (UAAs) are employed as industrial compounds that possess significant medicinal properties, enabling the synthesis of lucrative pharmaceutical chemicals in the industry. The most effective approach for the cost-effective production of L-alanine, the smallest chiral member of the amino acid family, and its derivatives is the utilization of enzymes with reductive amination process.

The L-alanine dehydrogenase enzyme (L-AlaDH), which relies on Nicotinamide Adenine Dinucleotide Hydrogen (NAD(H)) as a cofactor, catalyzes the oxidation of L-alanine and the reduction of pyruvate in both directions. It specifically recognizes L-alanine as its substrate. Despite obtaining numerous L-AlaDHs from various organisms, the studies on reductive amination reactions were significantly limited. No instances of successful application of protein engineering approaches have been found to enhance the reductive amination activities of L-AlaDHs, making them more effective than the only commercially available recombinant L-AlaDHs.

Researchers aim to develop next-generation enzymes with improved efficiency for UAA production by advancing protein engineering and gaining deeper insights into the structural and functional dynamics of L-AlaDH. This ongoing research is essential for overcoming the limitations of current enzymatic asymmetric synthesis of UAAs.

1.1. Amino Acids

Amino acids are universally recognized as the fundamental "building blocks" of biological systems, essential for the maintenance of cellular structure and function, as well as the repair of tissues. Apart from their basic functions, amino acids greatly support the immune system by generating antibodies against bacterial and viral infections. They are also essential parts of enzymatic and hormonal systems, participating in critical biochemical pathways that regulate metabolism and physiological homeostasis. Furthermore, amino acids are involved in the synthesis of nucleoproteins, support oxygen transport through hemoglobin, and play vital roles in muscle activity by enabling contraction and energy production [Grishin et al., 2020; Tonouchi and Ito, 2017].

Twenty-two coding amino acids occur naturally, with most proteins composed exclusively of their L-isomeric forms. This stereospecificity highlights their evolutionary significance and functional precision. Natural amino acids represent one of the most important families of industrial molecules, finding diverse applications in pharmaceuticals, food technology, agriculture, and biotechnology [Grishin et al., 2020; Tonouchi and Ito, 2017]. Amino acids play a crucial role in synthetic organic chemistry, especially in asymmetric synthesis. Their chiral and optically active properties make them ideal for building blocks for creating complex molecules, such as pharmaceutical intermediates and bioactive compounds. This unique feature has sparked considerable interest in advancing techniques for their asymmetric synthesis, particularly within the pharmaceutical industry, where amino acids and their derivatives are widely used [Patel, 2013].

In addition to their individual roles, amino acids are crucial precursors for peptide synthesis. Peptides, which are composed of artificially or naturally derived amino acids, have demonstrated immense potential for use in a wide range of applications, including therapeutic, diagnostic, and industrial fields. The limited industrial use of natural amino acids in peptide production can be attributed to challenges associated with their side chains. The structural diversity of amino acid side chains, while advantageous for biological functions, complicates their integration into industrial processes.

The broad applicability of both natural and unnatural amino acids extends to their inclusion in pharmacologically active products, molecular scaffolds, and as essential building blocks in organic synthesis. Unnatural amino acids have gained significant attention for their ability to enhance the chemical and functional diversity of peptides and proteins, thereby facilitating the development of novel therapeutics and biomaterials [Hoffmann et al., 2018; Martínez-Rodríguez et al., 2020].

1.2. Unnatural Amino Acids

The typical genetic code consists of 22 proteinogenic amino acids, which encompass selenocysteine and pyrrolysine. Nevertheless, scientific investigation has shown the existence of supplementary naturally existing amino acids, surpassing the initial count. Consequently, the whole tally of recognized naturally occurring amino acids has expanded to 800. UAAs are mostly generated endogenously in living organisms, particularly through the metabolic processes of plants and fungi. In addition to naturally occurring amino acids, a variety of unnatural or synthetic amino acids have been created in laboratory environments, although they do not exist in nature [Narancic et al., 2019; Xue et al., 2018].

UAAs cover a wide range of fields including several scientific disciplines. These compounds have possible uses ranging from the identification of new pharmacological compounds to their use as tools in scientific research and the synthesis of peptides and proteins with special characteristics or activities [Linser et al., 2014; Farmer et al., 2015; Xuan et al., 2016].

UAAs are employed as markers or labels in biological research, facilitating the tracking of specific molecules or cellular interactions [Iwata et al., 2003; Poppe et al., 2012; Linser et al., 2014; Hoffmann et al., 2018]. All of these diverse applications from biotechnology to materials science showcase UAAs' increasing scientific and industrial relevance as well as their capacity to transform sectors ranging from biotechnology to materials science and help to advance fields including synthetic biology, drug development, and bioengineering.

1.2.1. Synthesis of Unnatural Amino Acids

Despite their enormous potential and diverse applications, the asymmetric synthesis of UAAs remains a significant challenge. This synthesis can be categorized into two main approaches: enzymatic (green) synthesis and chemical synthesis. Chemically synthesized UAAs can be produced through various reactions, including alkylation [Narancic et al., 2019], cyclization [Nian et al., 2016], arylation [Brittain et al., 2020], acylation [Li et al., 2017], and side chain modification [Agirre et al., 2019]. Although these techniques are widely employed in industry, they pose significant environmental and safety concerns due to the use of transition metals and hydrogen gas under high pressure. Another limitation is their inability to induce chirality. Organocatalytic approaches have also been explored using hydrosilanes as hydrogen sources and employing chiral Brønsted acid catalysts, such as Akiyama-Terada catalysts, to induce chirality. While the optical purity of the products obtained from these processes is crucial, it has been noted that biocatalytic pathways should also be considered to achieve the desired compounds [Nian et al., 2016; Agirre et al., 2019; Narancic et al., 2019].

The enzymatic method for synthesizing chiral enantiopure UAAs is extremely appealing because of its quick and succinct reaction process. This technique frequently uses optimum buffers as the solvent for the reaction, thus removing the necessity for extra protecting groups and enhancing the energy efficiency, environmental friendliness, and cost-effectiveness of creating valuable UAAs. Enzymes including ammonia-lyases [Poppe et al., 2012], transaminases [Mathew and Yun, 2012; Isupov et al., 2019], and L-amino acid dehydrogenase [Brunhuber et al., 1994; Sharma et al., 2017] are commonly used to produce asymmetric UAAs. In addition, the multi-enzymatic cascade (MEC) is a biocatalytic method used for the production of UAA [Martínez-Rodríguez et al., 2020]. Enzymes such as acylases, lipases, nitrilases, and hydantoinases [Altenbuchner et al., 2001] have been used to create a wide variety of chiral amino acids through kinetic resolution [Xue et al., 2018]. Enzymes that have been specifically designed with distinct characteristics offer a flexible foundation for the process of biocatalytic hydroamination [Prier, 2021].

1.3. L-Alanine Dehydrogenase (L-AlaDH)

Enzymes are globular proteins and represent the largest group of proteins, like other proteins contain chains of long linear amino acids that joined together by peptide bonds with coupling the carboxylic acid (COOH) and amino (NH₂) residues between two amino acids. Each amino acid sequence creates a unique three-dimensional structure with its own characteristics. Enzymes are involved in the formation and degradation of all biological substances and plays an essential role in different processes including metabolism, gene expression and cell division [Blanco and Blanco, 2017; Porto de Souza Vandenberghe et al., 2020].

The reasons why enzymes have different properties can be explained as follows:

- Having different side chains of amino acids,
- Having different sequences of amino acids in their structure,
- Presence or absence of different metal ions in their structure
- Confirmation of the protein structure.

Enzymes are classified into 7 main groups, considering the reactions they catalyze with the recommendation of the International Union of Biochemistry and Molecular Biology (IUBMB). These groups are indicated by giving examples from **Table 1.1** class members.

Table 1.1: Classification of enzymes [Blanco and Blanco, 2017; Porto de Souza Vandenberghe et al., 2020].

IUBMB classification	Mechanism of action
EC1 Oxidoreductases	Catalyze redox reactions (e.g., dehydrogenases, oxidases)
EC2 Transferases	Catalyze the transfer of a certain group of atoms, such as an amine or carboxyl group (e.g., transaminases)
EC3 Hydrolases	Catalyze the hydrolytic cleavage of C—O, C—N, C—S, and O—P bonds (e.g., peptidases, nucleases)
EC4 Lyases	Catalyze the breaking of C—C, C—S, and C—N bonds (e.g., decarboxylases, dehydratases)
EC5 Isomerases	Catalyze the structural or geometrical rearrangement in one molecule (e.g., epimerase, racemase)
EC6 Ligases	Catalyze the joining of two molecules, at the end of reactions C—C, C—S, C—O, or C—N bonds forms occur. (e.g., ligases)
EC7 Translocases	Catalyze the transfer of ions and molecules across the membrane (e.g., ABC type transporter)

L-AlaDH (EC 1.4.1.1) belongs to the amino acid dehydrogenase group of the family of oxidoreductases. The enzyme is NAD(H) dependent and reversibly converts L-alanine to pyruvate. **Figure 1.1** illustrates the bidirectional reaction mechanism of L-AlaDH.

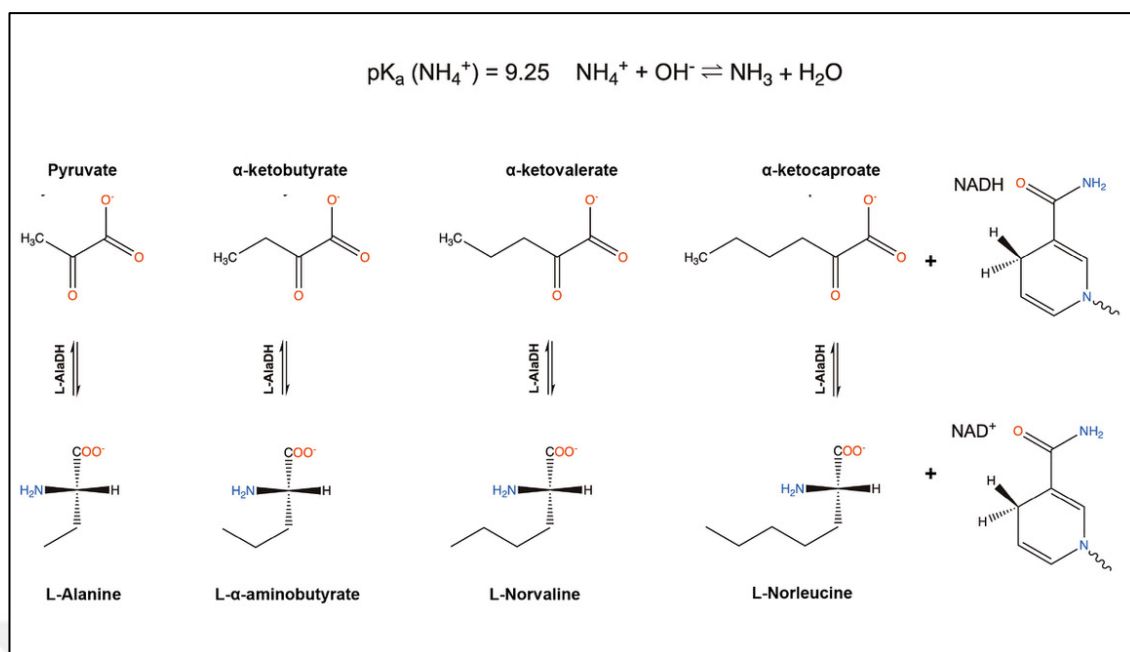


Figure 1.1: The reversible oxidative deamination and reductive amination reactions of L-AlaDH.

The reversible reaction of L-AlaDH is a reductive amination reaction in which L-alanine is synthesized from pyruvate. Many preferred asymmetric methods for UAA synthesis involve reductive amination reactions. Previous studies reveal that the kinetic parameters of L-AlaDH in reductive amination reactions have been scarcely investigated [Irwin et al., 2003; Seo and Yun, 2011; He et al., 2018]. In these limited studies, the characterization of the kinetic values for the reductive amination reaction of L-AlaDH in general has been reported. In addition, none of the L-AlaDH enzymes reported so far appear to show high catalytic efficiency against pyruvate. However, there are many reported oxidative deamination reaction kinetic constants and biochemical characterization reports. An alanine dehydrogenase enzyme designed through this reaction also has the potential to be used as a biosensor in medical applications [Dave and Kadeppagari, 2018].

Structural analysis of L-AlaDH proteins is crucial for predicting functional amino acid positions involved in the synthesis of chiral L-alanine derivatives. Furthermore, the functions of these positions must be validated through experimental studies. The identification of potential functional positions provides an invaluable foundation for the successful enzyme engineering of the target L-AlaDH. Looking at the L-AlaDH enzymes identified so far, most belong to *Bacillus* species [Dave and Kadeppagari,

2019]. Structure and sequence analysis studies at the molecular level were performed for L-AlaDHs belonging to *Mycobacterium tuberculosis* and *Phormidium lapideum* species [Sawa et al., 1994; Ågren et al., 2008]. In these two studies, sequence-level domains were defined for NAD⁺ binding, it was reported that the proposed catalytic mechanism for dehydrogenases was valid in L-AlaDHs computationally, and amino acid positions that could be functional were suggested from sequence analysis [Sawa et al., 1994]. However, these assumptions are still not confirmed experimentally.

The first L-AlaDH, to be produced using recombinant Deoxyribonucleic Acid (DNA) technologies, was isolated and cloned from *Bilophila wadsworthia* and expressed in *Escherichia coli*. This study reported the gene sequence of the L-AlaDH enzyme, its optimum pH, and its kinetic parameters [Laue and Cook, 2000]. The first L-AlaDH isolated from fungi belonged to *Thielaviopsis paradoxa* [Mona Al-Onazi et al., 2011]. However, this study does not focus on a molecular-level analysis of enzymes. Instead, it provides partial characterization, including kinetic evaluation, thermal stability assessment, and investigation of the effects of various metal ions on the activity of L-alanine derivatives. The first and only L-AlaDH enzyme cloned from archaea belongs to *Archaeoglobus fulgidus*. Nonetheless, it remains unclear whether this enzyme can be definitively classified as L-AlaDH, as it is smaller in size compared to known L-AlaDHs and exhibits lower amino acid sequence similarity. However, the kinetic parameters determined for L-alanine and pyruvate were comparable to those observed in other L-AlaDHs [Vadas et al., 2002].

The most striking finding in the crystal structure studies of *M. tuberculosis* L-AlaDH, whose three-dimensional structure was determined by experimental methods, was that the enzyme changed from the "open" position to the "closed" position by dinucleotide binding and contributed to the orientation of the substrate at a right angle for hydride transfer in this conformational transition. Binding sites and kinetic properties for the enzyme's NAD⁺ and L-alanine were determined [Ågren et al., 2008; Tripathi and Ramachandran, 2008].

1.4. L-AlaDH Enzyme Mechanism

Biochemical studies suggest that the L-AlaDH mechanism involves iminopyruvate and carbinolamine intermediates. The proposed strategy suggests that the carboxyl

group of the substrate is coordinated by both Lys74 and Arg15, leading to polarization of the carbonyl group and activation of the substrate. In the initial stage of the oxidative deamination reaction, a hydride is transferred from the C-alpha position of alanine to the nicotinamide ring. This transfer results in the formation of a protonated iminopyruvate. Subsequently, a conformational alteration occurs, enabling the ingress of a water molecule into the active site, which then proceeds to react with the iminopyruvate, resulting in the production of carbinolamine. The action is made easier by the hydrogen bonding of water to His94, which functions as a cationic acid. The movement of protons by His94 is facilitated by the close proximity of two acidic residues, Glu117 and Asp268. Ultimately, the carbinolamine decomposes to produce pyruvate and ammonia, thereby concluding the suggested reaction in **Figure 1.2**.

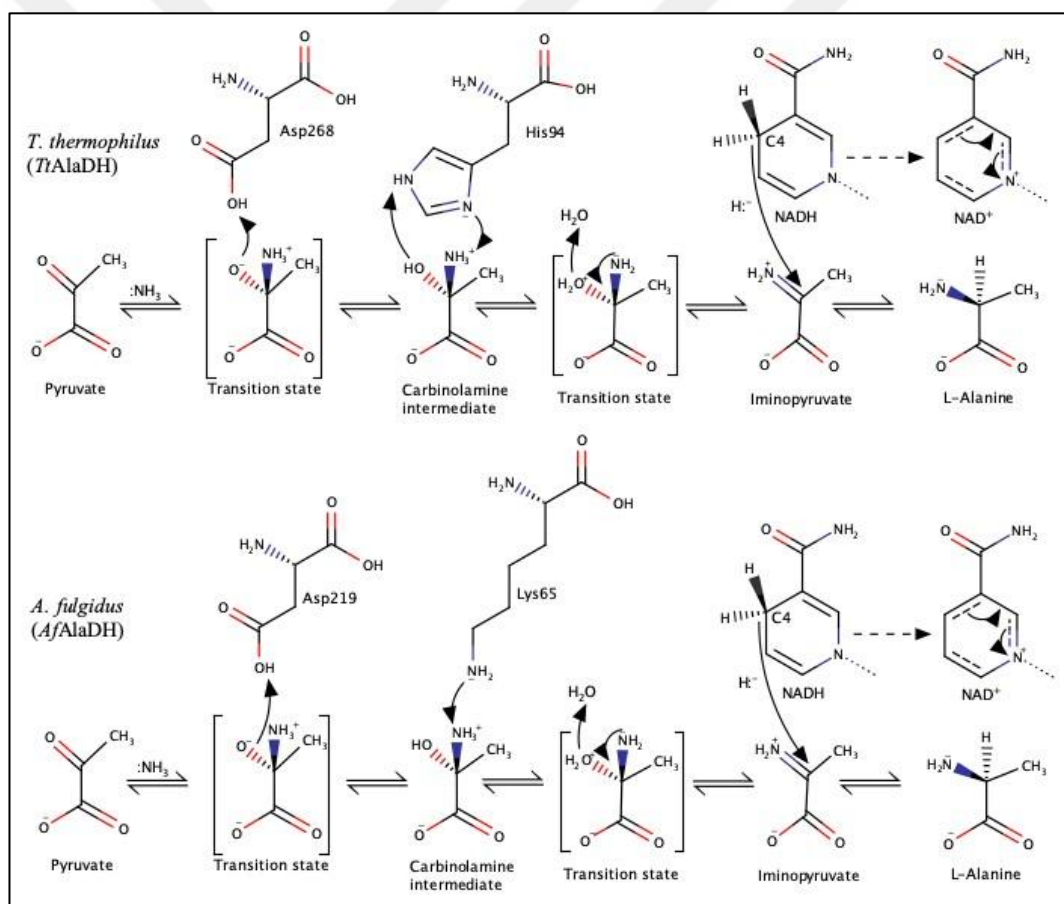


Figure 1.2: Catalytic mechanism in reverse reaction of *Tt* and *Af*AlaDH.

Gallagher et al. studied the crystal structure of *Af*AlaDH (Protein Databank Accession number: 1omo) and identified several amino acids present in the NAD⁺ binding site: Gly132, Gly134, Gln136, Arg108, Thr109, Ile216, Asp219, and Ser290 [Gallagher et

al., 2004]. Lys65 was identified as the main proton donor in the reaction, with other amino acids such as Lys41, Arg52, Asp297, and Arg108 playing active roles in binding alanine and pyruvate. Additionally, water molecules at positions 503 and 510 were observed to participate in binding interactions. These findings indicate that multiple residues and water molecules contribute to the stability and efficiency of the enzyme-substrate complex during the catalytic process [Gallagher et al., 2004].

1.5. L-AlaDH Mutation Studies for L-Alanine Derivatives

Because of their smallest chiral structure, alanine and its derivatives have great potential for use as precursors. Alanine is mainly used as a sweetener and flavor enhancer in the food industry, as well as a feed additive in animal foods. Alanine and its derivatives have a wide range of uses in the pharmaceutical industry, which can be summarized as components of infusion fluids and pharmaceutical products. Finally, alanine and its derivatives are used in cosmetics and washing products as a natural moisture stabilizer [Ikeda and Takeno, 2013; Tonouchi and Ito, 2017].

L-norvaline, one of the UAAs targeted for production in this study, functions as an inhibitor of arginase. Its inhibition of arginase enhances the production of nitric oxide (NO) in activated macrophages, as demonstrated by [Sosroseno 2006; Chang et al., 2024]. Studies indicate that the increased production of NO plays a crucial role in reducing hypertension by relaxing smooth muscle tissues, leading to improved blood vessel dilation, enhanced blood flow, and a reduction in overall cardiovascular strain. This process contributes to better regulation of blood pressure and supports overall vascular health. [Sosroseno 2006; Chang et al., 2024].

L-norleucine, an UAA, functions as a synthetic analog of methionine and has attracted considerable attention in experimental research. It is particularly utilized for its role in elucidating protein structure and function. Furthermore, L-norleucine has been identified as an effective antifibrinolytic agent, with multiple studies demonstrating its capacity to inhibit fibrinolysis and enhance clot stability. These properties underscore the potential of L-norleucine as a significant compound in both biochemical research and therapeutic applications, particularly in the modulation of hemostatic processes [Elisabetta Clementi and Misiti 2005; Mahdavi et al., 2014].

One additional UAA, L- α -aminobutyrate, is a pivotal compound targeted for synthesis in this thesis. It functions as a key chiral intermediate in the biosynthesis of various pharmaceutically relevant molecules. In particular, S-2-aminobutyramide, which can be synthesized from L- α -aminobutyrate, serves as a direct precursor for the antiepileptic drugs levetiracetam (commercially known as Keppra®) and brivaracetam. Moreover, L- α -aminobutyrate holds potential for conversion into S-2-aminobutanol, which is utilized in the production of the antituberculosis agent ethambutol [Zhang et al., 2010]. This highlights the significant role of L- α -aminobutyrate in the synthesis of compounds with notable therapeutic applications.

A comprehensive review of the literature highlights studies focused on improving the substrate and coenzyme utilization by L-AlaDHs. **Table 1.2** summarizes the mutations introduced in these studies, specifying the regions where modifications were made and detailing the resulting changes in enzyme functionality, including alterations in substrate specificity and coenzyme preference.

Table 1.2: Effect of mutations in L-AlaDH enzymes activity and substrate specificity.

Source Organism	Mutation site	Mutation and effect	References
<i>Mycobacterium tuberculosis</i>	His96	His96Ala inactive Enzyme	[Ågren et al., 2008]
	Asp270	Asp270Asn and Asp270Ala inactive enzyme	[Ågren et al., 2008]
<i>Mycobacterium tuberculosis</i>	Phe94	Phe94Ser and Phe94Ala showed activity toward a range of larger amino acids including L-norleucine, L-norvaline, at the cost of a large drop in activity against L-alanine.	[Fernandes et al., 2015]
		Phe94Ser, Tyr117Leu displaying more activity towards bigger amino acids than Phe94Ser single mutant.	
	Leu130	Insoluble protein	

Table 1.2: Continuine

<i>Bacillus subtilis</i>	Asp196Ala and Leu197Arg	Asp196Ala and Leu197Arg cofactor specificity, which is strictly dependent on NADH as redox cofactor, from NADH to NADPH via protein engineering by introducing two amino acid exchanges to amino acids positioned at 196 and 197.	[Lerchner et al., 2016]
<i>Bacillus pseudofirmus</i>	Lys75, Arg15, His95, Asp269	Arg15Ala, Lys75Ala, His96Ala and Asp269Ala Resulted in a complete loss in enzymatic activity, which signified that these predicted active sites are indispensable for OF4Ald activity	[He et al., 2018]
	Lys73	Lys73Ala 6-fold improvement in k_{cat}/K_M in forward reaction in the assimilation of L-alanine as compared to the wild type.	

The data in **Table 1.2** offers valuable insights into how protein engineering techniques can enhance the use of different substrates and co-enzymes for L-AlaDH enzymes. The mutants derived from L-AlaDHs are designed to be used in the synthesis of UAAs for applications in science and pharmacology, particularly with the incorporation of larger substrates. This approach aims to expand the range of UAAs that can be synthesized, potentially enhancing their applicability in drug development, enzyme engineering, and other biomedical fields.

1.6. Protein Engineering

The application of naturally occurring biocatalysts is often hindered by their restricted stability and activity under extreme industrial conditions, as well as their insufficient selectivity toward substrates or coenzymes. To address these challenges, protein engineering strategies are employed to enhance enzyme performance in non-native environments.

Two major categories of protein engineering are rational design and directed evolution. Rational design is dependent upon the understanding of the enzyme function/structure link and the utilization of structural knowledge. It entails directed alteration of specific amino acid residues through the site-directed mutagenesis technique. Unlike other methods, directed evolution does not rely on prior knowledge of the enzyme's structure. Instead, it harnesses the power of natural variation and selection to accomplish specific desirable qualities of the enzyme. This method usually includes the use of random mutagenesis and/or genetic recombination and then screening of mutant libraries with different directed evolution techniques in a high-throughput manner. [Chen, 2001; Williams et al., 2004; Rubin-Pitel et al., 2007].

Both rational design and directed evolution are effective approaches for enhancing enzymes in industrial settings. These methods can improve enzyme stability, enhance catalytic efficiency, fine-tune substrate specificity, and optimize coenzyme selectivity. However, each technique comes with its own set of challenges. Rational design is often constrained by the complex relationship between an enzyme's structure and its function, whereas directed evolution can face problems such as producing nonfunctional enzymes or relying on inefficient screening processes [Reetz and Carballeira 2007, Parra et al., 2013].

Although each method has its drawbacks, both have proven effective in enhancing enzyme characteristics and addressing the challenges associated with their industrial use. These approaches are crucial for advancing our understanding of enzyme mechanisms and catalytic processes, as well as for incorporating enzymes into a wide range of applications [Chen, 2001; Williams et al., 2004; Rubin-Pitel et al., 2007].

Site saturation mutagenesis and the Iterative Saturation Mutagenesis (ISM)/Combinatorial Active-site Saturation Test (CAST) methods are among the most used

techniques for introducing mutations in various genetic studies. [Reetz and Carballeira, 2007].

1.6.1. Site Saturation Mutagenesis

Site saturation mutagenesis technology is a method that allows creating a mutant library containing all possible predetermined amino acid changes at one or more target positions in a gene sequence.

The method entails genetic manipulation at the DNA level through the utilization of traditional DNA amplification techniques employing degenerate primer sets. These primer sets usually include 32 or 64 different codon variations for each amino acid residue. This approach enables the generation of a comprehensive library that includes every conceivable amino acid sequence at specific places [Li et al., 2018].

1.6.2. The Combinatorial Active Site Saturation Test Based Site Saturation Mutagenesis

The CAST based ISM method of the semi-rational design technique developed in recent years focuses on functional amino acid positions instead of one or all amino acid positions that make up the enzyme [Reetz and Carballeira, 2007]. Since directed mutation concentrates on fewer amino acids compared to other techniques; the mutant library obtained with semi-rational design is smaller and the probability of obtaining functional mutation(s) is quite high [Reetz and Carballeira, 2007].

Semi-rational design integrates rational design, which combines structural information of the enzyme with directed mutation to generate a mutant library. As a result, it is a successful blend of both basic methods, leading to less work and higher quality mutants. The selection of a candidate site or site for mutation is based on the hypothesis that it plays an important role in optimizing the desired properties of the enzyme. This process is central to semi-rational design, a strategy that bridges the gap between random mutagenesis and rational design. Identifying the appropriate candidate site or region for mutation requires a comprehensive understanding of the structure, function and dynamics of the enzyme, which is achieved through an integrated approach combining both experimental and computational methodologies. Techniques such as molecular docking, molecular dynamics simulations, and sequence alignment

analyses, combined with experimental methods like site-directed mutagenesis and functional assays, provide valuable insights into enzyme behavior. These insights enable researchers to pinpoint residues or domains that can be optimized for specific attributes high [Reetz and Carballeira, 2007].

Recent advances in semirational design have led to significant advances in engineering enzymes with improved properties specifically designed for industrial applications. By systematically targeting key sites, researchers have developed enzymes with improved thermal stability, increased catalytic efficiency, and broader substrate specificity. These optimized enzymes have proven invaluable in a variety of industrial processes, including biofuel production, drug synthesis, and the food and beverage industry, where robust and efficient biocatalysts are essential for sustainability and cost-effectiveness. The success of these efforts highlights the power of semirational design in creating enzymes that can meet the increasing demands of modern biotechnology [Reetz and Carballeira, 2007; Parra et al., 2013; Aslan et al., 2016; Qu et al., 2020;].

2. PURPOSE OF THE THESIS

Natural amino acids are used in different industrial fields. Their applications are particularly widespread in asymmetric synthesis in organic chemistry, where they serve as optically active, cost-effective and pure building blocks. In pharmaceutical chemistry, one of their most widespread uses, a strong emphasis is placed on the asymmetric synthesis of amino acid derivatives due to their fundamental biological role. Notably, non-proteinogenic/ UAAs are valuable precursors for producing important pharmaceutical chemicals and industrial molecules with pharmaceutical effects. The primary objective of this thesis is to characterize novel L-AlaDH enzymes and utilize mutant L-AlaDHs with improved reductive amination activity.

The purpose of this thesis is to develop an efficient semi-rational protein engineering approach aimed at producing more effective unnatural amino acids, particularly L-alanine derivatives such as L-norvaline, L- α -aminobutyrate, and L-norleucine. These amino acids will be synthesized asymmetrically as a single step to enhance enzyme activity and substrate specificity against ketoacids. Mutation studies will primarily focus on the Tyr92 and Tyr115 positions to optimize the catalytic efficiency and broaden the utility of the engineered enzymes in the synthesis of these compounds.

Structural and mechanistic insights will be gained by predicting the three-dimensional structures of the most catalytically efficient L-AlaDH enzymes using advanced computational techniques such as homology modeling. These methods will provide a detailed understanding of the enzyme, allowing the characterization of key structural features that contribute to its catalytic efficiency. In addition, this approach will facilitate the analysis of the structural consequences of specific mutations and reveal how changes in amino acid sequence can affect enzyme function and substrate affinity. This thesis will provide a comprehensive view of the factors that affect enzyme performance, which can be used to guide the design of more efficient mutants with improved properties for biotechnological applications.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment

This thesis has utilized a variety of instruments suitable for various analytical approaches. Each instrument has been selected to meet the specific needs of the studies and ensures accurate and reliable findings. Below is a complete list of each instrument and tool used along with their models and uses. **Table 3.1** lists the instruments used in the thesis.

Table 3.1: Equipments used in the thesis.

Equipment	Brand	Model
PCR	Techne	Prime
Refrigerated centrifuge	Hettich	Rotina 380 R
Microcentrifuge	Sigma	1-16 K
Microplate reader	BioTek	ELx808
pH meter	Hanna	H1221
Heating block	Stuart	CB300
Water bath	Stuart	SWB15D
Spectrophotometer	Shimadzu	UV-Pharma Spec 1700
Shaking incubator	BIOTEK	NB-205L
Incubator	Nüve	55L
Electrophoresis	Biorad Powerpac	Mini-PROTEAN
Precision Scale	Shimadzu	Atx224
Sonicator	BANDELIN	HD 2070
Biosafety Class II Cabin	N-Biotek	NB-604WS90
Horizontal mini gel system	BioRad	GT
Pipettes	IsoLab	Capp
Heated block	Denville Scientific	SBH130D/3
Ice maker	Brema	Ice maker
Sonicator	Branson	Digital Sonifier
Vortex	Heidolph	Reaxtop
Magnetic Stirrer	WiseStir	MSH-20A
Spectrophotometer	Shimadzu	UV 1800
Microwave Oven	Arçelik	MD20MB
Plate Reader	BioTek	Epoch2
Ultra-Pure Water Device	Milli-Q	Direct-Q® 5 UV
Pipettes	IsoLab	Capp
+4 °C and -20 °C Fridge	Vestel	20872323
-80 °C refrigerator	Arctico	ULTF

3.1.2. Enzymes Used in This Thesis

A comprehensive list of all the tools and equipment used in the thesis along with their respective models and purposes is provided. The equipment used in the thesis is listed in **Table 3.2**.

Table 3.2: Enzymes used in the thesis.

Enzyme	Supplier
<i>DpnI</i>	BioLabs
Phusion HF DNA Polymerase	BioLabs
Lysozyme	Sigma

3.1.3. Chemicals and Consumables

A detailed list of all chemicals and kits used in the experiments conducted within the scope of this thesis and their respective brand information is as follows. The relevant chemicals and kits were selected based on their applicability and reliability for the specific techniques used in the thesis and are shown in **Table 3.3**.

Table 3.3: The list of chemicals and consumables required for this thesis.

Equipment	Brand	Model
Acetic acid	Merc	45754
Agarose	Sigma-Aldrich	A9539
Kanamycin	Sigma-Aldrich	BP861
Auto Induction Medium	Formedium™	AIMLB0105
BCA Protein Assay Kit	Sigma-Aldrich	71285-M
EDTA	Sigma-Aldrich	EDS
Etanol	Sigma-Aldrich	493.511
Glycerol	Sigma-Aldrich	G5516
Glycine	Sigma-Aldrich	G8898
Hydrochloric Acid	Sigma-Aldrich	H1758
Hydrogen Peroxide	Sigma-Aldrich	216763
IPTG	Sigma-Aldrich	I6758
LB Agar	Lab M	NCM0236A

Table 3.3: continue

LB-Broth	Lab M	NCM0173A
Methanol	Sigma-Aldrich	34.860
Peptone	Lab M	NCM0246A
Phusion™ Site-Directed Mutagenesis Kit	Thermo Scientific	F541
Plasmid Purification Kit	Macherey-Nagel	740499.50
Potassium Chloride	Sigma-Aldrich	P9333
Potassium sodium tartrate	Merc	S2377
Sodium Acetate	Sigma-Aldrich	S2889
Sodium Chloride	Sigma-Aldrich	S7653
Sodium Hydroxide	Sigma-Aldrich	S8045
Sodium Phosphate	Sigma-Aldrich	S7907
Starch	Sigma-Aldrich	S2004
Tris	Roche	TRIS-RO
Yeast extract	Lab M	NCM0218A
Sodium Pyruvate	Sigma-Aldrich	P2250
α -Ketobutyric acid	Sigma-Aldrich	K401
α -Ketocaproic acid sodium salt,	Sigma-Aldrich	K6625
α -Ketovaleric acid	Sigma-Aldrich	75950

3.1.4. Purification Columns

A detailed list of the columns used during the isolation and purification processes in this thesis and their brand information are given below. The columns used in the thesis are listed in **Table 3.4**.

Table 3.4: Columns used for purifications.

Column	Supplier
Ni-NTA	GE Healthcare
PD10	Cytiva

3.1.5. Liquid and Solid Media

The list of all media prepared during the experiments, including their contents and detailed preparation information, is as follows. Each medium was prepared to meet the

specific requirements of the experimental protocols and ensure optimal conditions for the reactions and analyses conducted.

Luria-Bertani (LB) Medium: Dissolve 5 g yeast extract, 10 g peptone, 10 g NaCl in 1000 mL of autoclaved water (dH₂O). Sterilize the solution at 121°C for 15 min. under 1 atm pressure.

Luria-Bertani Agar (LB-Agar) Medium: Dissolve LB medium adds 15 g of the agar in 1000 mL of autoclaved water. Sterilize the solution at 121°C for 15 min. under 1 atm pressure.

Autoinduction Medium: Dissolve 34.85 g powdered medium (Foremedium, LB Broth Base w/o trace elements; 5 g yeast extract, 10 g tryptone, 5 g NaCl, 7.4 g Na₂HPO₄, 6.8 g KH₂PO₄, 0.5 g glucose, 0.15 g MgSO₄, 3.3 g (NH₄)₂SO₄, and 2 g lactose) in 1000 mL of autoclaved water. Sterilize the solution at 121°C for 15 min. under 1 atm pressure.

3.1.6. Solutions and Buffers

Lysis Buffer: Dissolve 2.76 g NaH₂PO₄·H₂O, 5.84 g NaCl, and 0.48 g imidazole in approximately 170 mL autoclaved water. Adjust the pH to 7.4 with NaOH and then make up the final volume to 200 mL with autoclaved water.

Solution A: Dissolve 2.76 g NaH₂PO₄·H₂O, 29.22 g NaCl, and 2.04 g imidazole in approximately 800 mL autoclaved water. Adjust the pH to 7.4 with NaOH and then make up the final volume to 1000 mL with autoclaved water.

Solution B: Dissolve 2.76 g NaH₂PO₄·H₂O, 29.22 g NaCl, and 34.04 g imidazole in approximately 800 mL autoclaved water. Adjust the pH to 7.4 with NaOH and then make up the final volume to 1000 mL with autoclaved water.

0.05 M Sodium Citrate Buffer: 0.05 M sodium citrate was calculated and dissolved in 100 mL of dH₂O. Buffers were prepared and adjusted to pH ranges between 4 and 6.5, and these buffers were used for activity and pH measurements.

0.05 M Tris Buffer: 0.05 M Tris-HCl and 0.05 M trizma-base were calculated and dissolved in 100 mL of dH₂O. Buffers were prepared and adjusted to pH ranges between 7 and 9, and these buffers were used for activity and pH measurements.

0.05 M Glycine-NaOH/Carbonate Buffer: 0.05 M glycine, 0.05 M carbonate, and 0.05 M NaOH were prepared by dissolving the calculated amounts in autoclaved water. Buffers were prepared and adjusted to pH ranges between 9 and 11, and these buffers were used for pH measurements.

Bicarbonate Buffer: 0.05 M bicarbonate and 0.05 M NaOH were prepared by dissolving the calculated amounts in autoclaved water. Buffers were prepared and adjusted to pH ranges for various measurements.

3.1.7. Solutions Used in Preparing Competent Cells

Solution A (0.02 M CaCl₂, 0.08 M MgCl₂): Dissolve 0.176 g CaCl₂ and 0.975 g MgCl₂ in approximately 50 mL autoclaved water. Then make up the final volume to 60 mL with autoclaved water.

Solution B (0.1 M CaCl₂): Dissolve 0.882 g CaCl₂ in 50 mL autoclaved water. The final volume is then completed to 60 mL with autoclaved water.

3.2. Methods

The methods used in the experiments conducted within the scope of the thesis are summarized below. These methods were selected and designed to achieve the research objectives efficiently and accurately and to obtain reliable results throughout the thesis. Each experiment was repeated at least three (3) times in biochemical characterization studies.

3.2.1. Selection and Bioinformatic Analysis of L-AlaDHs

To identify suitable L-AlaDH candidates, well-characterized L-AlaDHs reported in the literature were selected from the Protein Data Bank (PDB) and UniProt databases [Berman et al., 2000; Consortium, 2025]. This selection process ensured the inclusion of enzymes with established functionality and relevance for further analysis and application [Consortium, 2025]. Within the scope of this thesis, *M. tuberculosis* (UniProt Accession Number: P9WQB1), the most extensively studied organism in the L-AlaDH literature, was selected as the reference L-AlaDH for identifying bacterial candidate L-AlaDHs. Similarly, *A. fulgidus* (UniProt Accession Number: O28608)

was selected as the reference L-AlaDH for the identification of candidate L-AlaDHs from Archaea. These reference enzymes served as benchmarks for the selection and analysis of potential L-AlaDH candidates. The primary structures of candidate L-AlaDHs with sequence similarity to the reference L-AlaDHs were identified using the BLAST program [Camacho et al., 2009]. The selection process focused on ensuring that the sequence similarity of the candidate L-AlaDHs with the amino acid sequences of reference organisms ranged between approximately 40 and 65%. This interval was chosen to balance evolutionary differentiation with functional relevance, allowing the identification of novel L-AlaDHs with potential for further investigation. The chosen range both facilitates the selection of candidate L-AlaDH enzymes and contributes to the identification of new L-AlaDH enzymes. Additionally, using L-AlaDHs registered in the PDB; with the SWISS-MODEL program, the structures of candidate L-AlaDHs were tried to be predicted by homology modeling [Berman et al., 2000; Waterhouse et al., 2018]. The fit of the resulting models was evaluated, and candidate L-AlaDHs with expanded and differentiated sequence regions (with low GMQE values) that did not fit the experimentally determined template models were excluded. Additionally, the presence of the catalytic amino acids Arg15, Lys75, and His96 essential for forming the conserved active site of the L-AlaDH family was verified by aligning the primary structures of the candidate L-AlaDH enzymes. This process ensured the selection of candidates with both structural compatibility and functional conservation. The solubility of the candidate L-AlaDH protein sequences was evaluated using the SoluProt program [Hon et al., 2021]. Candidates exhibiting solubility predictions below the established threshold value of 0.5 were excluded, as they were deemed incompatible for expression within the *E. coli* system. The genes corresponding to the selected L-AlaDH candidates were optimized for *E. coli* codon usage and subsequently synthesized and ligated into the pET-28a(+) vector through commercial services. The detailed structure of the vector is provided in the **APPENDIX-A** for reference. The nucleotide sequences of the selected genes are provided in **APPENDIX-B**.

3.2.2. Expression and Purification of L-AlaDHs in *E. coli* BL21 (DE3) Cells

E. coli BL21 (DE3) cells will be used as the host to express L-AlaDH enzymes coding genes. The heat-shock transformation protocol was conducted as outlined in the following steps:

- . Thaw a tube of competent *E. coli* cells on ice for 10 minutes.
- . Add 1-5 μ L containing 1 pg-100 ng of plasmid DNA to the cell mixture.
- . Carefully flick the tube 4-5 times to mix cells and DNA.
- . Place the mixture on ice for 30 minutes.
- . Heat shock at exactly 42 °C for exactly 30 seconds. Place on ice for 5 minutes.
- . Pipette 250 μ L of room temperature Super Optimal broth with Catabolite repression (SOC) into the mixture.
- . Place at 37 °C for 60 minutes at (200 rpm). Spread 50-100 μ L of each dilution onto a selection plate and incubate overnight at 37 °C.

After overnight incubation, individual colonies will be picked from LB-agar plates supplemented with 50 μ g/mL kanamycin, inoculated into 5 mL of LB medium containing kanamycin, (50 μ g/mL) and incubated at 37 °C at 200 rpm for overnight. The overnight cultures were added to the large cultures (100 mL) Autoinduction medium than overnight incubated at 30 °C at 200 rpm.

Cell pellets resuspended in Buffer A: Tris-HCl, 500 mM NaCl, 30 mM imidazole pH 7.4, 1.0 mg/mL lysozyme) and incubated on ice for 45 mins. The resuspended cells will be disrupted by sonication, and clarified by centrifugation (10.000 rpm, 60 min). The lysate will be filtered through a 0.45 μ m filter then applied to a HisTrap column (GE) which will be pre-equilibrated with the Buffer A. The column will be washed with 5 mL of Buffer A and the L-AlaDH will be eluted with Buffer A containing different imidazole concentrations (100, 200, 400 and 500 mM of imidazole). Collected fractions will be analysed by SDS-PAGE to identify fractions containing pure protein, which will then be combined and diluted to 50 mL by addition of 100 mM of NaPi or Tris-HCl pH 7.4. Proteins will be concentrated with ultracentrifuge tubes (MM Amicon Ultracel-30). The concentration of the purified L-alanine

dehydrogenase will be determined by Bicinchoninic Acid Assay (BCA) Assay Kit. The expression efficiency will be monitored by SDS-PAGE.

Following the purification process, an SDS-PAGE experiment was conducted to observe the expression of the L-AlaDH protein. The proteins underwent denaturation prior to being placed onto the gel. The pre-prepared loading dye and samples were combined in eppendorfs at a ratio of 1:3 and then placed on a pre-heated heating block set to 95 °C. They were incubated for 15 minutes. Following the incubation period, the Eppendorf tubes were subjected to centrifugation at a speed of 10.000 rpm for a duration of 5 minutes. A 12% separating gel and an 8% stacking gel were prepared for the SDS-PAGE.

3.2.3. Enzyme Activity Assay of L-AlaDHs

Specific activities were determined as described. 5mM Substrate 0.5 M NH₄Cl in the indicated buffers and the appropriate amount of enzyme. The reaction was monitored by spectrophotometric analysis of NADH change at 340 nm within 10 min. An L-AlaDH unit (U) is defined as the amount of enzyme required to produce or consume 1 mM of NADH per min. [Gmelch et al., 2019].

3.2.4. Determination of Optimum pH and Temperature for L-AlaDH Enzymes

The activity of the purified L-AlaDH enzyme was assessed at different pH levels ranging from 4 to 11. For pH levels 4 to 6, a solution of 50 mM sodium citrate was used. For pH levels 7 to 9, a solution of 50 mM Tris-HCl was used. For pH levels 9.5 to 10.5, a mixture of 50 mM sodium carbonate and glycine was used, and for pH 11, sodium bicarbonate was used. The progress of the pyruvate reductive amination reaction was tracked by measuring the decrease in NADH concentration at a wavelength of 340 nm. In order to ascertain the ideal temperature for L-AlaDH activity, the reductive amination reaction of bacterial L-AlaDHs was examined within a temperature range of 25–70 °C, while the same reaction for archaeal L-AlaDHs was investigated within a temperature range of 25–90 °C. The enzyme was examined at its optimum pH.

3.2.5. Kinetic Analysis for L-AlaDH Enzymes

Multiple experiments were performed to ascertain the kinetic characteristics of the L-AlaDH enzymes. These measurements involved the use of pyruvate and its derivatives (α -ketobutyrate, α -ketovalerate, and α -ketocaproate) at varying concentrations. More precisely, the concentrations of pyruvate varied between 0.1 and 10 mM, while the concentrations of derivatives ranged from 0.1 to 50 mM. The tests were conducted using ideal pH and buffer conditions, with a concentration of 0.5 M NH_4Cl . The reaction was commenced by introducing 0.05 mM NADH and the pure L-AlaDH enzyme. The specific activity was determined by measuring the reduction in absorbance at 340 nm over a period of 10 minutes, using the extinction coefficient (ϵ) of $6220 \text{ M}^{-1} \text{ cm}^{-1}$ and by calculating the units per milligram (unit/mg^{-1}) of the enzyme. [Gmelch et al., 2019]. The k_{cat} and K_{M} values of the isolated enzymes were calculated by plotting Michaelis-Menten graphs using the provided activity values and applying GraphPad Prism 9 [GraphPad Software, 2022].

3.2.6. Structural Analysis and Modeling of *Tt*AlaDH

In this thesis *Tt*AlaDH, which exhibited the most favorable catalytic activity towards substrates among the L-AlaDHs obtained from different organisms, was selected for further investigation. It was computationally modeled in the presence of specific substrates and NADH. The goal of this modeling approach was to accurately identify and determine the most suitable amino acid positions for mutations, aiming to enhance the enzyme's catalytic efficiency and substrate specificity. Although the structure of *Tt*AlaDH is known, some amino acid positions (ValProGlyAlaLysAlaProLys between 238-246 and ValAspGlnGlyGlyCysValGluThrIleArgProThrThrHisAlaGluProThr between 266-286) are missing in the structure reported as PDB number: 2eez. The structural similarity between *Tt*AlaDH and the template *Mt*AlaDH is determined to be 57.4%.

Homology modeling was conducted using the *M. tuberculosis* crystal structure (PDB Accession Number: 2VOJ) as a template. The modeling process was performed with SWISS-MODEL to generate reliable structural models of the candidate L-AlaDHs [Waterhouse et al., 2018]. The positions of amino acids at the critical catalytic sites were visualized using PyMOL [Schrödinger and Delano, 2020] providing a detailed

representation of their spatial arrangement and confirming their alignment with the conserved active site of the L-AlaDH family. To incorporate the missing amino acids into the model, the molecular structures of *TtAlaDH* (2eez) and *MtAlaDH* (2voj) were compared. To incorporate the missing amino acids into the model, a comparative analysis was performed between the molecular structures of *TtAlaDH* (PDB Accession: 2eez) and *MtAlaDH* (PDB Accession: 2voj). The alignment of domain constructs ensured structural consistency, revealing that the missing amino acids were located within loop regions. To complete the model, the coordinates of the missing amino acids from the 2voj structure were employed, enabling accurate placement within the candidate model. Additionally, pyruvate and NADH were positioned within the candidate structure based on their spatial arrangement in the 2voj template, ensuring the functional relevance of the modeled active site.

The selected substrates were initially incorporated into the candidate structure using AutoDock Vina [Trott and Olson, 2010] for automated docking, followed by manual adjustments to the positioning of atoms in SwissPDB and PyMOL to refine their placement [Waterhouse et al., 2018; Schrödinger and Delano, 2020]. The candidate structure was then subjected to molecular dynamics simulation using the Nanoscale Molecular Dynamics (NAMD) protocol. In this protocol, all constructs were placed in a 12 Å thick TIP3P water box with a minimum distance of >1.2 nm from the box edges. The system was neutralized by adding Na⁺ ions (0.1 M) and underwent energy minimization at 300 K for 10 picoseconds (10,000 NAMD time steps). NAMD Version 2.15 ALPHA was used for both energy minimization and subsequent simulations [Phillips et al., 2020]. To convert the energy-minimized constructs into protein structure files, Visual Molecular Dynamics (VMD) version 1.9.4 was employed, enabling further analysis and visualization of the refined protein models.

3.2.7. Structural Analysis and Modeling of *TtAlaDH* Mutants

In the structural analysis of the Tyr92Ser mutant obtained from the 2eez models, the selected substrates were initially added automatically using Autodock Vina [Trott and Olson, 2010]. Subsequently, manual visualization was conducted to correct the positioning of atoms using SwissPDB [Waterhouse et al., 2018] and PyMOL [Schrödinger and Delano, 2020]. The NAMD simulation protocol was then applied to the estimated candidate structure. In this protocol, all structures were enclosed within

a 12 Å thick Transferable Intermolecular Potential With 3 Points (TIP3P) water box set to a dimension greater than 1.2 nm. Additionally, Na⁺ ions were added at a concentration of 0.1 M to neutralize negative charges, followed by energy minimization at 300 K for 10 ps (equivalent to 10,000 NAMD time steps). The minimizations and subsequent simulations were performed using NAMD version 2.15 ALPHA [Phillips et al., 2020]. Furthermore, the energy-minimized structures were converted into protein structure files using VMD version 1.9.4 [Humphrey et al., 1996]. Alphahold2 program was used to model the L-AlaDHs that have not been studied before and were selected within the scope of this thesis [Jumper et al., 2021].

3.2.8. Selection of the Candidate Positions for *TtAlaDH* Mutations

Previous studies have shown that the stabilization of pyruvate and NADH within the active site is facilitated by interactions with Tyr92 and Leu128. However, the side chains of these amino acids may induce steric hindrance, potentially obstructing the binding of pyruvate derivatives with larger side chains. This highlights the significant role that the side chain structures of these amino acids play in determining the enzymes ability to accommodate different pyruvate derivatives.

Based on the observations regarding the steric barriers created by the side chains of Tyr92 in accommodating larger pyruvate derivatives, it is hypothesized that mutations such as Ala, Ser, and Thr at the Tyr92 position could be promising. Gly was also considered as a potential candidate, although it may increase flexibility. The side chains of Ser and Thr at this position are predicted to rotate away from the substrate binding pocket, allowing for the formation of hydrogen bonds. Notably, the mutations of Ala, Gly, Ser, and Thr at Tyr92 can be manually modeled in a reactive conformation without causing steric clashes with larger substrates.

A study observed that certain substrates, such as L-norvaline, which is structurally larger than L-alanine, could be more efficiently utilized by L-AlaDH enzyme. This enhanced utilization was achieved through the introduction of the double mutation Tyr117Leu and Phe94Ser within the conserved IleAlaTyrThrGlu region of L-AlaDHs. This finding suggests that targeted mutations at critical amino acid positions can significantly enhance the enzyme's ability to accommodate and process larger

substrates, thereby improving its catalytic efficiency and broadening its functional scope [Fernandes et al., 2015].

Since such changes can significantly affect the three-dimensional conformation, hydrophobicity, and electrostatic characteristics of the binding sites, the consequences of mutations in amino acids close to the binding pocket have been well studied. Research at such sites has demonstrated that mutations at these sites can increase enzyme substrate selectivity, thermostability, and catalytic activity [Ali et al., 2020]. Thus, in this regard, knowing and analyzing particular amino acids can be rather helpful for improving the possible uses and functionality of L-AlaDH enzymes.

This information enhances the comprehensibility of selecting candidate amino acid positions and increases the efficiency of the semi-rational design technique ISM/CAST.

Table 3.5 outlines the amino acids linked to the degenerate codons utilized in the ISM/CAST method.

Table 3.5: Degenerate codons and encoded amino acids.

Degenerate Codon	Number of codons	Amino acids number	Number of stops	Encoded amino acids
NNK	32	20	1	20
NDT	12	12	0	RNDCGHILFSYV
DBK	18	12	0	ARCGILMFSTWV
NRT	8	8	0	RNDCGHSY

This semi-rational design approach was employed in the thesis to systematically assess the impact of individual amino acids on catalytic efficiency at the targeted positions. DBK degenerate codon encompasses a range of amino acids, including Ala, Arg, Cys, Gly, Ile, Leu, Met, Phe, Ser, Thr, Trp, and Val, which are considered appropriate for the targeted positions in the thesis. Consequently, the DBK degenerate codon was identified as the most suitable option for generating a mutation library for this particular position and thesis objective. **Table 3.6** provides details on the anticipated mutations and the library to be constructed.

Table 3.6: Expected mutational diversity of DBK for Tyr92 and Tyr115 positions of *TtAlaDH* and the library likely to occur at these positions.

Positions	Amino acids encoded by the selected degenerative primer	Explanation
Tyr92	Ala/Arg/Cys/Gly/Ile/Leu/Met/Phe/Ser/Thr /Trp/Val	-Removing the aromatic ring of Tyr at position 92 creates more space. It will be of interest for literature to see how it will affect K_M and k_{cat}. -It is predicted that Ser may facilitate the binding of larger substrates through potential interactions, thereby promoting their conversion into products through hydrogen bonding. -Gly flexibility is gained to the structure; more space/volume will be achieved. It will be interesting to see the effect of increasing the flexibility of the structure on both activity and protein. -By introducing Thr, additional space and volume can be provided for the substrate, allowing for better accommodation. The H-bonds formed by Thr can help regulate the movement and positioning of the substrate at the specific position.

Table 3.6: continue

Tyr115	Ala/Arg/Cys/Gly/Ile/Leu/Met/Phe/Ser/Thr /Trp/Val	- The conservation of position 115 in L-AlaDHs suggests its functional importance. Removing the aromatic ring at this position is expected to create additional space and volume, potentially accommodating larger keto acids more effectively. This modification aims to enhance the enzyme's ability to accommodate and interact with larger substrates. - The binary mutants generated at both candidate positions are anticipated to exhibit enhanced catalytic activity in converting larger-volume target substrates, specifically keto acid derivatives, into L-alanine derivatives. These mutations are expected to optimize the active site of the enzyme, allowing for improved binding and conversion of larger substrates.
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3.2.9. Designing Degenerative Primers

Degenerate primers targeting the Tyr92DBK and Tyr115DBK positions of *TtAlaDH* were designed using the Sigma OligoEvaluator program [OligoEvaluator, 2023]. These primers were commercially synthesized to create a mutant library containing amino acids encoded by the DBK codon. The design of the primers was based on the reference enzyme and gene sequences of *TtAlaDH*, as depicted in **Figures 3.1** and **3.2**.

10	20	30	40	50	60
MVIGVPKEIK	TLENRVALTP	GGVESLVRRG	HTVLVERGAG	EGSGLSDAEY	ARAGAEVLGR
70	80	90	100	110	120
EEAWGAEMVV	KVKEPLPEEY	GFLREGLILF	TYLHLAADRG	LTEAMLRSGV	TGIA ^Y ETVQL
130	140	150	160	170	180
PDGTLPLLVP	MSEVAGRMAP	QVGAQFLEKP	KGGRGVLLGG	VPGVAPASVV	ILGGGTVGTN
190	200	210	220	230	240
AAKIALGMGA	QVTILDVNHK	RLQYLDDVFG	GRVITLTATE	ANIKKSVQHA	DLIGAVLVP
250	260	270	280	290	300
GAKAPKLVTR	DMLSLMKEGA	VIVDVAVDQG	GCVETIRPTT	HAEPTYVVDG	VVHYGVANMP
310	320	330	340	350	360
GAVPRTSTFA	LTNQTLPYVL	KLAEKGLDAL	LEDAALLKGL	NTHKGRLTHP	GVAEAFGLPY
TPPEEALRG					

Figure 3.1: The protein sequence of *TtAlaDH* (Targeted Tyr92 and 115 are marked in yellow).

10	20	30	40	50	60
ATGGTTATTG	GTGTGCCGAA	AGAAATTAAAG	ACCCTGGAAA	ATCGTGTTGC	CCTGACCCCG
70	80	90	100	110	120
GGCGGCGTTG	AAAGTCTGGT	GCGCCGTGGC	CATACCGTGC	TGGTTGAACG	CGGCGCAGGT
130	140	150	160	170	180
GAAGGTAGCG	GTCTGAGCGA	TGCAGAAATAT	GCACGTGCAG	GCGCAGAACT	GGTGGGTCTG
190	200	210	220	230	240
GAAGAAGCAT	GGGGCGCAGA	AATGGTGGTT	AAAGTTAAAG	AACCGCTGCC	GGAAGAATAT
250	260	270	280	290	300
GGCTTTCTGC	GTGAAGGCCT	GATTCTGTTT	ACCTATCTGC	ATCTGGCCGC	CGATCGCGGT
310	320	330	340	350	360
CTGACCGAAG	CCATGCTGCG	TAGCGGTGTG	ACCGGTATTG	CCATGAAAC	CGTGCAGCTG
370	380	390	400	410	420
CCGGATGGTA	CCCTGCCGCT	GCTGGTCCG	ATGAGCGAAG	TTGCCGGTGC	TATGGCCCCG
430	440	450	460	470	480
CAGGTGGGCG	CACAGTTTCT	GGAAAAACCG	AAAGGCGGCC	GCGGTGTGCT	GCTGGGTGGC
490	500	510	520	530	540
GTGCCTGGTG	TGGCACCGGC	TAGCGTGGTT	ATTCTGGGCG	GCGGTACCGT	TGGTACCAAT
550	560	570	580	590	600
GCCGCAAAAA	TTGCACTGGG	TATGGGTGCC	CAGGTGACCA	TTCTGGATGT	TAATCATAAA
610	620	630	640	650	660
CGCCTGCAGT	ATCTGGATGA	TGTTTTTGGT	GGCCGCGTGA	TTACCCTGAC	CGCCACCGAA
670	680	690	700	710	720
GCCAATATTA	AGAAAAGCGT	GCAGCATGCC	GATCTGCTGA	TTGGCGCAGT	GCTGGTGCCG
730	740	750	760	770	780
GGTGCAAAAG	CACCGAAACT	GGTTACCCGT	GATATGCTGA	GCCTGATGAA	AGAAGGCGCC
790	800	810	820	830	840
GTGATTGTTG	ATGTTGCCGT	TGATCAGGGT	GGTTGCGTTG	AAACCATTGC	CCCGACCACC
850	860	870	880	890	900
CATGCAGAAC	CGACCTATGT	GGTTGATGGC	GTTGTTTATT	ATGGTGTTGC	AAATATGCCG
910	920	930	940	950	960
GGTGCAAGTT	CGCGTACCAG	CACCTTTGCA	CTGACCAATC	AGACCCTGCC	GTATGTTCTG
970	980	990	1000	1010	1020
AAACTGGCAG	AAAAAGGCCT	GGATGCACTG	CTGGAAGATG	CCGCACTGCT	GAAAGGTCTG
1030	1040	1050	1060	1070	1080
AATACCCATA	AAGGTCGTCT	GACCCATCCG	GGTGTGGCCG	AAGCCTTTGG	TCTGCCGTAT
1090	1100				
ACCCCGCCCG	AAGAAGCCCT	GCGCGGC			

Figure 3.2: The nucleotide sequence encoding *TtAlaDH* (Targeted Tyr92 and 115 the nucleotide codons (TAT) encoding are indicated in red).

The primer designed for Tyr92 is as follows.

Forward Primer: 5'CTG ATT CTG TTT ACC **DBK** CTG CAT CTG GCC'3

Reverse Primer: 5'GGC CAG ATG CAG **MVH** GGT AAA CAG AAT CAG'3

The analysis results obtained using the Sigma OligoEvaluator for this primer set are given in **Figure 3.3** [OligoEvaluator, 2023].

1. Analysis Results												
Sequence: 5' CTGATTCTGTTTACCCDBKCTGCATCTGGCC 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 = 3, U = 0, G = 5, C = 9, T = 10, I = 0, B = 1, D = 1, K = 1, Total = 30	9108.2	261.8	No Mod	34.8	30	73.5	46.7	4	3	Very Weak	Yes	Sequence

Figure 3.3: The analysis results of the primers designed for Tyr92.

The primer designed for Tyr115 is as follows.

Forward Primer: 5'GGT ATT GCC **DBK** GAA ACC GTG CAG CTG'3

Reverse Primer: 5'CAG CTG CAC GGT TTC **MVH** GGC AAT ACC CAG'3

The analysis results obtained using the Sigma OligoEvaluator for this primer set are given in **Figure 3.4** [OligoEvaluator, 2023].

1. Analysis Results												
Sequence: 5' GGTATTGCCDBKGAAACCGTGACGCTG 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 = 5, U = 0, G = 8, C = 6, T = 5, I = 0, B = 1, D = 1, K = 1, Total = 27	8333.6	253.8	No Mod	32.8	27	77.2	51.9	2	3	Weak	No	Sequence

Figure 3.4: The analysis results of the primers designed for Tyr115.

The Polymerase Chain Reaction (PCR) was set up as follows: The plasmid containing the *TtalD* gene was first transferred to competent *E.coli* DH5α (DE3) cells and amplified. Plasmid purification was then carried out using the MN_NucleoSpin® kit. For the DBK mutation at the Tyr92 and Tyr115 positions, the specific reagents listed

in **Table 3.3** were added to the PCR tube, and the PCR reaction was conducted at a temperature of 65 °C, as indicated in **Table 3.4**.

Table 3.7: Reaction content and quantities used for the Tyr92DBK and Tyr115DBK mutations.

Components	μL
Buffer 5X HiFi FID	10
Template plasmid DNA	2
F primer	2.5
R primer	2.5
dNTPs 10mM	1.5
Phusion™ Hi-Fi DNA polymerase	1
H2O	30.5
Total Volume	50

For the DBK mutation of the Tyr115 position, the reagents taken in the amounts shown in **Table 3.7** were added to the PCR tube and the PCR established at 68 °C (**Table 3.8**).

Table 3.8: Program detail for PCR.

Teperature (°C)	Time	Repeat
95	2 min	
98	30 sec	X 25
65 /68	20 sec	
72	30 sec/Kb	
72	5 min	
4	∞	

3.2.10. Screening the Mutant Library

For screening the mutant library, LB (Luria-Bertani) liquid medium supplemented with 50 μg/mL kanamycin was dispensed into three (3) 96-well plates, with 400 μL in each well. The wild-type colonies were transferred to the "A" row of the 96-well plates, while colonies obtained from the mutant library were transferred to the remaining rows. Subsequently, these deep-well plates were subjected to incubation at 30 °C with

agitation at 220 rpm for a duration of 18 hours. The designed master plate is illustrated in **Figure 3.5**.

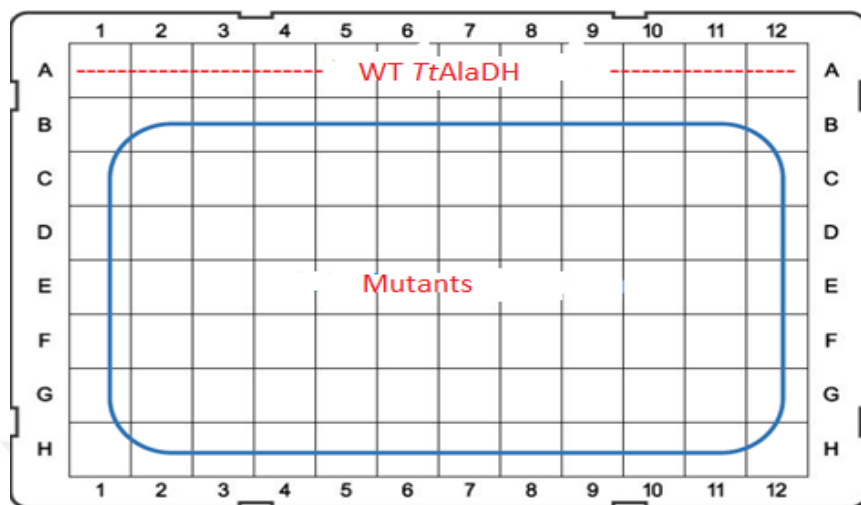


Figure 3.5: Layout of wild-type and *TtAlaDH* variants in a deep-well plate for screening.

At the end of the incubation period, 10 μ L samples were extracted from these wells and transferred to another 96 deep-well plate containing 400 μ L of autoinduction medium, supplemented with 50 μ g/mL kanamycin. This process was repeated to generate duplicate plates of the master plates. The duplicate plates were then subjected to incubation at 30 $^{\circ}$ C for 18 hours to facilitate protein expression.

Glycerol stocks were prepared by adding 390 μ L of glycerol to the wells of deep-well master plates containing LB medium. These glycerol-supplemented master plates were subsequently stored at -80 $^{\circ}$ C for future use.

The cell culture in autoinduction medium obtained after incubation in replicate plate was centrifuged at 4,000 rpm for 20 minutes. After discarding the supernatant, 300 μ L of 1 mg/mL lysozyme, 50 mM pH 8.5 Tris-HCl buffer was added to the resulting precipitate. The resulting samples were allowed to shake at +4 $^{\circ}$ C 900 rpm for 30 min. They were then centrifuged at 4,000 rpm for 45 min. Using the supernatant as an enzyme source, 50 μ L of it was added to the reaction mixture containing 80 μ L sodium Tris-HCl (50 mM, pH 8.5), 10 μ L NADH (0.5 mM) and 10 μ L ketoacid substrate (5 mM) and the mutants were screened for enzyme activity by monitoring NADH depletion at 340 nm in a microplate spectrophotometer for 10 min. A similar reaction was set up for the wild-type enzyme in the microplate.

Throughout the experiment, no detectable activity was observed against the keto acids specifically chosen for the wild type. This suggests that the supernatant obtained from the microplate lacked sufficient enzyme concentrations when used as the enzyme source. To mitigate the challenges associated with establishing cell culture in microplates, volume expansion and efficient cell lysis methods were employed; 100 μ L of the starter culture was transferred to 5 mL of autoinduction medium supplemented with 50 μ g/mL kanamycin and grown at 30 °C 220 rpm for 18 hours. The resulting culture was then centrifuged at 4,500 rpm for 15 minutes. After discarding the supernatant, the cell pellet was resuspended in 3 mL of a solution containing 50 mM Tris-HCl pH 8.5 1 mg/mL lysozyme and allowed to shake at +4 °C 900 rpm for 2 hours. It was then centrifuged at 10,000 rpm for 20 minutes and the obtained supernatant was used as an enzyme source.

The reaction mixture using the supernatant as enzyme source consisted of 50 μ L enzyme solution, 80 μ L sodium Tris-HCl (50 mM, pH 8.5), 10 μ L NADH (0.5 mM) and 10 μ L ketoacid substrate (5 mM). Reactions were monitored for 20 min. Libraries were screened by observing the decrease in absorbance of NADH at 340 nm in the spectrophotometric plate reader in which this reaction was set up.

Protein analysis was carried out on samples collected from each stage of the process using 12% SDS-PAGE to assess protein expression, purity, and molecular weight. In the final stage, purified samples were concentrated using an Amicon Ultra-15 30K filter in a Tris-HCl buffer solution. Subsequently, imidazole and salts were removed by passing through a PD-10 (GE Healthcare) column. Protein quantity was determined using the BCA protein kit (Pierce™ BCA Protein Assay).

3.2.11. DNA Sequence Analysis

20 μ L of the isolated plasmid samples were sent for sequence analysis. For sequence analysis, the T7 promoter primer with 5' TAATACGACTCACTATAGGG 3' nucleotide sequence contained in the selected plasmid was used. Sequence results were visualized using the BioRender program and compared with wild-type *TtAlaDH* using the Clustal Omega program [Sievers et al., 2011] . Since the significant sequence lengths obtained were of different sizes, the results were edited in MS Word.

4. RESULTS

4.1. Bioinformatic Analysis and Selection of L-AlaDH Encoding Genes

In the preliminary preparation phase of this thesis, well-studied bacterial L-AlaDHs with known properties were selected from the PDB and UniProt databases [Berman et al., 2002; Consortium, 2025]. Within the scope of the thesis, *M. tuberculosis* (UniProt Accession Number: P9WQB1), which is the most extensively studied organism in the L-AlaDH literature, and the archaeal L-AlaDH from *A. fulgidus* (Af) (UniProt Accession Number: O28608) were selected as the starting organisms. These sequences were subsequently used for unreviewed enzyme mining via the Basic Local Alignment Search Tool (BLAST) [Altschul et al., 1990].

Putative *Melghiribacillus thermohalophilus* (MetAlaDH) UniProt Accession Number: (A0A4R3N762), *Ammoniphilus* sp. CFH 90114(AcfhAlaDH) UniProt Accession Number (A0A4Q1SWL0), *Vagococcus lutrae* (Vl) (UniProt accession number: A0A429ZGT5) and *Candidatus Bathyarchaeota archaeon* (Cb) UniProt Accession Number (A0A7J3W6F5) L-AlaDHs amino acid sequences were selected.

The selected sequences were confirmed to be L-AlaDH by homology modeling (SWISS-MODEL, [Schwede et al., 2003]). The multiple sequence alignment of selected L-AlaDHs can be seen in **Figure 4.1**.

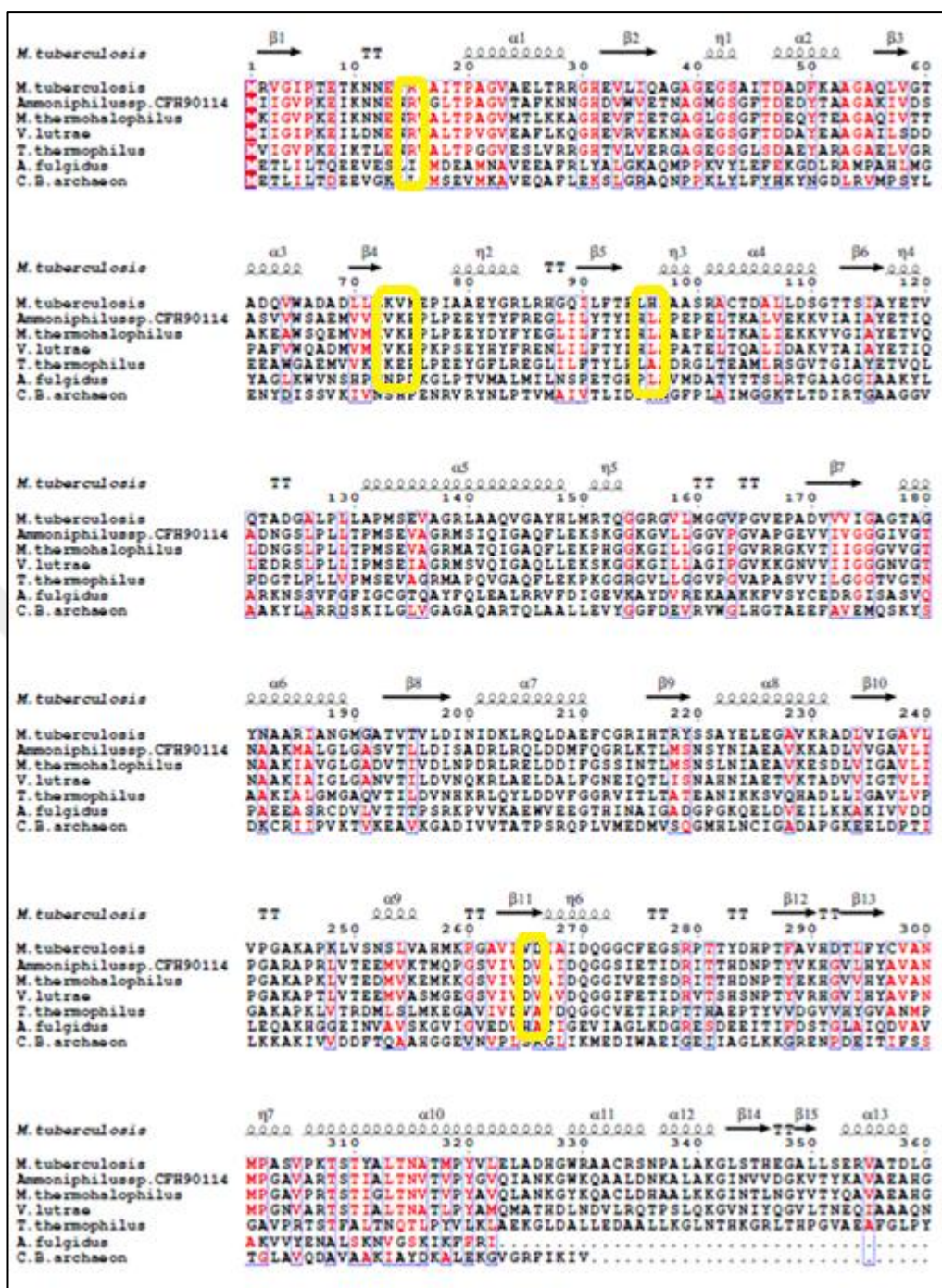


Figure 4.1: Alignment of primary structures of selected L-AlaDHs in this thesis with respect to *M. tuberculosis* L-AlaDH (Conserved amino acid positions shown in yellow).

The α -helical structures are visually represented as helices, while the β -sheet structures are depicted as arrows. Amino acid positions are numbered with reference to L-AlaDH obtained from *M. tuberculosis* organism, which is extensively studied in scientific literature. The predicted secondary structure of L-AlaDHs is presented above the alignment.

For bacterial L-AlaDHs, the conserved amino acid positions Arg15, Lys75, His96, and Asp270, compared to *MtAlaDH*, are highlighted in yellow, indicating their conservation across bacterial L-AlaDH candidates. However, the corresponding positions in *AfAlaDH* and *CbAlaDH*, which belong to archaea, display different amino acids, reflecting variations in these positions between bacterial and archaeal enzymes. In addition, to avoid any solubility issues, the solubility of the selected sequences in *E. coli* was assessed using the SoluProt program [Hon et al., 2021]. The relevant values and Similarity scores for the selected putative *MetAlaDH*, *VIAlaDH* and *AcfhAlaDH* are provided in **Table 4.1**, while the data for *CbAlaDH* can be found in **Table 4.2** [Hon et al., 2021; Sievers et al., 2011].

Table 4.1: Similarity score, Solubility score, molecular weight and Uniprot Accession Number of *M.tuberculosis* to *VIAlaDH*, *MetAlaDH* and *AcfhAlaDH*.

L-AlaDH	% similarity <i>M.tuberculosis</i>	Solubility score	Molecular weight (Da)	Uniprot Accession number
<i>MetAlaDH</i>	51.62	0.716	39564.56	A0A4R3N762
<i>AcfhAlaDH</i>	51.35	0.682	39688.76	A0A4Q1SWL0
<i>VIAlaDH</i>	48.11	0.726	39730.65	A0A429ZGT5

As shown in **Table 4.1**, candidate L-AlaDH amino acid sequences exhibiting approximately 40-65% sequence similarity with *M. tuberculosis* were selected to obtain bacterial-origin L-AlaDHs with different biochemical properties.

Table 4.2: Similarity score, Solubility score, molecular weight and Uniprot Accession Number *A. Fulgidus* and *CbAlaDH*.

L-AlaDH	% similarity <i>A. fulgidus</i>	Solubility score	Molecular weight (Da)	Uniprot Accession number
<i>CbAlaDH</i>	56.44	0.614	35914.64	A0A7J3W6F5

In order to obtain arkea originated L-AlaDH as can be seen from **Table 4.2** *CbAlaDH* was chosen. *CbAlaDH* has 56% sequence similarity with *A. fulgidus*, which is the only known archaeal L-AlaDH.

The 3D structure models of the *MetAlaDH*, *AcfhAlaDH*, *VIAlaDH* and *CbAlaDH* obtained by using amino acid sequences with the Alphafold2 program, are shown in **Figure 4.2- 5** [Jumper et al., 2021].

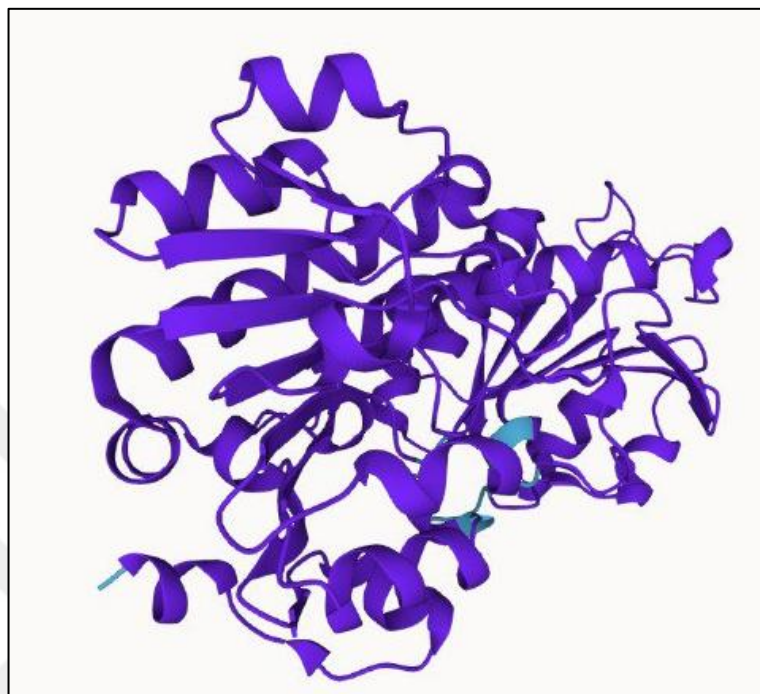


Figure 4.2: The representation of 3D model of predicted structure of *MetAlaDH* by Alphafold2.

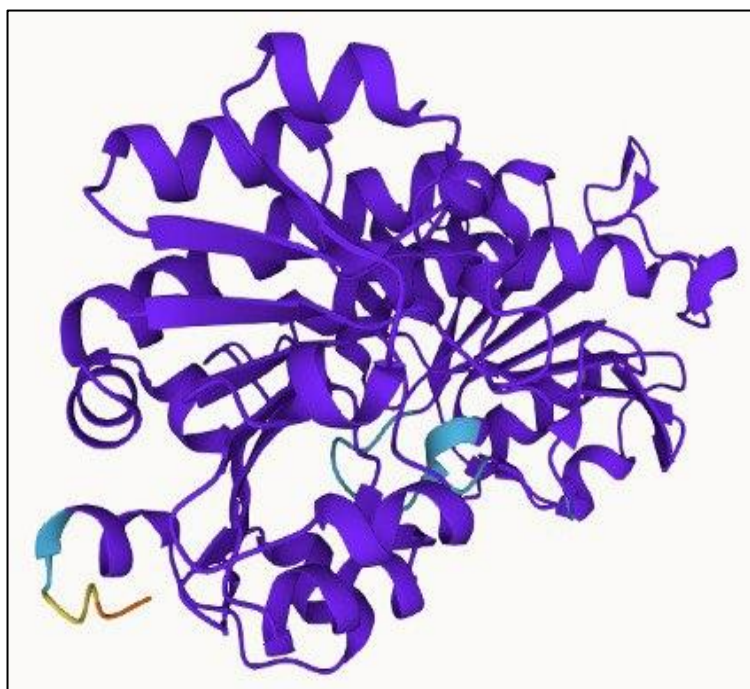


Figure 4.3: The representation of 3D model of predicted structure of *AcfhAlaDH* by Alphafold2.

As can be seen in **Figure 4.3**, the purple-colored regions are expected to be modeled with high accuracy, the blue regions with good accuracy, and the orange-yellow regions with low confidence. The presence of an orange-yellow region indicates that the modeling in this area may be inaccurate.

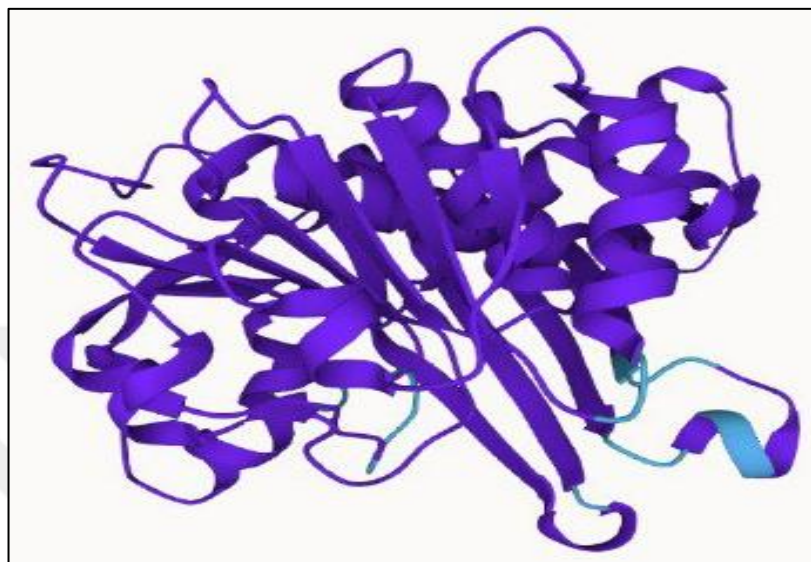


Figure 4.4: The representation of 3D model of predicted structure of *CbAlaDH* by AlphaFold2.

As shown in **Figure 4.4**, the predicted 3D model structures of *CbAlaDH* do not contain any yellow-orange regions, indicating that the structure has been largely modeled correctly.

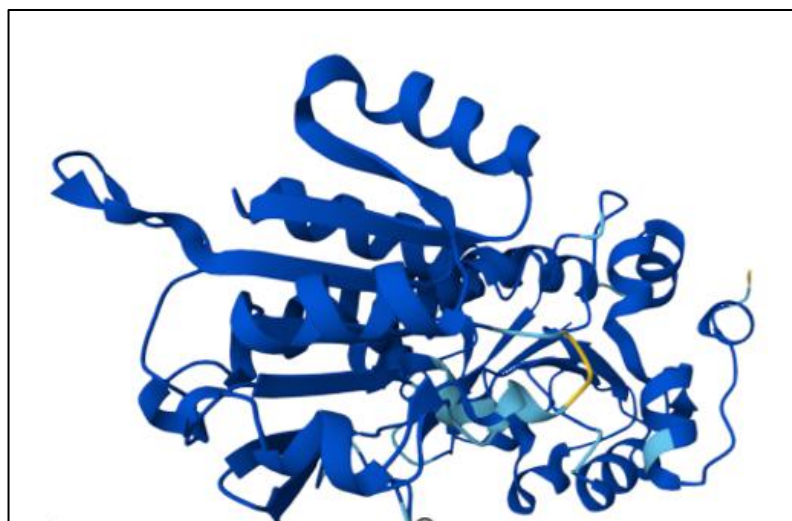


Figure 4.5: The representation of 3D model of predicted structure of *VIAlaDH* by AlphaFold2.

As shown in **Figure 4.5**, the purple-colored regions are expected to be modeled with high accuracy, the blue regions with good accuracy, and the orange-yellow regions with low confidence. The orange-yellow region indicates that the modeling in this area may be inaccurate.

4.2. Expression and Purification of L-ALaDHs in *E. coli* BL21 (DE3) cells

The design of recombinant vectors was successfully completed. Following the transformation of the plasmids into *E. coli* DH5- α cells, plasmid isolation was carried out, and the constructions were confirmed using sequence analysis.

The recombinant plasmid containing the *metaladh* gene was transformed into *E. coli* BL21 (DE3) cells as described in the method section. As can be seen in **Figure 4.6** *MetAlaDH* protein was expressed and purified.

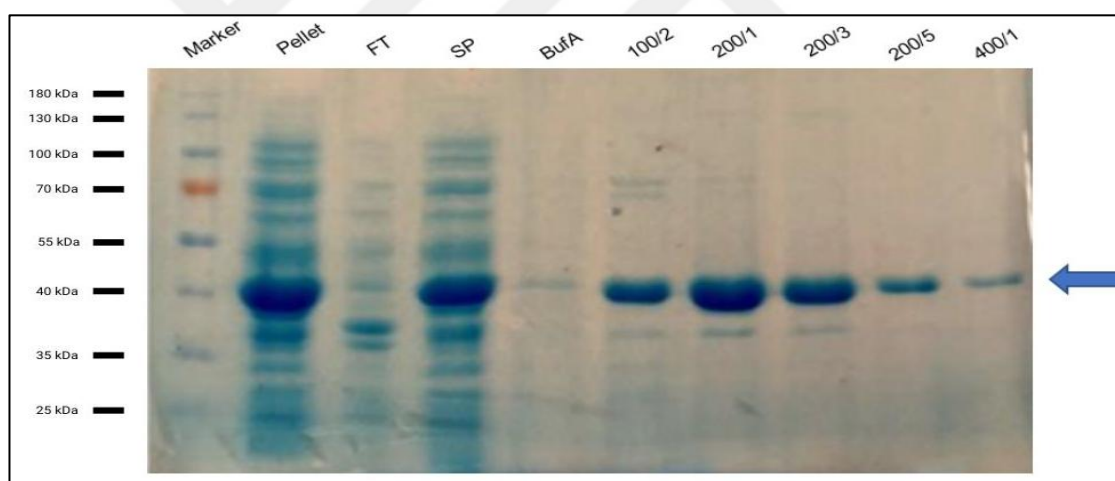


Figure 4.6: SDS-PAGE gel analysis of the purified fractions of *MetAlaDH*. Lanes: **Marker**, **Pellet**, **FT** (solution eluted from the column), **SP** (supernatant), Buffer A (**BufA**), and elution fractions **100/2**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1**:400 mM imidazole.

SDS-PAGE was performed to analyze the proteins in the samples obtained after purification. The fraction obtained by washing the purification column is shown in the wells marked with the letter BufA. The elution fraction containing 100 mM imidazole, the elution fraction containing 200 mM imidazole, and the elution fraction containing 400 mM imidazole are shown in the named wells.

MetAlaDH enzyme was successfully purified the obtained samples were analysed by SDS-PAGE. The fractions obtained between these two, including wells 200/1 and 200/5, were combined and salts and other impurities were removed using an ultracentrifuge tube (with a pore diameter of 35 kDa). The resulting concentrated enzyme sample was used for activity and kinetics studies.

The recombinant plasmid containing the *acfhaldh* gene was transformed into *E. coli* BL21 (DE3) cells as described in the method section. *AcfhAlaDH* proteins were expressed and purified. SDS-PAGE was performed to analyse the proteins in the samples obtained after purification. The fraction obtained by washing the purification column is shown in the wells marked with the letter BufA. Elution fractions containing 100 mM, 200 mM and 400 mM imidazole are shown in the named wells (**Figure 4.7**).

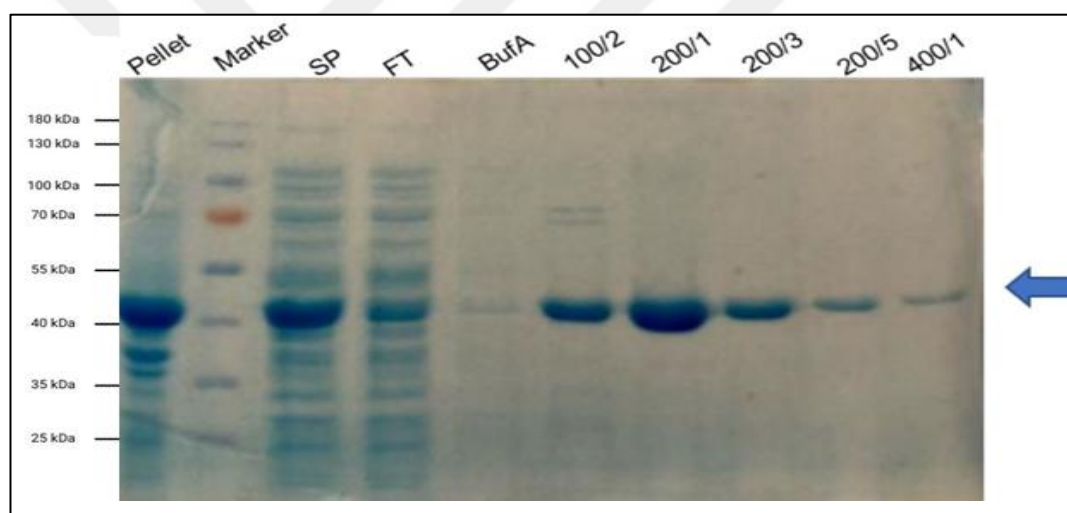


Figure 4.7: SDS-PAGE gel analysis of the purified fractions of *AcfhAlaDH*. Lanes: **Pellet**, **Marker**, **SP** (supernatant), **FT** (solution eluted from the column), **BufA** (Buffer A), and elution fractions **100/2**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1**:400 mM imidazole.

The *AcfhAlaDH* enzyme was successfully purified the obtained samples were analyzed by SDS-PAGE. The fractions obtained between these two, including the 200/1 and 200/5 wells seen in **Figure 4.7** were combined and salts and other impurities were removed using an ultracentrifuge tube (with a pore diameter of 35 kDa). The resulting concentrated enzyme sample was used for activity and kinetics studies.

The recombinant plasmid containing the *cbaladh* gene was transformed into *E. coli* BL21 (DE3) cells. *CbAlaDH* protein was expressed in Studier auto-induction medium at 30 °C, purified, and then analyzed using SDS-PAGE. SDS-PAGE (**Figure 4.8**).

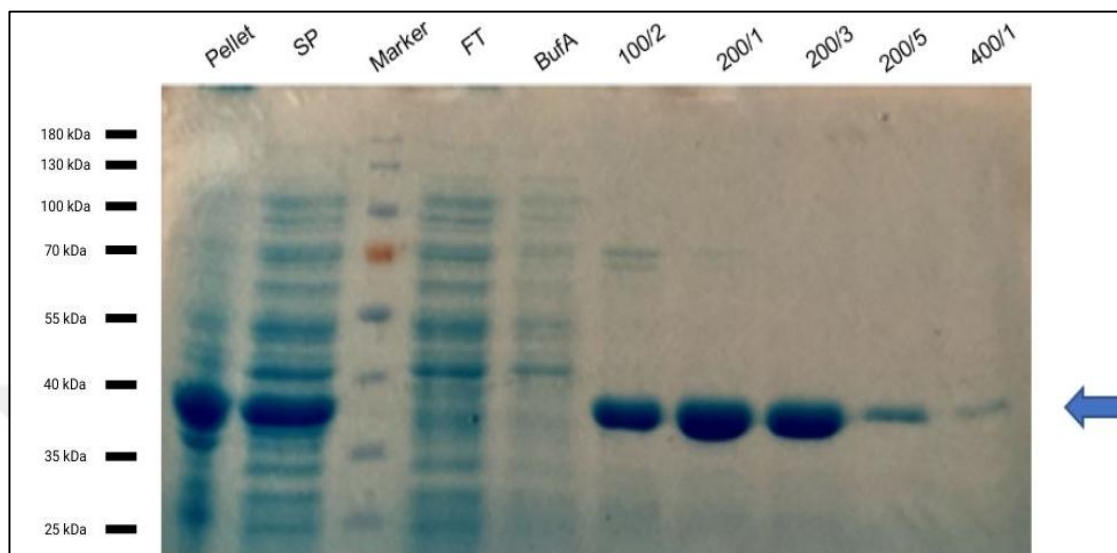


Figure 4.8: SDS-PAGE gel analysis of the purified fractions of *CbAlaDH*. Lanes: **Marker**, **Pellet**, **SP** (supernatant), **FT** (solution eluted from the column), **BufA**: (Buffer A) and elution fractions **100/2**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1**:400 mM imidazole.

The fractions obtained between these two, including the 200/1 and 200/5 wells seen in **Figure 4.8** were combined and salts and other impurities were removed using an ultracentrifuge tube (with a pore diameter of 35 kDa). The resulting concentrated enzyme sample was used for activity and kinetics studies.

The recombinant plasmid containing the *vlaladh* gene was transformed into *E. coli* BL21 (DE3) cells as described in the method section. *VIAlaDH* was expressed in Studier auto-induction medium at 30 °C, purified, and then analyzed using SDS-PAGE (**Figure 4.9**).

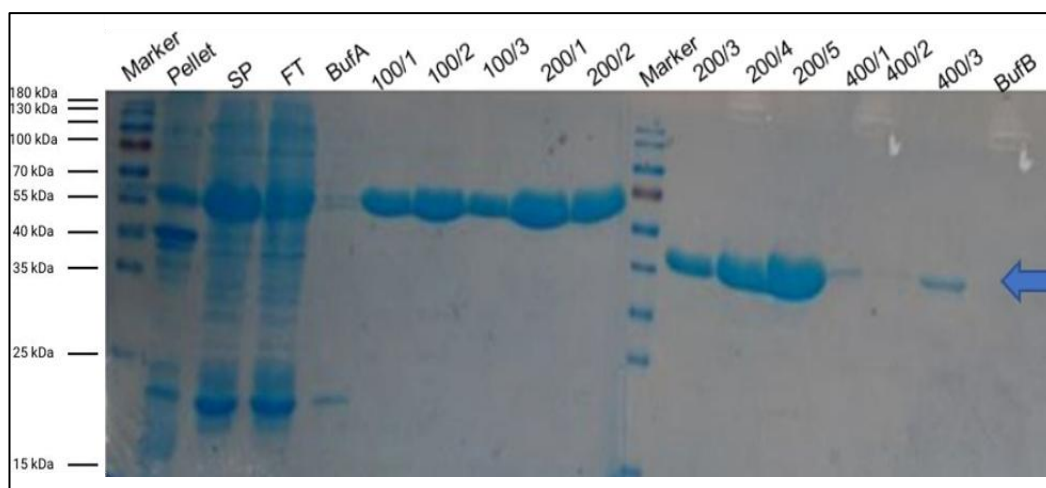


Figure 4.9: SDS-PAGE gel analysis of the purified fractions of *VIAlaDH*. Lanes: **Marker**, **Pellet**, **SP** (supernatant), **FT** (solution eluted from the column), **BufA**: Buffer A, elution fractions **100/1-3**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1-3**:400 mM imidazole. **BufB**: 500 mM imidazole.

The fractions obtained from wells from 100/1 to 200/5 were combined and salts and other impurities were removed using an ultracentrifuge tube (with a pore diameter of 35 kDa) as shown in **Figure 4.9** The resulting concentrated enzyme sample was used for activity and kinetics studies.

The recombinant plasmid containing the *afaladh* gene was transformed into *E. coli* BL21 (DE3) cells; *AfAlaDH* was expressed in Studier auto-induction medium at 30 °C, purified, and then analyzed using SDS-PAGE. (**Figure 4.10**).

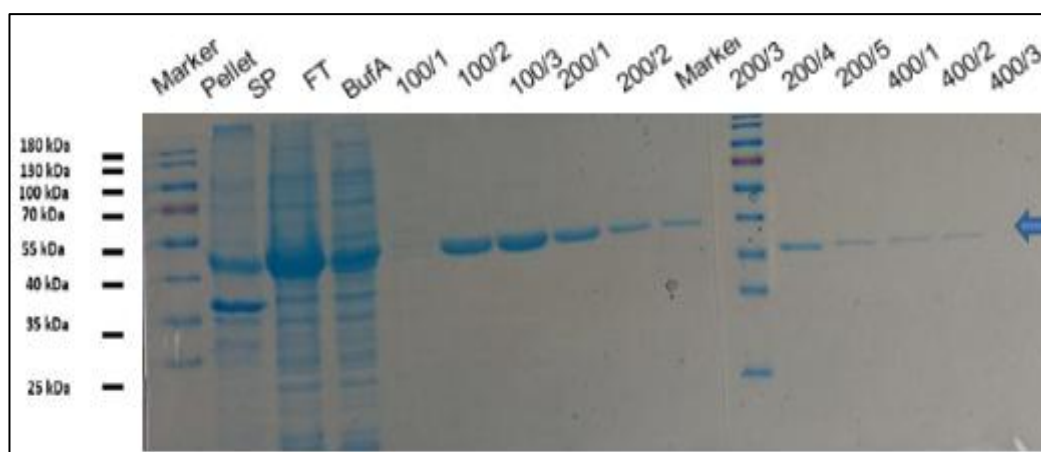


Figure 4.10: SDS-PAGE gel analysis of the purified fractions of *AfAlaDH*. Lanes: **Marker**, **Pellet**, **SP** (supernatant), **FT** (solution eluted from the column), **BufA** (Buffer A), elution fractions **100/1-3**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1-3**:400 mM imidazole.

The fractions obtained from the 100/1, 100/2 and 100/3 wells shown in **Figure 4.10**. were combined and salts and other impurities were removed using an ultracentrifuge

tube (with a pore diameter of 35 kDa). The resulting concentrated enzyme sample was used for activity and kinetics studies. When the obtained SDS-PAGE figures were examined, it was seen that the target proteins were successfully purified, the purity level achieved and the amount obtained were sufficient for the next steps.

The recombinant plasmid containing the *ttaladh* gene was transformed into *E. coli* BL21 (DE3) cells as described above; *TtAlaDH* was expressed in Studier auto-induction medium at 30 °C, purified, and then analyzed using SDS-PAGE (**Figure 4.11**).

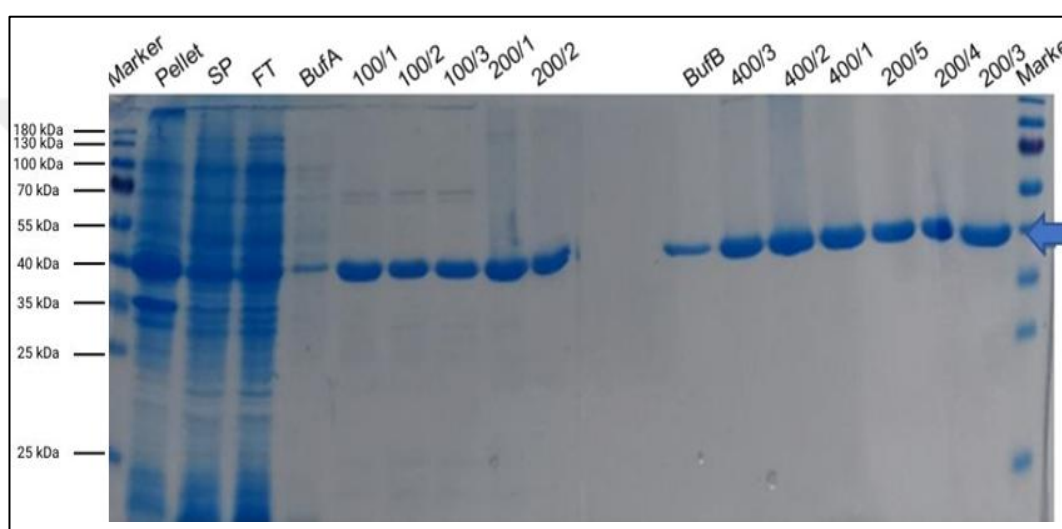


Figure 4.11: SDS-PAGE gel analysis of the purified fractions of *TtAlaDH*. Lanes: **Marker**, **Pellet**, **SP** (supernatant), **FT** (solution eluted from the column), **BufA** (Buffer A), elution fractions **100/1-3**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1-3**:400 mM imidazole. **BufB**: 500 mM imidazole.

The fractions obtained from the 200/1, 200/2, 200/3, 200/4 and 200/5 wells shown in **Figure 4.11** were combined and salts and other impurities were removed using an ultracentrifuge tube (with a pore diameter of 35 kDa). The resulting concentrated enzyme sample was used for activity and kinetics studies.

4.3. Effect of pH and Temperature on L-AlaDHs Activity

The activity of L-AlaDH enzymes were influenced by both pH (4–11) and temperature (25–90 °C) when pyruvate was utilized as the substrate. The pH values that yielded the best results for reductive amination were obtained at a temperature of 25 °C for the

enzymes *AcfhAlaDH*, *AfAlaDH*, *CbAlaDH*, *MetAlaDH*, *VIAlaDH*, and *TtAlaDH* (Figure 4.12.).

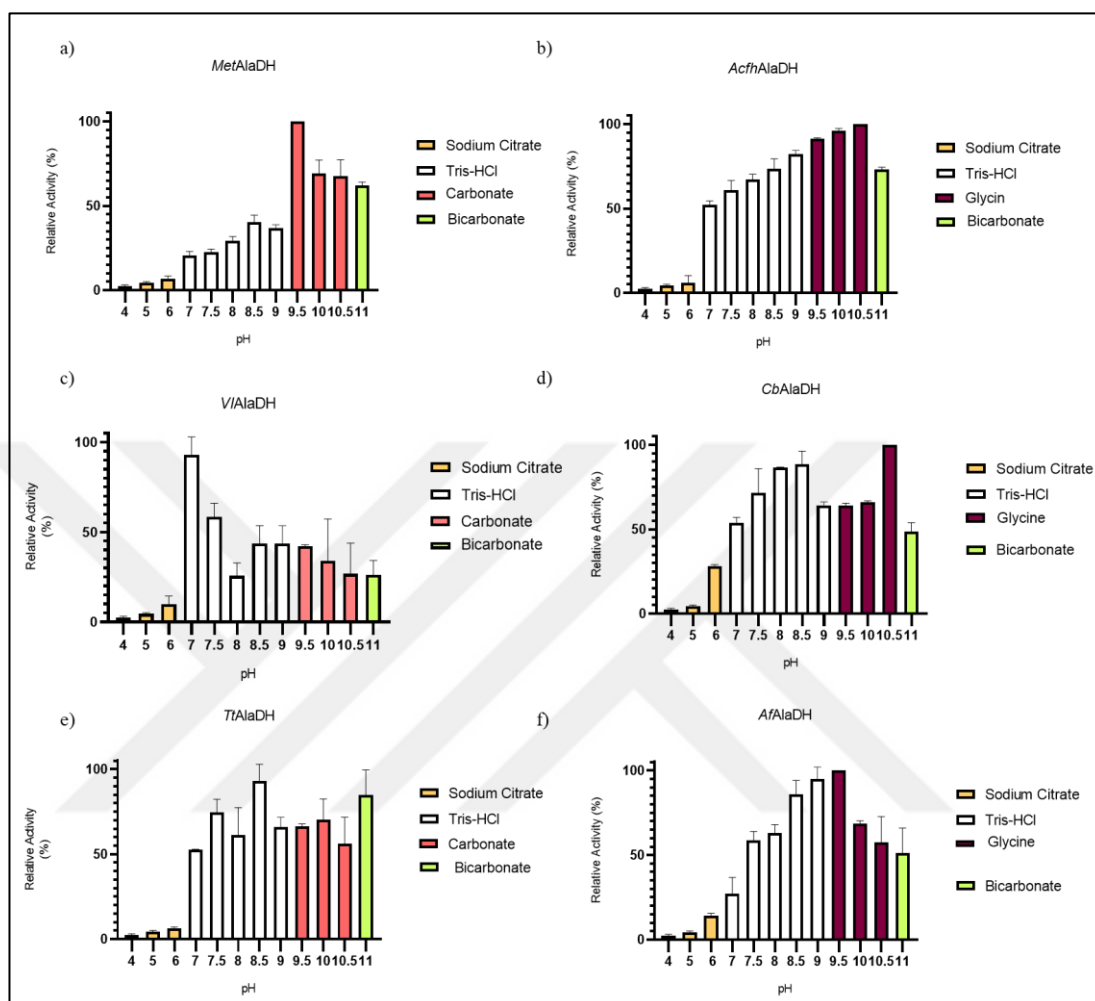


Figure 4.12: Effect of pH on the activity of L-AlaDH enzymes in reductive amination reactions.

A notable characteristic of this group of L-AlaDH enzymes was the significant variation in their pH profiles. The enzymes demonstrated optimal activity at pH levels above 7 (Figure 4.12), while exhibiting minimal activity at pH levels below 7. Notably, *CbAlaDH* displayed the highest activity at pH 6. Among the enzymes tested, only *VIAlaDH* exhibited a peak in activity at pH level at neutral, with the maximum level of activity seen at pH 7 in the Tris buffer. The activity maxima of all other enzymes were observed within the pH range of 8.5–10.5.

As shown in Figure 4.12, *TtAlaDH* showed its peak activity at pH 8.5 in the Tris buffer, *AfAlaDH* and *MetAlaDH* exhibited their peak activity at pH 9.5 in the glycine

and carbonate buffer, and *Acfh*AlaDH displayed its peak activity at pH 10.5 in the glycine buffer. The *Cb*AlaDH enzyme exhibited the greatest level of activity when tested in a glycine buffer solution with a pH of 10.5. The *Tt*AlaDH enzyme displayed a secondary peak of catalytic activity at pH 11 when analyzed using bicarbonate buffer, indicating its capacity to function in highly alkaline environments. This finding emphasizes how flexible the enzyme is to different pH levels. Moreover, the pH activity profiles showed that the L-AlaDH enzymes show a strong sensitivity to exact pH values, implying that even little changes from their ideal pH range might greatly affect their catalytic effectiveness. These results underline the need of pH optimization in enzymatic uses and offer understanding of the biochemical characteristics of L-AlaDHs under several environmental settings.

The impact of temperature on the reductive amination activity of L-AlaDH exhibited significant variation among the enzymes examined. Except for *Cb*AlaDH, which had its optimal activity at 60 °C, all the enzymes analyzed in this investigation exhibited their best activity at 50 °C (**Figure 4.13**).

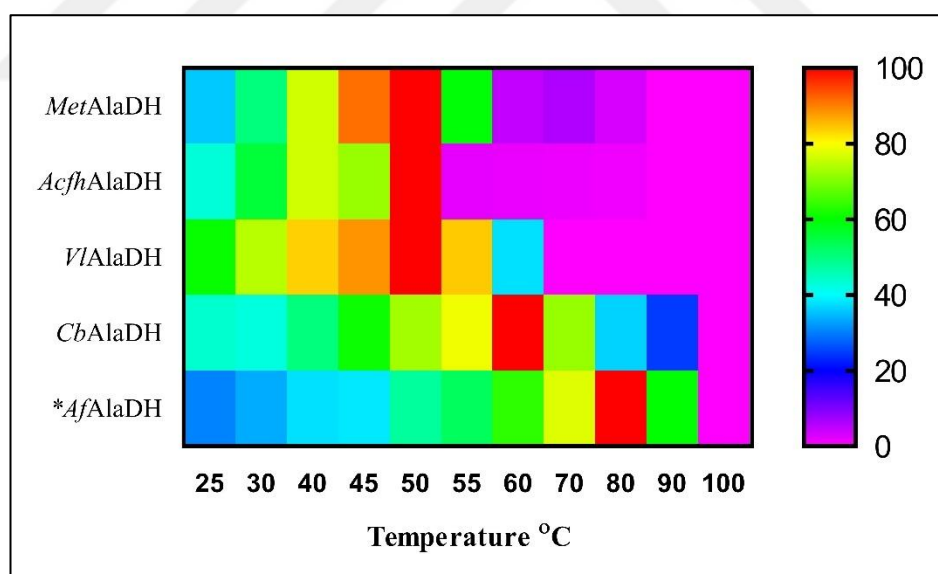


Figure 4.13: Effect of temperature on the activity of L-AlaDH enzymes in reductive amination reactions. *Data taken from the literature [Schröder et al., 2004].

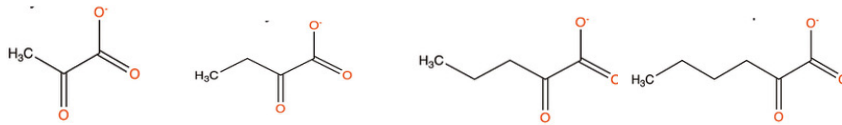
On the other hand, *Acfh*AlaDH exhibited a rapid decline in activity when exposed to temperatures above this threshold. *Met*AlaDH showed almost no activity at 60 °C, but *VI*AlaDH exhibited the greatest temperature tolerance among bacterial L-AlaDHs, with activity only ceasing at 70 °C. *Af*AlaDH enzyme demonstrated its peak activity

at a temperature of 82 °C [Schröder et al., 2004]. The enzyme *CbAlaDH*, derived from archaeal sources, demonstrated its peak activity at a temperature of 60 °C. Among the enzymes examined in our study, *CbAlaDH* had the greatest capacity to withstand high temperatures, remaining functional at temperatures as high as 90 °C. It is important to mention that the solubility of ammonia decreases with increasing temperature. This leads to a decrease in the concentration of ammonia in the reaction when a constant concentration of ammonium ions is utilized in the assays. This behavior likely decreases the apparent temperature optimum of L-AlaDH enzymes to some extent.

4.4. Determination of Kinetic Parameter of L-AlaDHs

Upon examining the specific activity levels provided in **Table 4.3**, it was found that all the L-AlaDH enzymes examined exhibited activity against pyruvate, α -ketobutyrate, and α -ketovalerate substrates. *MetAlaDH* had the highest specific activity for pyruvate, whereas *CbAlaDH* displayed the lowest specific activity for pyruvate (**Table 4.3**).

Table 4.3: Optimum pH and specific activity constant (U/mg) of purified L-AlaDH enzymes with regard to different substrates.

Enzyme	pH				
		Pyruvate	α -ketobutyrate	α -ketovalerate	α -ketocaproate
<i>MetAlaDH</i>	9.5	172.5 $\pm$ 5.0	3.7 $\pm$ 0.4	0.9 $\pm$ 0.1	ND*
<i>AcfhAlaDH</i>	10.5	154.2 $\pm$ 8.0	6.7 $\pm$ 0.3	1.2 $\pm$ 0.1	ND*
<i>VlAlaDH</i>	7.0	134.8 $\pm$ 3.0	13.2 $\pm$ 0.5	1.9 $\pm$ 0.1	ND*
<i>TtAlaDH</i>	8.5	127.0 $\pm$ 3.0	1.7 $\pm$ 0.3	1.5 $\pm$ 0.0	0.3 $\pm$ 0.0
<i>CbAlaDH</i>	10.5	3.2 $\pm$ 0.3	1.6 $\pm$ 0.2	0.4 $\pm$ 0.0	ND*
<i>AfAlaDH</i>	9.5	114.2 $\pm$ 6.0	4.3 $\pm$ 0.3	0.5 $\pm$ 0.1	2.8 $\pm$ 0.3

* None determined.

All L-AlaDH enzymes demonstrated significantly higher activity when utilizing pyruvate as a substrate compared to other tested substrates. Furthermore, among the examined L-AlaDH enzymes, α -ketobutyrate was identified as the preferred substrate following pyruvate. Notably, only the *AfAlaDH* and *TtAlaDH* enzymes exhibited

measurable activity toward α -ketocaproate, whereas the other L-AlaDH candidates showed no activity for this substrate under the experimental conditions. These findings underscore the substantial influence of substrate carbon backbone length on the enzyme's ability to recognize and utilize substrates. Additionally, the results highlighted variations among L-AlaDH enzymes derived from different sources, emphasizing that the enzymatic preferences are influenced by their origin and structural properties. The Michaelis-Menten plots were generated by analyzing the L-AlaDH enzymes for each substrate under controlled conditions of 25 °C and an optimal pH level. This analysis aimed to discover the kinetic characteristics of the enzyme. The software GraphPad Prism 9 [GraphPad Software, 2022] was employed to calculate the kinetic parameters, namely the k_{cat} and K_{M} values. The K_{M} and k_{cat} graphs obtained for the substrates used are presented in **APPENDIX-C**. The summary of the computed kinetic parameters for pyruvate, as shown in **Table 4.4** demonstrated notable variations in both K_{M} and k_{cat} values.

Table 4.4: Kinetic constant of obtained L-AlaDH enzymes with regard to pyruvate.

Enzyme	Pyruvate		
	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{mM}^{-1}$)
<i>MetAlaDH</i>	139.6 ± 19.7	1.30 ± 0.4	108.2 ± 12.0
<i>AcfhAlaDH</i>	112.2 ± 6.6	0.70 ± 0.2	148.2 ± 13.5
<i>VIAlaDH</i>	62.5 ± 3.4	0.30 ± 0.0	170.0 ± 6.7
<i>TtAlaDH</i>	87.0 ± 5.1	0.63 ± 0.2	137.8 ± 15.7
<i>CbAlaDH</i>	0.5 ± 0.1	0.40 ± 0.0	1.3 ± 0.1
<i>AfAlaDH</i>	15.4 ± 1.0	0.20 ± 0.1	66.7 ± 10

The K_{M} values for the enzymes examined ranged from 0.2 to 1.3 mM, while the k_{cat} values ranged from 0.5 to $139.6 \text{ s}^{-1} \text{mM}^{-1}$. *MetAlaDH* demonstrated the highest catalytic efficiency (k_{cat}) compared to the other enzymes tested, and it also had the highest substrate affinity (K_{M}). *VIAlaDH* demonstrated the highest catalytic efficiency among the L-AlaDHs, whereas *CbAlaDH* exhibited the lowest.

The K_{M} values with regard to α -ketobutyrate varied widely: *AfAlaDH* had the lowest K_{M} value (0.2 mM), while *VIAlaDH* exhibited the highest value (5.8 mM) (**Table 4.5**). Of the L-AlaDHs evaluated in this thesis, *AfAlaDH* displayed the most favourable catalytic efficiency with regard to α -ketobutyrate, with a value of $34 \text{ s}^{-1} \text{mM}^{-1}$, while

CbAlaDH, an archaeal L-AlaDH, displayed the least favourable catalytic efficiency ($0.3 \text{ s}^{-1} \text{ mM}^{-1}$).

Table 4.5: Kinetic constant of obtained L-AlaDH enzymes with regard to α -ketobutyrate.

Enzyme	α -ketobutyrate		
	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{ mM}^{-1}$)
<i>MetAlaDH</i>	3.3 ± 0.0	3.8 ± 1.2	0.8 ± 0.0
<i>AcfhAlaDH</i>	5.4 ± 0.5	1.3 ± 0.4	4.0 ± 1.4
<i>VlAlaDH</i>	10.7 ± 0.4	5.8 ± 1.0	1.8 ± 0.2
<i>TtAlaDH</i>	1.1 ± 0.0	0.4 ± 0.1	3.0 ± 0.7
<i>CbAlaDH</i>	0.7 ± 0.2	2.2 ± 1.0	0.3 ± 0.2
<i>AfAlaDH</i>	7.5 ± 0.1	0.2 ± 0.0	34.0 ± 0.1

The kinetic parameters with regard to α -ketovalerate are shown in (Table 4.6).

Table 4.6: Kinetic constant of obtained L-AlaDH enzymes with regard to α -ketovalerate.

Enzyme	α -ketovalerate		
	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{ mM}^{-1}$)
<i>MetAlaDH</i>	0.7 ± 0.0	1.9 ± 0.0	0.4 ± 0.0
<i>AcfhAlaDH</i>	1.0 ± 0.0	5.2 ± 0.8	0.2 ± 0.0
<i>VlAlaDH</i>	1.9 ± 0.3	3.3 ± 0.2	0.6 ± 0.0
<i>TtAlaDH</i>	1.2 ± 0.0	3.3 ± 1.1	0.4 ± 0.0
<i>CbAlaDH</i>	0.17 ± 0.1	0.8 ± 0.0	0.21 ± 0.1
<i>AfAlaDH</i>	0.9 ± 0.2	0.3 ± 0.0	2.7 ± 0.3

Similar to the observations with ketobutyrate, the results exhibited a broad variation in K_{M} values, with the highest value (5.2 mM) observed in *AcfhAlaDH*, and the lowest value observed in *AfAlaDH* (0.3 mM). While *AfAlaDH* displayed the highest catalytic efficiency ($2.7 \text{ s}^{-1} \text{ mM}^{-1}$), the lowest catalytic activities were observed in *CbAlaDH* and *AcfhAlaDH*, with values of 0.21 and $0.2 \text{ s}^{-1} \text{ mM}^{-1}$, respectively.

The study found that only *TtAlaDH* and *AfAlaDH* were active with regard to ketocaproate. Although *AfAlaDH* showed some activity with regard to α -ketocaproate, its kinetic values could not be determined due to difficulties in obtaining values that fitted the Michaelis-Menten model. Only the catalytic efficiency of *TtAlaDH* was

measured, which was $0.6 \text{ s}^{-1} \text{ mM}^{-1}$. The K_M for *TtAlaDH* was measured at 0.4 mM, which indicated a strong affinity of the enzyme for this large substrate.

4.5. Structural Analysis and Modeling *TtAlaDH* for Mutation Studies

The selected substrates were first added automatically to the candidate structure with Autodockvina and then visualized manually in SwissPDB and PyMOL by making corrections in the positioning of the atoms [Trott and Olson, 2010]. The predicted candidate structure underwent a molecular dynamics simulation using the NAMD protocol. In this protocol, all constructs were placed within a 12 Å thick TIP3P water box set to a minimum distance of >1.2 nm. The system was subjected to energy minimization at 300 K for 10 picoseconds (10,000 NAMD time steps) following the addition of Na^+ ions (0.1 M) to neutralize negative charges. NAMD version 2.15 ALPHA was employed for both minimizations and subsequent simulations [Phillips et al., 2020]. To convert the energy-minimized constructs into protein structure files, VMD Version 1.9.4 was utilized [Humphrey et al., 1996]. The results of the simulations were visualized and analyzed using the PyMOL as shown in **Figure 4.14** [Schrödinger and Delano, 2020].

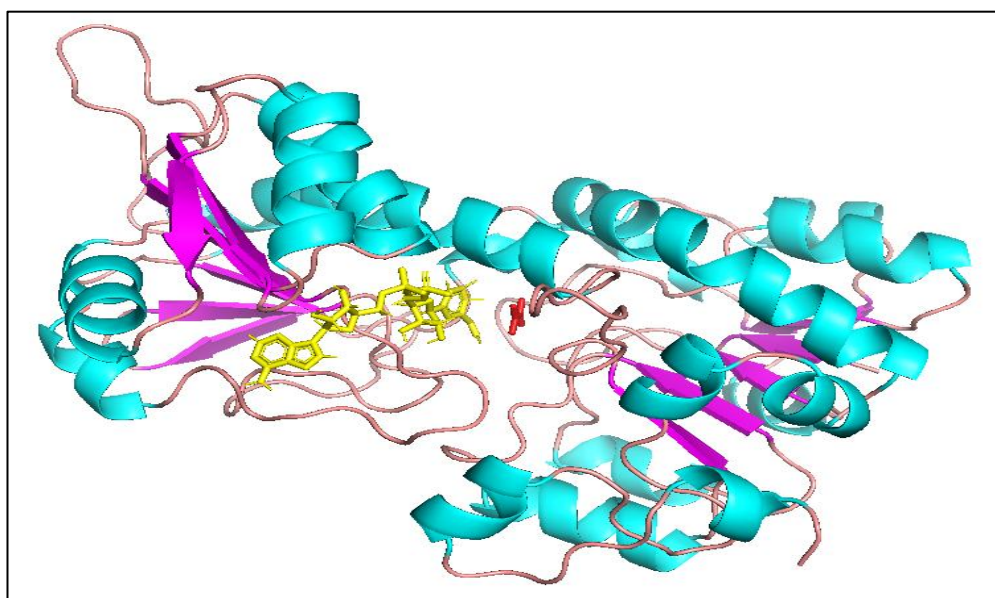


Figure 4.14: The predicted structure of the active site of *TtAlaDH* in the presence of pyruvate (red) and NADH (yellow).

To identify the amino acid positions directly involved in ligand binding, *TtAlaDH* was coupled with the substrate using Autodockvina [Trott and Olson, 2010]. This automatic substrate docking process at the molecular level enabled the successful identification of amino acids located within the enzyme's binding pocket that interact directly with the substrate. These identified amino acid positions, which are predicted to have functional significance and potential interactions with substrates, are shown in **Figure 4.15** [Schrödinger and Delano, 2020].

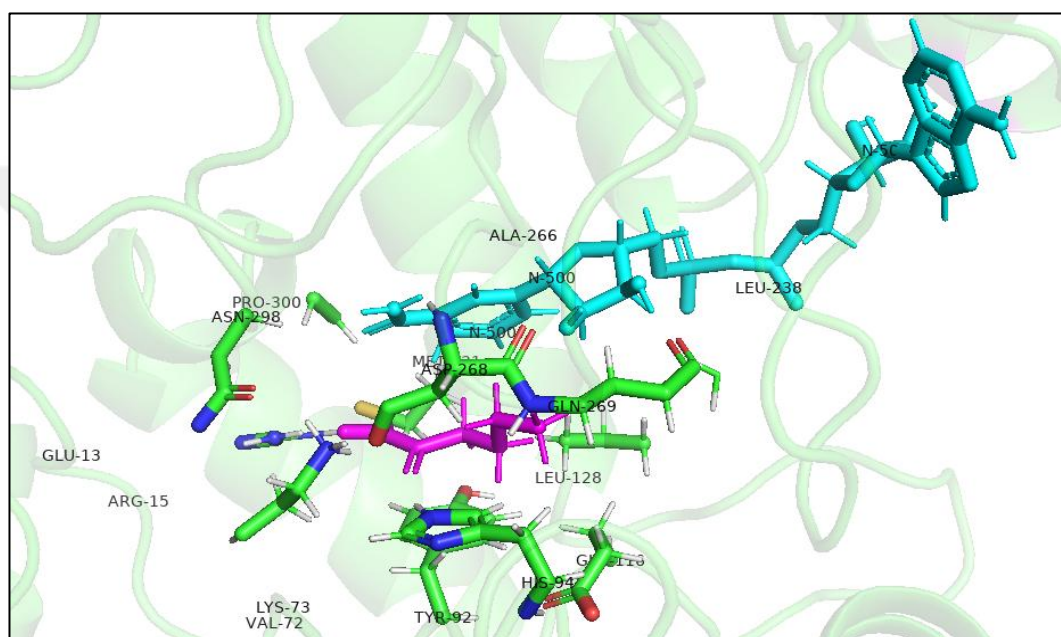


Figure 4.15: The predicted structure of the active site of *TtAlaDH* in the presence of ketocaproate (purple) and NADH (turquoise).

The identical protocol was applied to other substrates, and the corresponding results are presented in **Figures 4.16** and **4.17** [Schrödinger and Delano, 2020]. These figures show the outcomes of the application of the aforementioned protocol for each respective substrate.

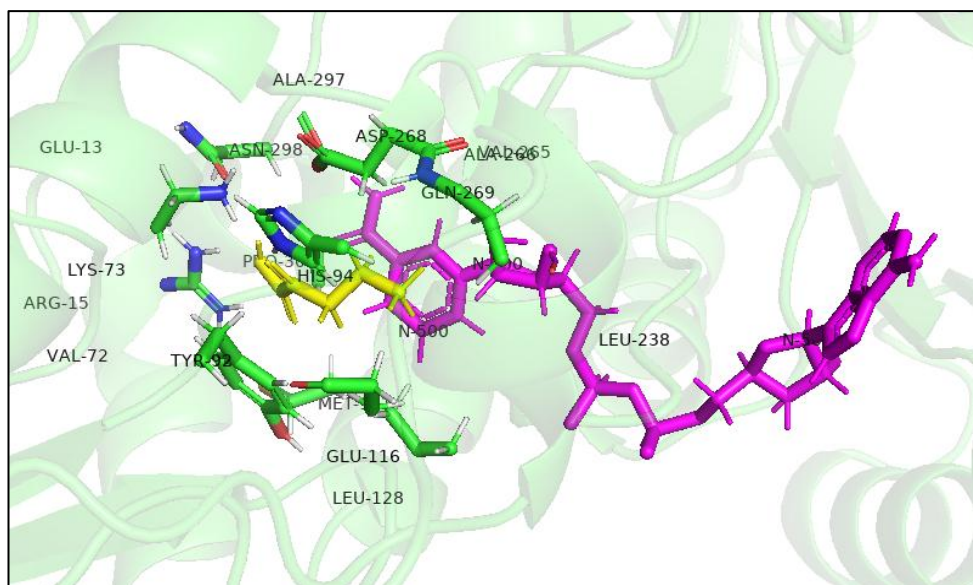


Figure 4.16: The predicted structure of the active site of *TtAlaDH* in the presence of ketovalerate (yellow) and NADH (purple).

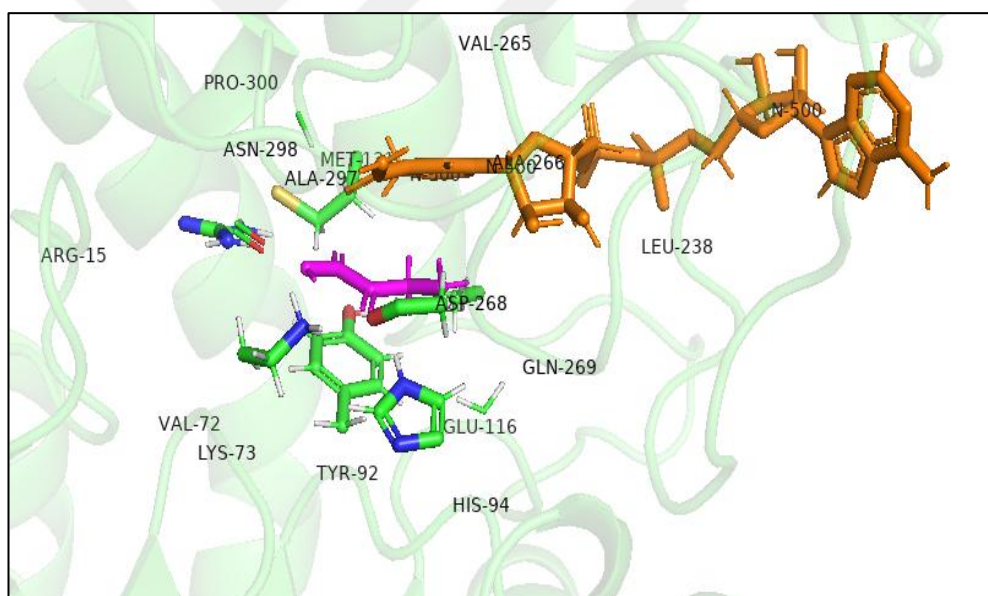


Figure 4.17: The predicted structure of the active site of *TtAlaDH* in the presence of ketobutyrate (purple) and NADH (orange).

The model strongly suggests the functionality of the active region, primarily due to its high conservation within the predicted structure. Specifically, the correct placement of Arg15, Lys75, His96 (Lys73, His94 in the *TtAlaDH* model), and Asp270 (Asp268 in the *TtAlaDH* model) further supports their functional relevance. The interactions between the selected target substrates and the side chains of two amino acids (Arg15 and Lys73) appear to play a crucial role in positioning the substrates within the

enzyme's active site, facilitating their conversion into products. Following the utilization of the *TtAlaDH* crystal structure, the missing regions were completed, and molecular modeling techniques were applied. Subsequently, substrate placement was carried out. According to literature findings, the interaction between pyruvate and NADH, situated in the active site, involves stabilization through the interaction with Tyr92 and Leu128. However, it has been observed that the side chains of amino acids at these positions can pose steric barriers, hindering the binding of larger side chain pyruvate derivatives. This information suggests that the specific side chain configurations of these amino acids may influence the binding capabilities of different pyruvate derivatives.

Based on the observations regarding the steric barriers created by the side chains of Tyr92 in accommodating larger pyruvate derivatives, it is hypothesized that mutations such as Ala, Ser, and Thr at the Tyr92 position could be promising. Gly was also considered as a potential candidate, although it may increase flexibility. The side chains of Ser and Thr at this position are predicted to rotate away from the substrate binding pocket, allowing for the formation of H-bonds. Notably, the mutations of Ala, Gly, Ser, and Thr at Tyr92 can be manually modeled in a reactive conformation without causing steric clashes with larger substrates.

Due to its essential function in protein stability, it is noteworthy that a mutation at the Leu130 position in *TtAlaDH*, corresponding to position 128 in *MtAlaDH*, has been documented to lead to a total loss of solubility [Fernandes et al., 2015]. Since this position is located within the β -sheet structure, it was considered too risky and was therefore excluded as a candidate for mutation in the present study.

In a previous study it was observed that certain substrates, such as L-norvaline (larger than L-alanine), could be utilized more effectively by the enzyme through a double mutation of Tyr117Leu and Phe94Ser in the conserved IleAlaTyrThrGlu region of L-AlaDHs. This indicates that specific mutations in key amino acid positions can enhance the enzyme's efficiency in utilizing larger substrates [Fernandes et al., 2015].

In **Figure 4.18**, it is demonstrated that the amino acids Tyr92 and Tyr115 in the candidate *TtAlaDH* interact with each other, based on our analysis using the Protein Contact Atlas [Kayikci et al., 2018].

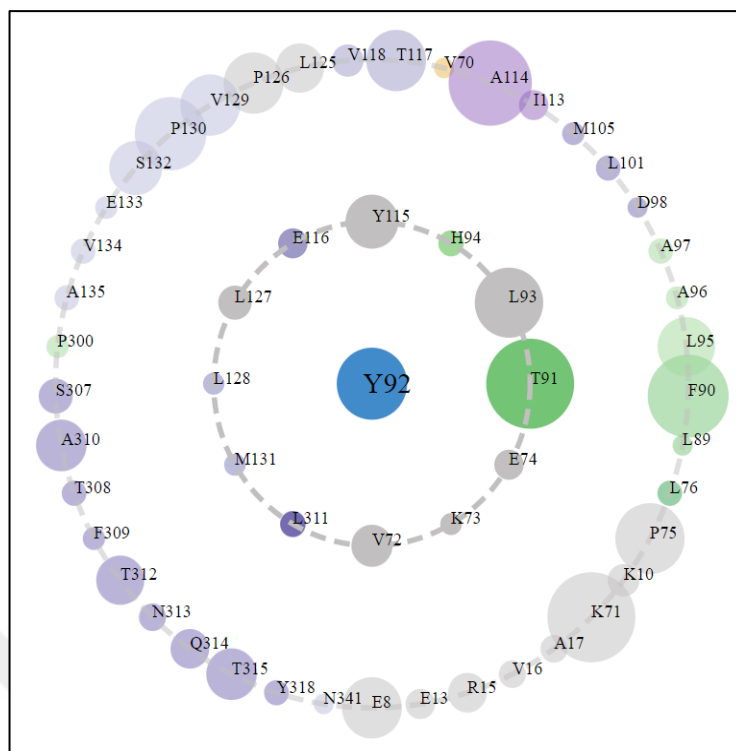


Figure 4.18: The illustration of the amino acids that interact with Tyr92 in *TtAlaDH*.

Additionally, non-polar interactions are observed in the enzyme and other neighboring positions. These interactions involve the side chains of amino acids such as Val72, Tyr92, Leu128, Met131, and Asn298. These interactions play a vital role in facilitating the conversion of pyruvate to the product through the reductive amination reaction, enabling the enzyme to efficiently carry out its catalytic function [Kayikci et al., 2018].

Based on this information, further analysis was conducted on the Tyr115 position, and it is predicted that mutations at both the Tyr115 and Tyr92 positions may enhance the enzyme's affinity for larger substrates. **Figure 4.1** illustrates the preservation of the LeuPheThr-Phe/Tyr-LeuHis region among the L-AlaDHs studied in the thesis. When comparing the K_M values of L-AlaDHs with Tyr amino acid in this region towards pyruvate, it is observed to range from 0.3 to 1.3 mM. Therefore, it is predicted that this position may have an impact on the K_M . Moreover, mutations in a conserved position like Tyr115 are expected to yield interesting results.

The impact of amino acid mutations near the binding pocket is extensively studied, as these changes can significantly influence the three-dimensional structure, hydrophobicity, and charge distribution of the binding sites. Research has shown that mutations in these regions can enhance enzymatic properties, including catalytic

activity, thermostability, and substrate specificity [Ali et al., 2020]. Thus, in this regard, knowing and analyzing particular amino acids can be rather helpful for improving the possible uses and functionality of L-AlaDH enzymes.

This information improves the clarity of choosing candidate amino acid positions and boosts the effectiveness of the semi-rational design technique ISM/CAST.

Figure 4.19 shows a comparison between the native structure of *TtAlaDH* and one of the anticipated mutations [Schrödinger and Delano, 2020]. The mutation alters the amino acid residues, therefore affecting the volume and surface area of the active site. This comparison offers insightful analysis of the structural changes the mutation could cause.

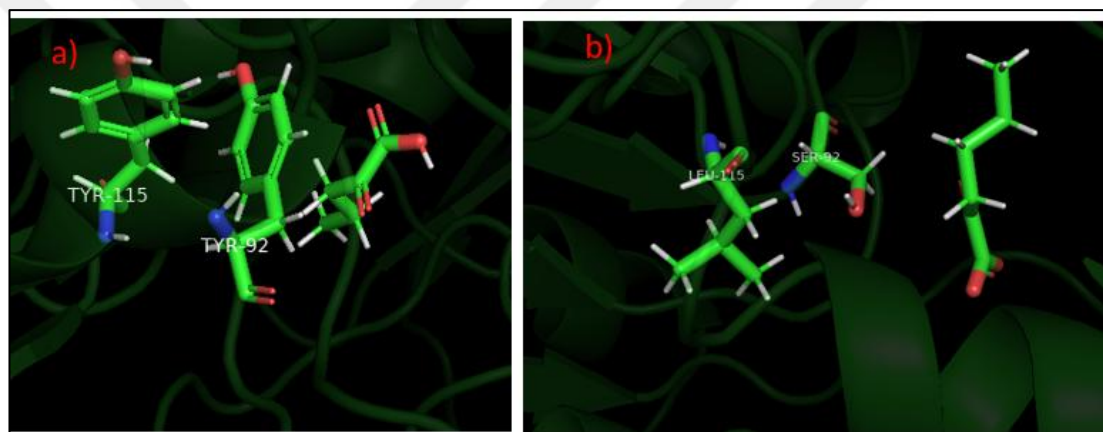


Figure 4.19: a) Wild type *TtAlaDH* b) Tyr92Ser and Tyr115Leu mutation of *TtAlaDH*.

4.6. Mutation Studies of *TtAlaDH*

4.6.1. Semi-Rational Design Technique

The gel imaging of the PCR results, as shown in **Figure 4.20**, reveals the presence of bands in both the Tyr92DBK and control wells. The expected size of the pET-28a(+) vector containing the mutation bands is approximately 6488 bp. The presence of these bands indicates the successful completion of the PCR reaction. Subsequently, the PCR products of the Tyr92DBK mutation were incubated with *DpnI* enzyme for overnight at 37 °C, and the resulting digestion products were visualized on an agarose gel.

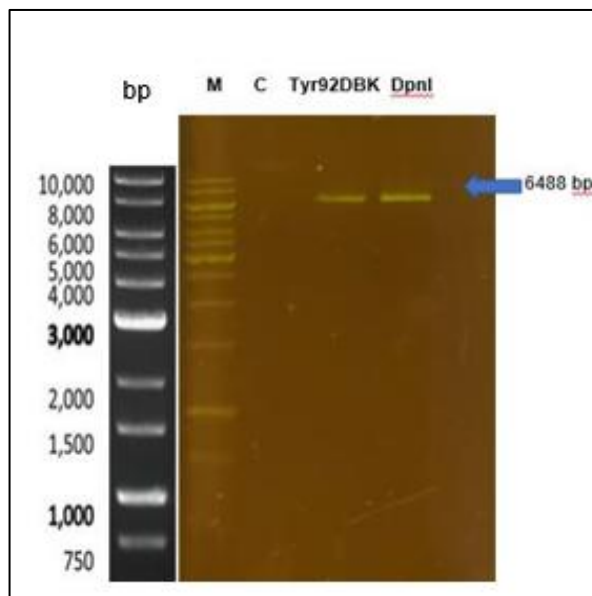


Figure 4.20: Agarose gel image of PCR samples. **M:** Reference protein (**Marker**), **C:** *TtAlaDH* plasmid, **Tyr92DBK:** Example of PCR amplified recombinant plasmid pET-28a(+), **DpnI:** Example of the recombinant plasmid pET-28a(+) cut with *DpnI*.

pET-28a(+) plasmids which contain *T. thermophilus* alanine dehydrogenase(*ttald*) gene were transformed into host *E.coli* BL21 (DE3) and *E. coli* TOP10 cells via heat-shock transformation protocol from NEB[®]. After overnight incubation at 37 °C, individual colonies were observed LB agar plates supplemented with 50 µg/mL kanamycin (**Figure 4.21, 4.22**).

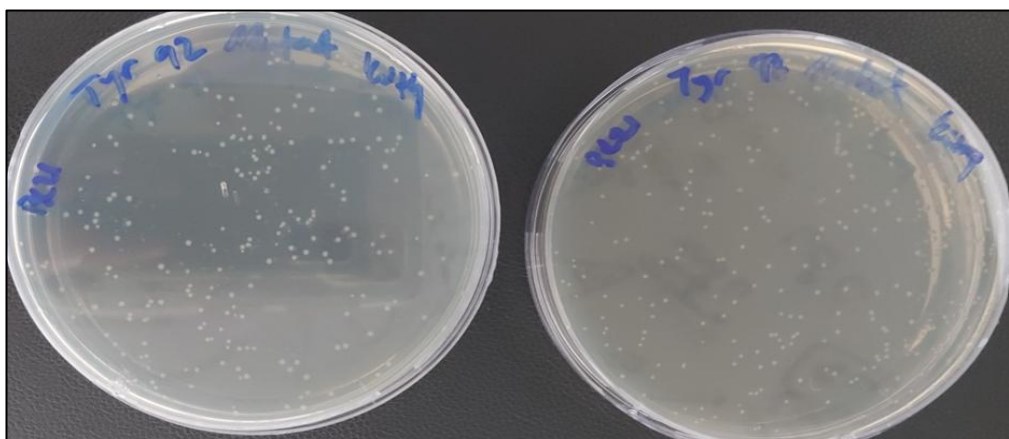


Figure 4.21: Recombinant colonies of *E.coli* BL21 after transformation.



Figure 4.22: Recombinant colonies of *E.coli* Top10 after transformation.

Since a sufficient number of colonies are obtained with *E.coli* BL21 and Top10 cells, plasmid isolation will be made from these cells and sent for sequencing to determine whether the relevant mutations have occurred.

To confirm the mutation at the specified Tyr92 location, colonies were handpicked from the Petri plate, and subsequent plasmid isolation procedures were executed. The plasmid samples, obtained for mutation validation, were dispatched to company for comprehensive sequence analysis. For the sequencing process, the T7 promoter primer with the nucleotide sequence 5' TAATACGACTCACTATAGGG 3' was employed. The sequencing outcomes were visualized using the BioRender software and are exhibited in **Figure 4.23** [BioRender, 2023].

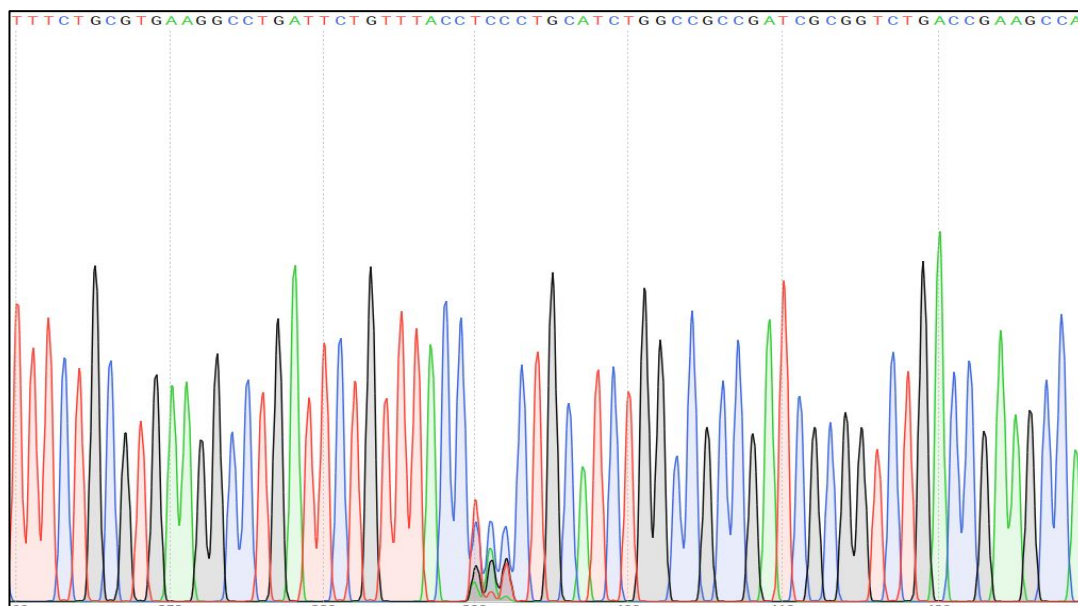


Figure 4.23: Sequence analysis result of the Tyr92DBK mutation.

The results displayed in BioRender show each nucleotide represented by a specific color: cytosine in blue, thymine in red, adenine in green, and guanine in black as can be seen in **Figure 4.23**.

The plasmid library, confirmed to contain the targeted mutation (Tyr92DBK) by sequence analysis, was transformed into *E. coli* BL21 (DE3) [Mandel and Higa, 1970]. The transformed mutant library containing more than 100 colonies in a Petri plate is shown in **Figure 4.24**.

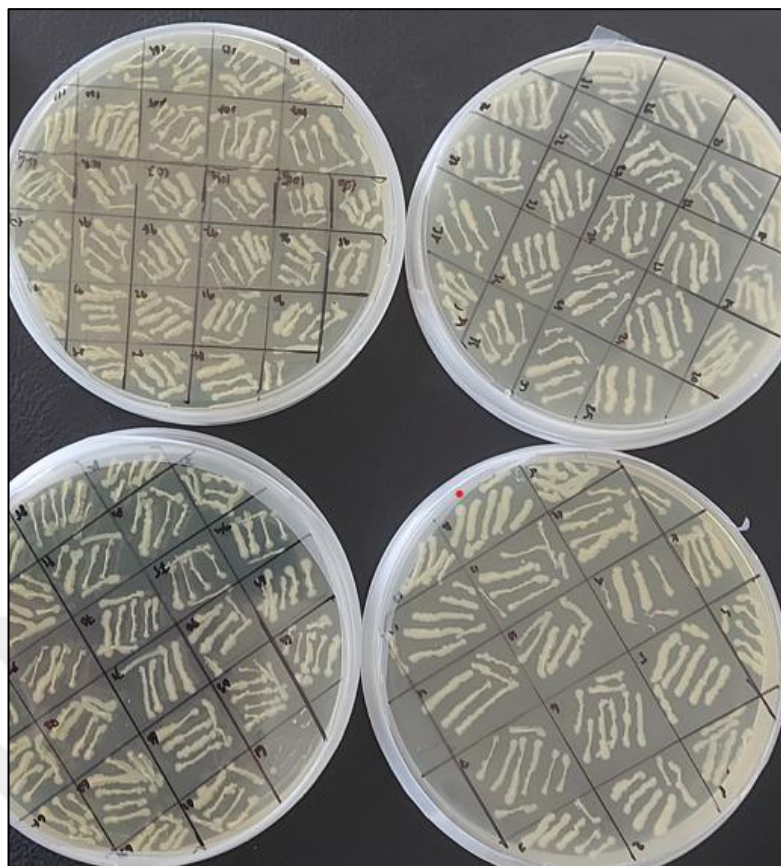


Figure 4.24: Mutant library obtained for *TtAlaDH* Tyr92 position.

As depicted in **Figure 4.24** the presence of over 100 colonies signifies that the requisite number of colonies needed to cover all amino acids encoded by the DBK degenerate codon at the Tyr 92 position has been attained [Reetz and Carballeira, 2007]. With a sufficient number of colonies to fulfill the objectives of this work package, we proceeded with screening this mutant library.

As a result of the analysis, Ala/Asp/Cys/Gly/Pro/Ser/Thr/Val mutations were identified at position Tyr92. The amino acids that can be assigned with the DBK degenerative codon are Ala/Arg/Cys/Gly/Ile/Leu/Leu/Met/Phe/Ser/Thr/Trp/Val. It is speculated that the Pro amino acid could have been introduced as an "error" during the translation/transcription phase or during the acquisition of Ser or Thr obtained with the DBK degenerative codon. The sequence analysis results for the region of interest are shown in **Figure 4.25**.

Tyr92	CTG ATT CTG TTT ACC TAT CTG CAT CTG GCC
Tyr92Ser	CTG ATT CTG TTT ACC TCC CTG CAT CTG GCC
Tyr92Ala	CTG ATT CTG TTT ACC GCG CTG CAT CTG GCC
Tyr92Gly	CTG ATT CTG TTT ACC GGG CTG CAT CTG GCC
Tyr92Thr	CTG ATT CTG TTT ACC ACC CTG CAT CTG GCC
Tyr92Val	CTG ATT CTG TTT ACC GTT CTG CAT CTG GCC
Tyr92Pro	CTG ATT CTG TTT ACC CCC CTG CAT CTG GCC
Tyr92Asp	CTG ATT CTG TTT ACC AAC CTG CAT CTG GCC
Tyr92Cys	CTG ATT CTG TTT ACC TGT CTG CAT CTG GCC

Figure 4.25: Sequence analysis results of wild-type and obtained mutant *TtAlaDH*s.

As shown in **Figure 4.25**, after screening over 100 colonies from the mutation library and analyzing a large number of plasmid sequences, only variants containing Ala/Asp/Cys/Gly/Pro/Ser/Thr/Val mutations were obtained.

4.6.2. Purification of Mutant L-AlaDHs

The mutant L-AlaDH enzymes were purified using the method described in the methodology section, and their purity was evaluated by SDS-PAGE analysis. (**Figure 4.26**).

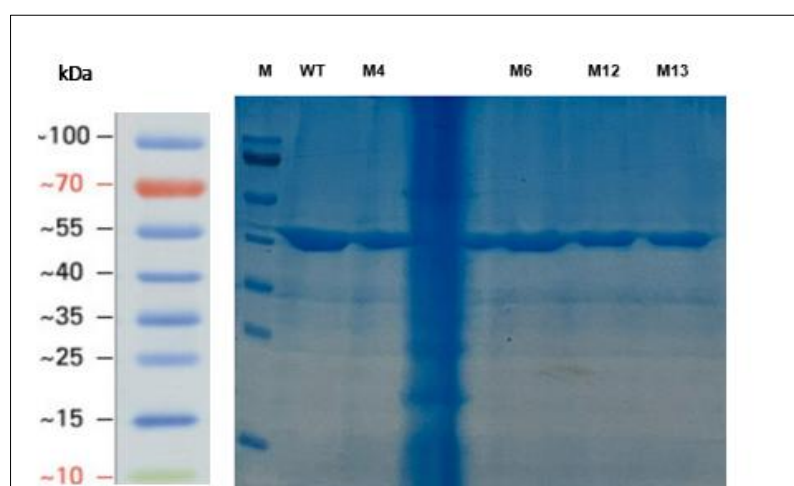
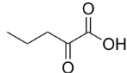
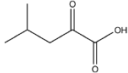


Figure 4.26: SDS-PAGE gel analysis of the purified proteins from mutant colonies (**M**: Marker, **WT**: wild type, **M4**: Tyr92Gly, **M6**: Tyr92Ser, **M12**: Tyr92Ala, **M13**: Tyr92Ser).

Figure 4.26 shows that the bands obtained for the wild type and mutant enzymes were sufficiently pure for kinetic studies. Specific activity assays for the purified wild type and mutant enzymes were carried out as previously described. The calculated specific activities (U/mg) for the high purity wild type mutant enzymes are shown in **Table 4.7**.

Table 4.7: Specific activity constant (U/mg) of high purity enzymes obtained from wild type and mutant *TtAlaDH*s with regard to different substrates.

			
Enzyme	α -ketobutyrate	α -ketovalerate	α -ketocaproate
Wild type	6.40 $\pm$ 0.30	0.72 $\pm$ 0.12	0.01 $\pm$ 0.00
Tyr92Ser	0.85 $\pm$ 0.25	1.00 $\pm$ 0.20	2.80 $\pm$ 0.15
Tyr92Ala	0.83 $\pm$ 0.15	0.40 $\pm$ 0.00	2.10 $\pm$ 0.30
Tyr92Gly	0.27 $\pm$ 0.06	0.30 $\pm$ 0.10	1.20 $\pm$ 0.27
Tyr92Thr	0.10 $\pm$ 0.00	0.50 $\pm$ 0.13	0.60 $\pm$ 0.15
Tyr92Pro	0.12 $\pm$ 0.00	0.14 $\pm$ 0.01	0.80 $\pm$ 0.23
Tyr92Val	0.22 $\pm$ 0.04	0.35 $\pm$ 0.04	0.97 $\pm$ 0.20

As can be seen in **Table 4.7.**, when the specific activities of the mutant *TtAlaDH*s obtained were compared with wild type *TtAlaDH*; Ala/Gly/Pro/Ser/Thr/Val mutation increased the activity against at least one substrate. Total activity analysis showed that Asp and Cys mutations decreased the activity against the respective substrates.

4.6.3. Purification and Kinetic Characterization of Selected Mutant L-AlaDH Enzyme Against Selected Substrates

As a result of the analyses, the Tyr92Ser mutant was purified and its kinetic constants were calculated, since it showed better activity against α -ketovalerate and α -ketocaproate substrates than the wild type. The purification protocol described below was followed to purify the mutant enzyme. Buffer A and buffer B (20 mM NaPi, 500 mM NaCl, 500 mM imidazole pH 7.4) containing 100 mM, 200 mM, 400 and 500 mM imidazole were used separately for purification only. Target proteins were eluted from the column by creating an imidazole gradient (100-500 mM). The purity of the obtained samples was analyzed by SDS-PAGE (**Figure 4.27**) and the fractions obtained between these two, including wells 200/1 and 400/3, were combined and salts and other impurities were removed using an ultracentrifuge tube (35 kDa pore

diameter). The resulting concentrated enzyme sample was used for activity and kinetic studies.

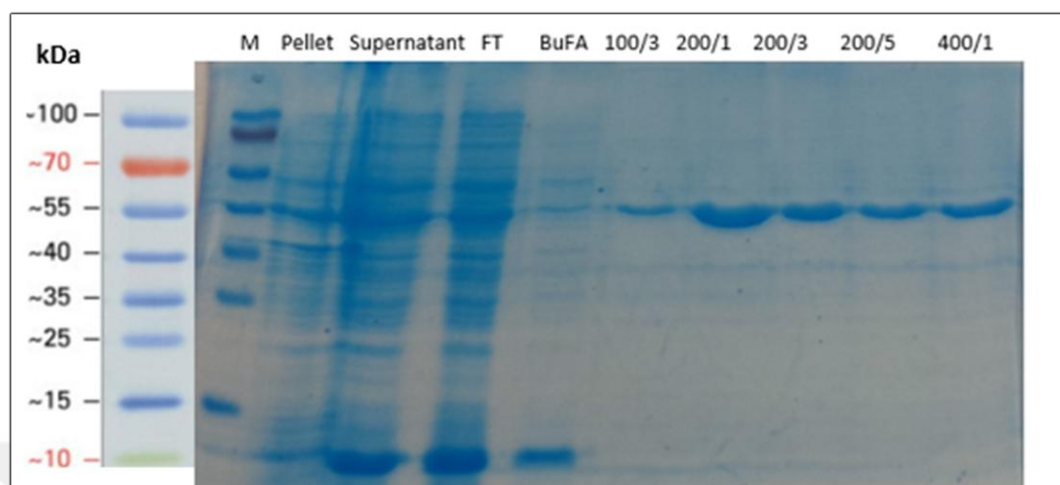


Figure 4.27: SDS-PAGE gel analysis of Tyr92Ser after purification (**M**: Marker, **Pellet**: Precipitate, **Supernatant**: Supernatant, **FT**: sample through the column, **BuFA**: Wash fraction, **100/3**: 100 mM imidazole, **200/1-5**: 200 mM imidazole **400/1**: 400 mM imidazole (elution fractions).

The gel picture in **Figure 4.27** shows that the purity level of the mutant was sufficient to perform specific activity and kinetic studies.

In order to determine the optimum pH values of the reactions catalyzed by the selected mutant Tyr92Ser enzyme, 50 mM Tris-HCl buffer at pH 7-9 and carbonate/bicarbonate buffer at pH 9-11 were used at different pHs. The candidate enzyme was assayed in 5 mM ketovalerate 0.5 M NH_4Cl in the indicated buffers and the appropriate amount of enzyme was calculated as relative activity using $\epsilon=6220 \text{ M}^{-1}\text{cm}^{-1}$, followed by absorbance decrease based on NADH depletion at 340 nm for one (1) minute. [Gmelch et al., 2019]. The effect of different pH on Tyr92Ser activity is shown below (**Figure 4.28**).

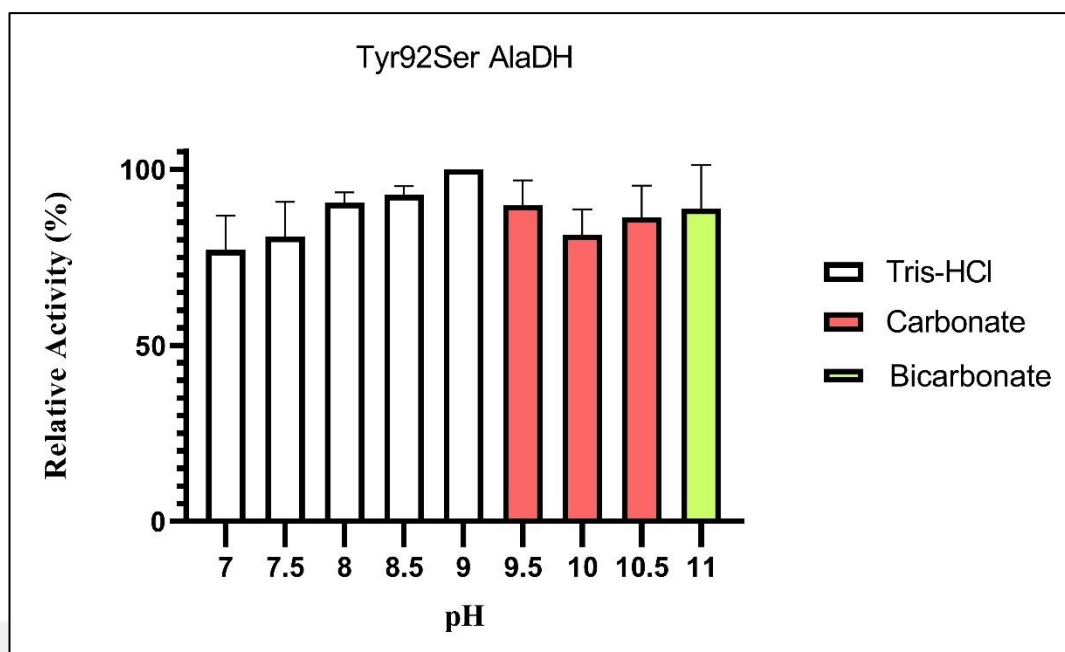


Figure 4.28: Effect of different pH on Tyr92Ser activity.

As presented in **Figure 4.28**, the Tris-HCl buffer at pH 9 exhibited the most optimal results in the investigation aimed at determining the optimal pH for the Tyr92Ser mutant. Using a range of buffer solutions, a thorough assessment of several pH values was carried out with the matching standard deviations to guarantee the accuracy and dependability of the results.

In order to determine the kinetic parameters of the purified Tyr92Ser enzyme, measurements were taken against different concentrations of pyruvate derivatives (α -ketovalerate, α -ketobutyrate and α -ketocaproate) at optimum pH/buffer. 0.5 M ammonium chloride was added as NH_4^+ source. 0.5 mM NADH and pure L-AlaDH enzyme were added and the reaction was initiated. Specific activity was calculated in units/mg using $\epsilon=6220 \text{ M}^{-1} \text{ cm}^{-1}$ by monitoring the absorbance increase based on NADH consumption at 340 nm for 10 min. [Gmelch et al., 2019]. Michaelis-Menten plots were drawn using the determined activity values (**APPENDIX-D**) and the $V_{\max}$ (maximum velocity) and K_M of the purified enzyme were determined using GraphPad Prism 9 [GraphPad Software, 2022] as shown at **Table 4.8**. Each experiment was repeated at least three (3) times in biochemical characterization studies.

Table 4.8: Kinetic constants of Tyr92 wild type and Tyr92Ser mutant with regard to ketoacids.

Substrate	Tyr92		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
α - ketocaproate	0.3 ± 0.0	0.5 ± 0.2	0.6 ± 0.1
α -ketovalerate	1.2 ± 0.0	3.3 ± 1.1	0.4 ± 0.0
α -ketobutyrate	1.1 ± 0.0	0.4 ± 0.1	3.0 ± 0.7

Substrate	Tyr92Ser		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
α - ketocaproate	29.7 ± 3.2	13.14 ± 2.5	2.25 ± 0.50
α -ketovalerate	2.47 ± 0.3	3.91 ± 0.70	0.67 ± 0.10
α -ketobutyrate	0.06 ± 0.0	3.10 ± 0.30	0.02 ± 0.00

Table 4.8 shows that the K_M value of the Tyr92Ser mutant enzyme increased for the three substrates when the mutant enzyme was compared to the wild type. However, the catalytic efficiencies of the Tyr92Ser enzyme against α - ketocaproate and α - ketovalerate increased due to a much higher increase in the k_{cat} constant. Compared to the wild type, the k_{cat} value of Tyr92Ser enzyme against α - ketocaproate increased about 99-fold, while the K_M value increased about 26-fold. The catalytic efficiency increased by about 6 times. The k_{cat} of the enzyme against α -ketovalerate increased approximately 2.2-fold, while the K_M value increased 1.8-fold. The catalytic efficiency of the Tyr92Ser enzyme against α -ketovalerate increased approximately two (2) fold. The k_{cat} of Tyr92Ser enzyme against α -ketobutyrate decreased approximately 18-fold, while the K_M increased approximately 8 times. This resulted in a 150-fold decrease in catalytic efficiency.

4.6.4. Modeling Studies and Examination of the Effects of Mutations on *TtAlaDH* Structure

The structure In the structural analysis of the Tyr92Ser mutant obtained from the 2eez models acquired in the previous reporting period, the selected substrates were initially added automatically using Autodock Vina [Trott and Olson, 2010]. Subsequently, manual visualization was conducted to correct the positioning of atoms using SwissPDB and PyMOL [Waterhouse et al., 2018; Schrödinger and Delano, 2020]

Structural modeling studies were conducted through molecular dynamics simulations of the Tyr92Ser mutant in the presence of α -ketovalerate, α -ketobutyrate, and α -ketocaproate. Autodock Vina was employed to dock Tyr92Ser with the substrate to identify amino acid positions directly involved in ligand binding [Trott and Olson, 2010]. Through automated substrate docking at the molecular level, amino acids at amino acid positions that directly interact with the substrate in the binding pocket of the enzyme were successfully identified. The predicted functional amino acid positions and possible interactions with substrates were obtained as shown in **Figure 4.29-4.31** [Schrödinger and Delano, 2020)]. Distances are in Ångström.

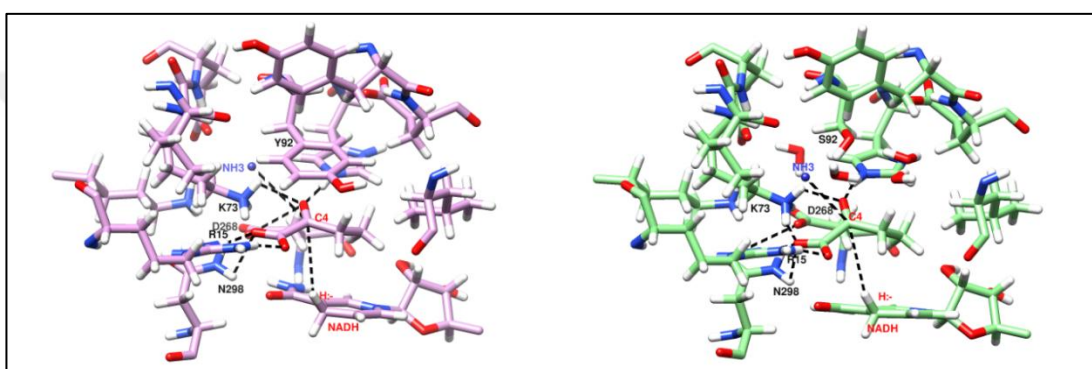


Figure 4.29: Modeling of wild type (Lilac) and Tyr92Ser (Green) with α -ketobutyrate including the positions of catalytic amino acids within the active site.

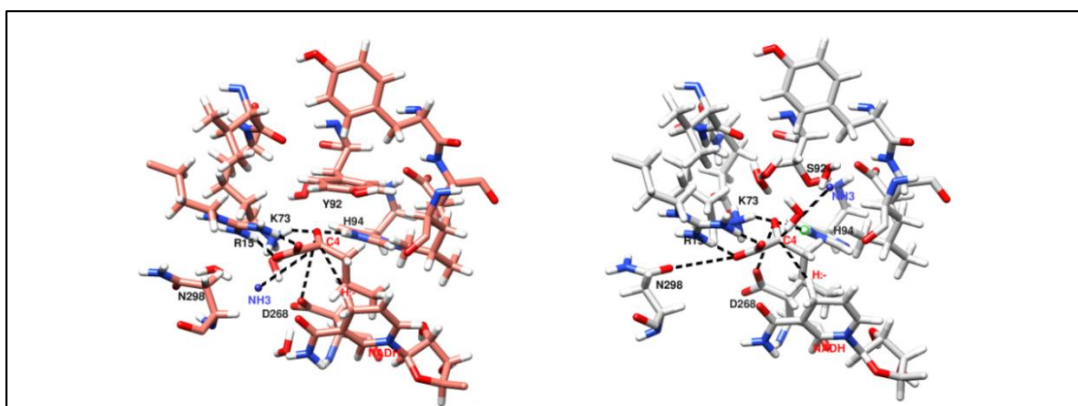


Figure 4.30: Modeling of wild type (Red) and Tyr92Ser (White) with α -ketovalerate including the positions of catalytic amino acids within the active site.

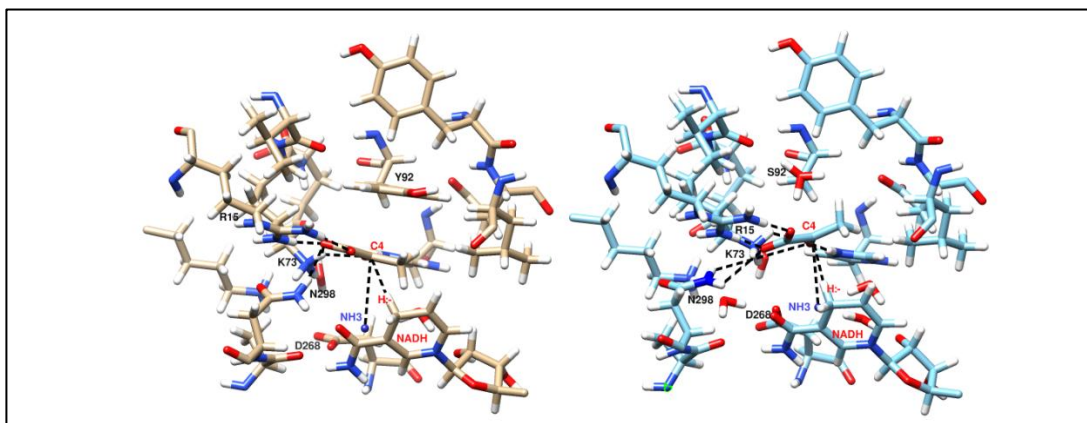


Figure 4.31: Modeling of wild type (Beige) and Tyr92Ser (Turquoise) with α -ketocaproate including the positions of catalytic amino acids within the active site.

The active site models of the wild-type and mutant enzyme are shown with different substrates and amino acids in the active site: Oxygen atoms are shown in red and nitrogen atoms in blue. H-bonds are shown between hydrogen and the electronegative atom.

As shown in the modeling figures, the Tyr92Ser mutation led to an increase in the volume of the L-AlaDH enzymes active site. By comparing the positioning of the aliphatic side chains of the target substrates within the active site and their size relative to the additional space created by the mutation, it becomes possible to understand why the mutation results in reduced activity against α -ketobutyrate, which has the smallest side chain. The expanded active site may reduce the enzyme's ability to properly orient or stabilize smaller substrates, leading to decreased catalytic efficiency. The enlargement of the "pocket" in the active site does not prevent the substrate from being directed to this site for conversion into the product. However, the mutation-induced volumetric growth of the "pocket" is likely to cause overfilling/occupying the site with water molecules before the substrate can bind. This situation can be seen as a filling or bridge formation between the substrate and the functional groups of the amino acids at the positions forming the active site. In other words, the substrate can occupy the active site only when it is surrounded by water molecules. Therefore, the lifetime of the active transition complex formed by enzyme-substrate interaction is significantly longer, making its conversion into the product either impossible or delayed over time. Therefore, an increase in both K_M and k_{cat} values was observed, indicating changes in the enzyme substrate affinity and catalytic turnover rate.

The opposite of what was predicted for α -ketobutyrate occurs for α -ketovalerate and α -ketocaproate. The side chains of these substrates easily fit into the additional volume created by the Tyr92Ser mutation and can interact with the catalytic amino acids to form products. The changes observed in the Michaelis-Menten kinetic constants obtained (as shown in **Table 4.8**) reinforce all these predictions.

However, it's crucial to consider that other substances dissolved in the water environment, such as $\text{NH}_3/\text{NH}_4^+$, can also access the enzyme's active site. The mutation from the large Tyr side chain to the smaller Ser not only provides more space for solutes but also alters the positions of other amino acids in the active site. Specifically, the Tyr92Ser mutation does not appear to affect the binding of the side chains of Arg15 and Lys73, located in the enzyme's active site, to the substrate, as their movement is restricted in the binding environment due to hydrogen bonding. This positioning ensures that the substrate remains in a conformation conducive to effective catalysis, thereby enhancing the catalytic reaction.

In contrast to the wild type, the activity of His94 on the substrate is weakened concerning the C=O group of the reacting substrate in the mutated structure, as evidenced by shorter hydrogen bonds measured in the mutated structure compared to the wild-type structure. Consequently, it is expected that the mutated enzyme structure will exhibit higher activity against α -ketovalerate and α -ketocaproate compared to α -ketobutyrate, a hypothesis supported by the calculated specific activity values in **Table 4.7** and the Michaelis-Menten kinetic constants in **Table 4.8**

Furthermore, Asp268 and Asn298 possess mobile side chains, which positively affect $\text{NH}_3/\text{NH}_4^+$ interactions, particularly in the presence of α -ketovalerate and α -ketocaproate. The positioning of $\text{NH}_3/\text{NH}_4^+$ and the different approach angles exhibit noticeable disparities compared to the measured values. However, proving all of these hypotheses solely through computational geometric analysis is challenging.

4.6.5. The Second Round Mutation Studies for Tyr92Ser *TtAlaDH*

A mutant enzyme containing Tyr92Ser mutation with increased activity against ketocaproate and ketovalerate compared to the wild type was obtained. It was decided to continue PCR studies by targeting new functional amino acid positions (such as Tyr115) on the most active mutant (Tyr92Ser) obtained.

There are bands in the Tyr115DBK and Ser92 wells. The pET-28a(+) vector containing the mutation bands was expected to be approximately 6488 bp in size. These bands indicate that the PCR was successful. The PCR products of the mutation Tyr115DBK were incubated with *DpnI* at 37 °C overnight and visualized on agarose gel (**Figure 4.32**).

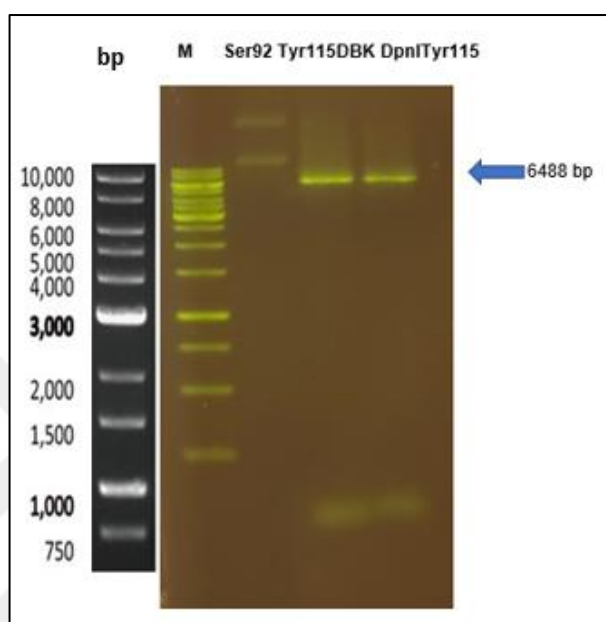


Figure 4.32: Agarose gel image after *DpnI* incubation of Tyr115. **M:** (Marker), **Ser92:** Ty292 mutant plasmid pET-28a (+), **Tyr115DBK:** PCR amplified Ser92 recombinant plasmid pET-28a (+), **DpnITyr115:** *DpnI* digested recombinant plasmid pET-28a (+) sample.

Clear visualization of the PCR output as a single band on an agarose gel is critical for gene sequence analysis and to obtain the number of colonies that will form the library containing the expected mutant diversity upon transformation into the host cell. PCR samples containing recombinant plasmids, confirmed by agarose gel image after incubation with *DpnI* enzyme, were transferred by heat shock into *E. coli* Top10 and BL21 (DE3) competent cells [Mandel and Higa, 1970]. The transformed cells were seeded on LB agar plates containing 50 µg/mL kanamycin and incubated at 37 °C for 16 hours. The colonies obtained are shown in **Figure 4.33**.

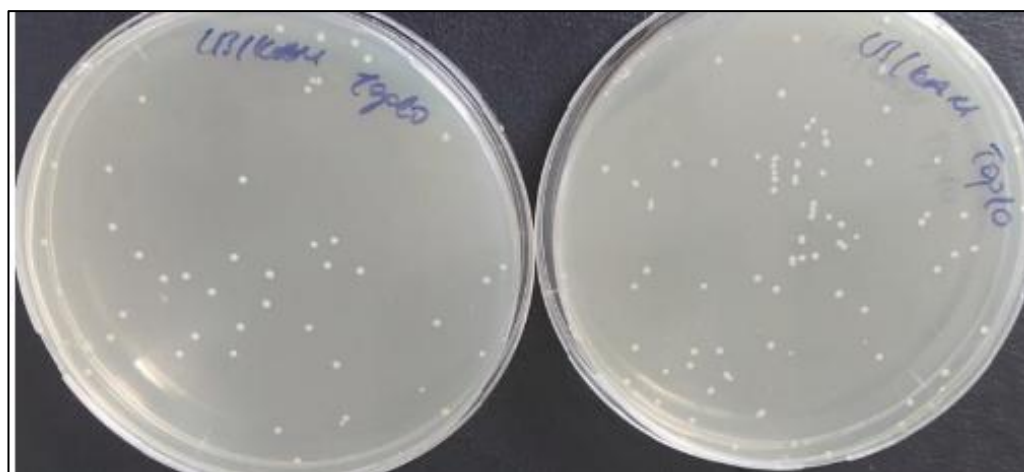


Figure 4.33: Recombinant colonies containing the Ser92 Tyr115 transformation

As shown in **Figure 4.33**, a sufficient number of colonies were obtained to analyze whether the relevant mutation occurred.

To confirm the mutation for the targeted Tyr115 position, colonies were selected from Petri plates and plasmid isolation was performed. The plasmid samples obtained to confirm the mutations were sent to for sequence analysis. T7 promoter primer with 5' TAATACGACTCACTATAGGG 3' nucleotide sequence was used for sequence analysis. The sequencing result was visualized with BioEdit program (BioEdit, 2023) since the free use of BioRender program has been discontinued and is shown in **Figure 4.34**.

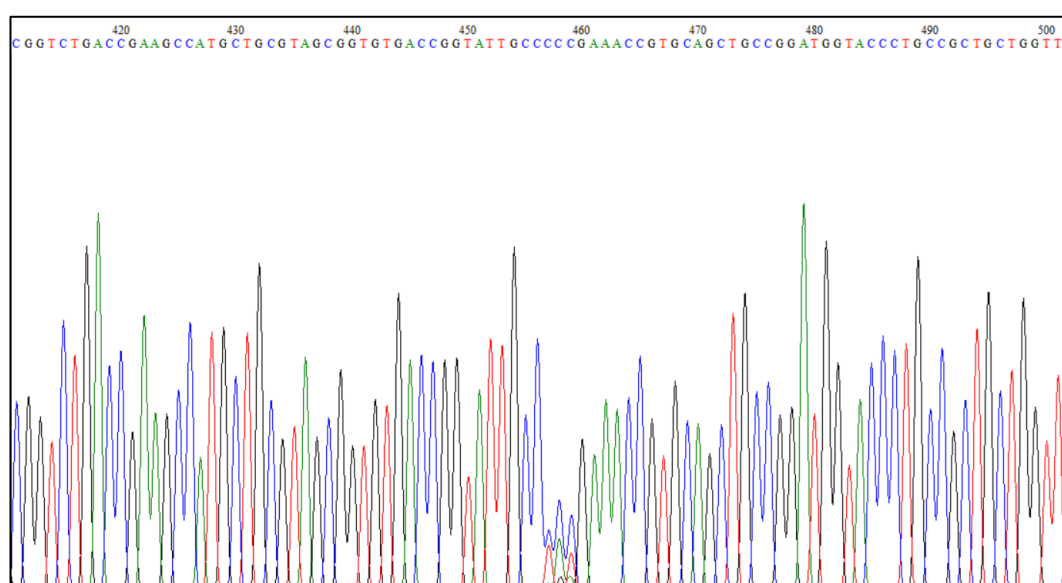


Figure 4.34: Screenshot of Bioedit program for the Tyr115DBK mutation sequence analysis results.

Each nucleotide represents a color, with blue representing cytosine, red thymine, green adenine and black guanine. Nucleotides numbered 457-458-459 in **Figure 4.34** correspond to the Tyr115 amino acid position. The numbering is based on the sequencing data. Each nucleotide is represented by a specific color, with cytosine shown in blue. In this region, the nucleotides from the DBK codon appear as blue peaks because cytosine predominates in all three positions. The presence of peaks of various colors along the 115 positions (5'GGT ATT GCC DBK GAA ACC GTG CAG CTG '3) where the mutation was introduced indicates different mutations originating from different colonies.

The plasmid library confirmed to contain mutations by sequence analysis was transformed into *E. coli* BL21 (DE3) [Mandel and Higa, 1970]. The transformed mutant library containing more than 100 colonies in a Petri plate is shown in **Figure 4.35**.

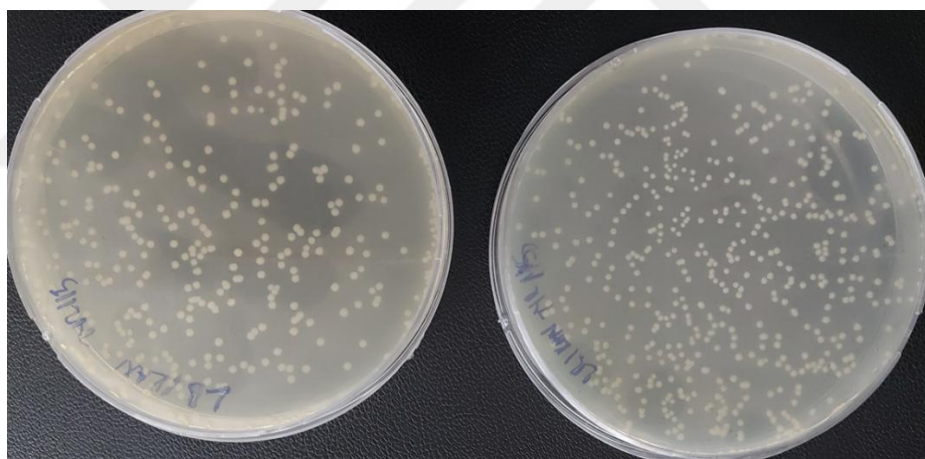


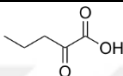
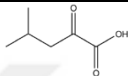
Figure 4.35: Mutant library for *TtAlaDH* Tyr115.

Mutant screening was performed as previously reported. Plasmids of colonies with high activity compared to wild-type enzyme were isolated and sent for sequencing.

As depicted in **Figure 4.35**, the presence of over 100 colonies signifies that the requisite number of colonies needed to cover all amino acids encoded by the DBK degenerate codon at the Tyr115 position has been attained [Reetz and Carballeira, 2007] With a sufficient number of colonies to achieve the objectives, we proceeded with screening the mutant library.

As a result of the analysis, Ala/Asp/Cys/Gly/Pro/Ser/Met/Lys mutations were identified at position Tyr115. The amino acids that can be assigned with the DBK degenerative codon are Ala/Arg/Cys/Gly/Ile/Leu/Leu/Met/Phe/Ser/Thr/Trp/Val. It is speculated that the Pro, Met and Lys amino acid could have been introduced as an "error" during the transcription phase or during the acquisition of Ser or Thr obtained with the DBK degenerative codon. The calculated specific activities (U/mg) for the high purity wild type mutant enzymes are shown in **Table 4.9**.

Table 4.9: Specific activity constant (U/mg) of high purity enzymes obtained from mutant *TtAlaDHs* against different substrates.

			
Enzyme	α -ketobutyrate	α -ketovalerate	α -ketocaproate
Ser92	0.80 ± 0.20	1.00 ± 0.15	2.50 ± 0.15
Tyr115Met	0.82 ± 0.15	0.05 ± 0.01	5.00 ± 0.10
Tyr115Lys	0.70 ± 0.15	0.04 ± 0.00	3.10 ± 0.30

When the activities of the mutants were examined, it was found that the Lys and Met mutants increased their activity against ketocaproate but largely lost their activity against ketovalerate. Pro activity was completely lost, and the other mutants did not show as much affinity toward substrates as the Tyr92Ser mutant. Specifically, the Tyr92Ser/Tyr115Met mutant exhibited a 2-fold increase in activity against ketocaproate, a 20-fold decrease in activity against ketovalerate, and no significant change in activity against ketobutyrate **Table 4.9** For definitive results, the optimal pH must be determined, and k_{cat}/K_M values need to be obtained.

In order to determine the optimum pH values of the reactions catalyzed by the selected mutant Tyr92Ser enzyme, 50 mM Tris-HCl buffer at pH 7-9 and carbonate/bicarbonate buffer at pH 9-11 were used at different pHs. The candidate enzyme was assayed in 5 mM ketovalerate 0.5 M NH_4Cl in the indicated buffers and the appropriate amount of enzyme was calculated as relative activity using $\epsilon=6220 \text{ M}^{-1}\text{cm}^{-1}$, followed by absorbance decrease based on NADH depletion at 340 nm for one (1) minute. [Gmelch et al., 2019], The effect of different pH on Tyr92Ser activity is shown below (**Figure 4.36**).

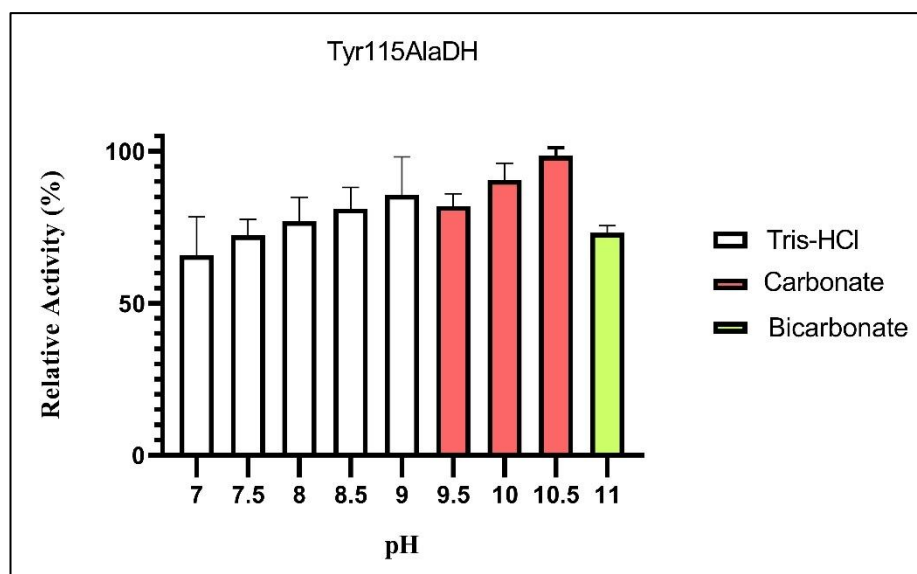


Figure 4.36: Effect of different pH on Tyr92Ser/Tyr115Met double mutant activity. As can be seen in **Figure 4.36**, the carbonate buffer solution at pH 10.5 yielded the most favorable results in the study to determine the optimal pH for the Tyr115Met mutant. Various pH values were tested using different buffer solutions, and the corresponding standard deviations are also presented.

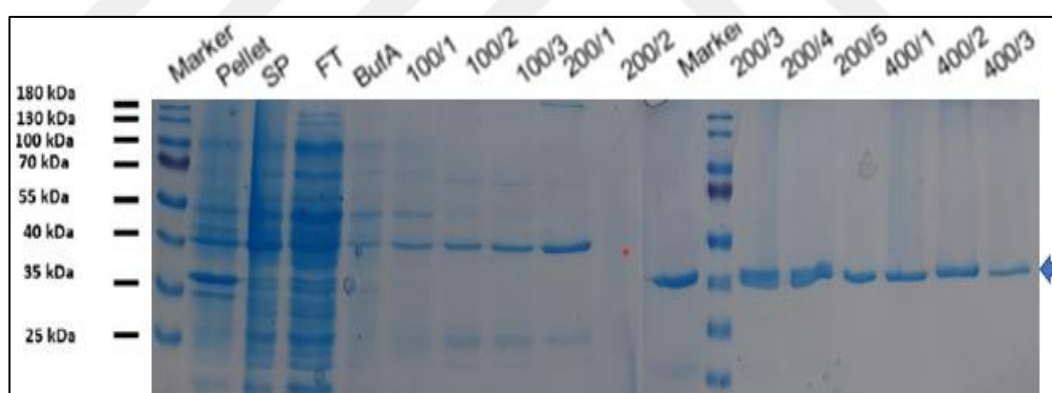


Figure 4.37: SDS-PAGE gel analysis of Tyr115Met after purification. Lanes: **Pellet**, **Marker**, **SP** (supernatant), **FT** (solution eluted from the column), Buffer A (**BufA**), and elution fractions **100/1-3**:100 mM, **200/1-5**: 200 mM imidazole. And **400/1-3**:400 mM imidazole.

As can be seen in **Figure 4.37**, the purity level of the mutant protein was sufficient for performing specific activity and kinetic studies. During the protein collection process, three 1 ml fractions were obtained with 100 mM imidazole, five 1 ml fractions with 200 mM imidazole, and three 1 ml fractions with 400 mM imidazole.

To determine the kinetic parameters of the purified Tyr115Met enzyme, measurements were performed using varying concentrations of pyruvate derivatives (α -ketovalerate, α -ketobutyrate, and α -ketocaproate) under optimal conditions. Michaelis-Menten plots were generated based on the measured activity values (**APPENDIX-E**), and the V_{max} and K_M values of the purified enzyme were calculated using GraphPad Prism 9 [GraphPad Software, 2022]. All biochemical characterization experiments were conducted in triplicate to ensure accuracy and reliability.

Table 4.10: Kinetic constant of Tyr115Met mutant with regard to ketoacids.

Substrate	Tyr115Met		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
α - ketocaproate	46.40 ± 5.200	18.61 ± 2.5	2.500 ± 0.22
α -ketovalerate	0.30 ± 0.015	15.50 ± 3.2	0.020 ± 0.00
α -ketobutyrate	0.17 ± 0.000	19.44 ± 5.8	0.008 ± 0.00

Table 4.10 shows that the catalytic activity of the Tyr115Met enzyme toward α -ketocaproate exhibited a significant increase compared to both the wild-type enzyme and the Tyr92Ser mutant. Specifically, the k_{cat} value increased approximately 155-fold relative to the wild type, accompanied by a 37-fold increase in the K_M value. Overall, the catalytic efficiency of the Tyr115Met mutant was enhanced by 4-fold compared to the wild type. In comparison to the Tyr92Ser mutation, the Tyr115Met enzyme showed a 1.5-fold increase in k_{cat} and a 1.4-fold increase in K_M , resulting in a 1.2-fold improvement in catalytic activity.

The catalytic activity of the Tyr115Met enzyme toward α -ketovalerate was significantly reduced compared to both the wild-type enzyme and the Tyr92Ser mutant. The k_{cat} value decreased by approximately 4-fold relative to the wild type, while the K_M value increased by 5-fold. Overall, the catalytic efficiency of the Tyr115Met mutant was reduced by 20-fold compared to the wild type. In comparison to the Tyr92Ser mutant, the k_{cat} value decreased by 8.2-fold, and the K_M value increased by 4-fold, leading to a 33.5-fold reduction in catalytic activity. Similarly, the catalytic activity of the Tyr115Met enzyme against α -ketobutyrate showed a significant decrease when compared to both the wild-type enzyme and the Tyr92Ser mutant. The k_{cat} (s^{-1}) value decreased by approximately 6.5-fold relative to the wild type, accompanied by a 49-fold increase in the K_M (mM) value. Overall, the catalytic

efficiency of the Tyr115Met mutant was reduced by 375-fold compared to the wild type. In comparison to the Tyr92Ser mutant, the k_{cat} (s^{-1}) value increased by 2.8-fold; however, the K_{M} (mM) value simultaneously increased by 6.2-fold, leading to an overall 2.5-fold reduction in catalytic activity.



5. DISCUSSION

In the initial phase of this thesis, the structural and kinetic properties of L-AlaDH from six different bacterial and archaeal sources were characterized to evaluate their potential in UAA production. Following this, the best mutants obtained from mutation studies were characterized, and changes in UAAs production were observed. Experimental studies provided significant insights into the potential of these enzymes for synthesizing UAAs.

The need for UAA manufacturing is rising as there is a growing need for amino acids and their derivatives in the development of functional foods and in the pharmacochemical industry. The current estimated market value of the amino acid industry is US\$25 billion [Wendisch, 2020]. Therefore, enzymes that are capable of being utilized in the production of UAA possess significant promise. The literature has described various methods for the chemical and enzymatic synthesis of L-amino acids. Because of their ecological sustainability and low cost, microbial synthesis has drawn most interest. [Sharma et al., 2017; Martínez-Rodríguez et al., 2020; Liu et al., 2022]. L-AlaDH is a significant enzyme involved in the synthesis of L-alanine, which is an essential intermediary in the biosynthesis of peptidoglycans in microorganisms. It also plays a vital role in maintaining balance in nitrogen-carbon metabolism [Giffin et al., 2012; Dave and Kadeppagari, 2019]. Given this feature, efforts have been made to produce several L-alanine derivatives with different ketoacids. Still, the yield has stayed modest, maybe in line with a poor conversion rate. The strong selectivity of the L-AlaDH enzyme when pyruvate is utilized as a substrate has been linked to this [Schröder et al., 2004; Dave and Kadeppagari, 2018; Hu et al., 2020; Van Wieren et al., 2020].

This thesis evaluated the ability of L-AlaDHs to perform reductive amination by utilizing pyruvate and its derivatives, including α -ketobutyrate, α -ketovalerate, and α -ketocaproate. Information on the reductive deamination reactions of L-AlaDHs is scarcely available in the literature. Ammonia is crucial in the reductive process of L-AlaDH enzymes because its unpaired electron pair enables it to create bonds with the carbonyl group of the substrate. Ammonia is expected to exert its influence in the catalytic reaction through nucleophilic substitution (SN1). The occurrence of the amination reaction requires the reaction between ammonia and the reactive carbonyl

of the substrate. This process might be facilitated by the presence of the acidic aspartate near the active site [Ågren et al., 2008; Ling et al., 2014]. While the distances between the substrates and cofactors in our models were of average magnitude, they nonetheless offer useful insights when comparing the interactions that bind the substrate. The substrate mostly forms hydrogen bonds with charged amino acids, such as arginine, histidine, and lysine. These interactions play a crucial role in maintaining its stability. *TtAlaDH* models can be employed for the purpose of comparing the mechanism of reverse reactions. In the *AfAlaDH* enzyme, pyruvate is bound by the amino acids Lys41, Lys65, Arg52, and Arg108. The catalytic activity of the enzyme is most likely facilitated by Lys65 and Asp219, as they are positioned in a way that promotes catalysis. The initial stage of the reaction involves the attachment of ammonia to the carbonyl moiety of pyruvate. Asp219 functions as an acid donor by transferring a proton to the first transition state. Lys65 functions as a base and accepts the proton. The carbinolamine intermediate is positively charged, resulting in an unfavorable repulsion of positive charges. During another transitional state, the water molecule is removed, resulting in the production of iminopyruvate. Subsequently, the hydride is transferred from NADH to iminopyruvate, resulting in the formation of L-alanine. The catalytic residues of *TtAlaDH* include His94 and Asp268, while the residues involved in pyruvate binding are Arg15, Lys73, and Asp298. Conformational modifications in the active site are necessary for the production of catalytic intermediates. They facilitate the nucleophilic attack of ammonia on the carbonyl group of pyruvate and the subsequent elimination of a water molecule. Furthermore, the structure of the nicotinamide ring of the NADH cofactor undergoes conformational changes during reaction [Gallagher et al., 2004]. Additionally, *AfAlaDH* has a higher alkaline optimal pH compared to *TtAlaDH*, leading to a slower protonation step at elevated pH values. The *AfAlaDH* and *TtAlaDH* models align with the finding that lysine serves as a significant acid-base catalyst [Grimshaw and Cleland, 1981]. The reverse reaction of *AfAlaDH* and *TtAlaDH* involves the keto acid substrate, ammonia, and NADH.

In the hydride transfer reaction, the carbonyl group that is involved is typically oriented towards the hydrogen atom of the C4 carbon in the NADH ring. The overall stability of the NADH structure significantly affects the end phase [Tripathi and Ramachandran, 2008]. Given the suggestion that the last stage of the substrate

reduction involves a hydride shift, it is clear that the reaction conditions must be completely free of water (anhydrous) [Cook et al., 1981; Gallagher et al., 2004]. Tyr92 in *TtAlaDH* has a role in maintaining the active site in a closed conformation and also helps align the carbonyl group of substrates with hydrogen in the NADH ring. The substrates aliphatic side chain interacts with hydrophobic amino acids in the active site. The ketocaproate molecule's biggest side chain occupies the smallest amount of area at the active site. The amino acids that impose the greatest limitations include Leu128 in *TtAlaDH*.

The optimal temperatures documented in prior research range from 25 to 82 °C (**Table 5.1**). The majority of enzymes demonstrate an optimum temperature at or below 50 °C, except for *AfAlaDH* [Schröder et al., 2004] and L-AlaDHs from *Thermus caldophilus* [Cho et al., 2003], and *Helicobacter aurati* [Hu et al., 2020]. Our study revealed that *MetAlaDH*, *VIAlaDH*, and *AcfhAlaDH* exhibit peak performance at a temperature of 50 °C. In our investigation, the *CbAlaDH* enzyme from archaea exhibited the highest level of thermotolerance, with an optimal temperature of 65 °C. It also shown considerable activity even at temperatures as high as 80–90 °C, as shown in **Figure 4.13**. Notably, the optimal value discovered was lower than the temperature at which *AfAlaDH* its highest activity. *AfAlaDH* the only archaeal L-AlaDH previously studied in the scientific literature, displayed its highest activity at 82 °C. [Schröder et al., 2004].

Table 5.1 demonstrates that the enzymes analyzed in prior studies have an ideal pH range that is predominantly alkaline, ranging from pH 7.0 to 10.5. The enzymes examined in our study had a comparable pH range of 7.0–10.5. The mean pH optimum ranged from 8.0 to 9.5, with only a small number of enzymes having an optimal value at pH 7.0 (**Table 5.1**). When examining the pH values of the obtained *TtAlaDH* mutants, it is observed that the optimal pH of the Tyr92Ser mutant increases to 9, whereas the optimal buffer for the Tyr92Ser/Tyr115Met double mutant changes to carbonate and the optimal pH increases to 10.5 (**Figures 4.28 and 4.36**). These findings suggest that the alkaline preference of L-AlaDHs is a common trait among these enzymes.

Table 5.1: Optimum pH, temperature and kinetic constant for the reported reductive amination reactions of L-AlaDH from different species with regard to pyruvate.

Source organism of AlaDH	Host Cell	Reductive amination			Temp.(°C)	Ref.
		pH	k_{cat} (s^{-1})	K_M (mM)		
<i>Thermus thermophilus</i>	<i>Thermus thermophilus</i>	8.5	-	0.75	25	[Vali et al., 1980]
<i>Phormidium lapideum</i>	<i>Phormidium lapideum</i>	8.4	-	0.33	-	[Sawa et al., 1994]
<i>Mycobacterium tuberculosis</i>	<i>E.coli</i> CAG629	7.5	-	1.45	-	[Hutter and Singh, 1999]
<i>Thermus caldophilus</i>	<i>E. coli</i> MV1184	8.0	-	0.20	60	[Cho et al., 2003]
<i>Vibrio proteolyticus</i>	<i>E.coli</i> TG1	8.0	-	0.61	50	[Kato et al., 2003]
<i>Archaeoglobus fulgidus</i>	<i>E.coli</i> BL21 (DE3)	7.0	118.0	0.16	82	[Schröder et al., 2004]
<i>Streptomyces anulatus</i>	<i>E.coli</i> BL21 (DE3)	8.5	-	0.80	40	[Dave and Kadeppagari, 2018]
<i>Aphanothece halophytica</i>	<i>E.coli</i> BL21 (DE3)	9.0	-	0.22	25	[Phogosee et al., 2018]
<i>Bacillus pseudofirmus</i>	<i>E.coli</i> BL21 (DE3)	10.5	18.0	1.00	40	[He et al., 2018]
<i>Streptomyces coelicolor</i>	<i>E.coli</i> BL21 (DE3)	9.0	$k_{cat} / K_M (s^{-1} mM)$ = 1.90		30	[Van Wieren et al., 2020]
<i>Helicobacter aurati</i>	<i>E.coli</i> BL21 (DE3)	8.0	-	0.56	55	[Hu et al., 2020]
<i>Amycolatopsis sulphurea</i>	<i>E.coli</i> BL21 (DE3)	-	-	-	25	[Aktaş, 2021]
<i>Thermomicrobium roseum</i>	<i>E.coli</i> BL21 (DE3)	9.5	35.0	1.9	25	[Dedeakay oğulları et al., 2023]

All L-AlaDHs exhibited enzymatic activity towards pyruvate, α -ketobutyrate, and α -ketovalerate, as indicated in **Table 4.3** *Met*AlaDH had the highest specific activity in relation to pyruvate compared to all other enzymes that were investigated. All exhibited higher levels of activity towards pyruvate compared to other substrates. Out of all the L-AlaDHs, α -ketobutyrate was the most favored substrate following pyruvate. The results of our study demonstrated that L-AlaDHs were capable of

catalyzing the reaction involving α -ketobutyrate and α -ketovalerate (as shown in **Table 4.3**). However, these enzymes did not display noteworthy activity towards α -ketocaproate, which could be attributed to the enlarged size of the aliphatic sidechain. Only *TtAlaDH* and *AfAlaDH* exhibited measurable activity when exposed to α -ketocaproate. According to current knowledge, it is clear that α -ketocaproate is unable to correctly place or orient itself within the active site of L-AlaDHs in order to perform catalytic conversion. Our analysis indicated that the K_M values for pyruvate (**Table 4.4**) varied between 0.2 and 1.3. The findings of our study indicate that the K_M value for *MetAlaDH* was greater than the K_M values reported in prior research for most of the examined L-AlaDH enzymes (**Table 5.1**). Furthermore, our investigation uncovered that the K_M values for pyruvate, which varied from 0.2 to 1.3, are consistent with those reported in earlier studies (**Tables 4.4 and 5.1**). The k_{cat} values for pyruvate exhibited a significant range of variation, ranging from 0.5 to 139.6 (**Table 4.4**). It is important to mention that the k_{cat} value of *MetAlaDH* (**Table 4.4**) is greater than the values reported in earlier research (**Table 5.1**). In our work, *VIAlaDH* exhibited the highest catalytic efficiency ($170 \text{ s}^{-1} \text{ mM}^{-1}$) for pyruvate, followed by *AcfhAlaDH* ($148.2 \text{ s}^{-1} \text{ mM}^{-1}$). Given that the K_M value remained rather consistent across the different enzymes, it is clear that the variance in catalytic efficiency can be attributed to significant differences in the catalytic rate of the enzyme process. *VIAlaDH* demonstrated the greatest specific activity among the enzymes examined for α -ketobutyrate and α -ketovalerate, with values of 13.2 U/mg and 1.9 U/mg, respectively (**Table 4.3**). The catalytic efficiency of *AfAlaDH* is higher for α -ketobutyrate and α -ketovalerate, with values of $34.0 \text{ s}^{-1} \text{ mM}^{-1}$ and $2.7 \text{ s}^{-1} \text{ mM}^{-1}$, respectively (**Tables 4.5 and 4.6**). Although the majority of enzymes did not show a reaction with α -ketocaproate, most likely because of its large molecular size, the *AfAlaDH* enzyme shown the highest specific activity towards α -ketocaproate ($2.8 \pm 0.3 \text{ U/mg}$) among the two enzymes that accepted it as a substrate (Table 1). Unfortunately, the lack of suitable data prevented the calculation of K_M and k_{cat} values according to the Michaelis-Menten model. Among the tested enzymes, only *TtAlaDH* exhibited quantifiable K_M and k_{cat} values for α -ketocaproate, measuring 0.5 mM and 0.3, respectively.

Modeling studies have suggested that a mutation at position Tyr92 will result in an increase in volume/space in the active region, enhancing its efficiency against larger

substrates such as ketocaproate and ketovalerate. It was anticipated that a mutation at the Tyr115 position would yield a comparable outcome. The DBK mutations were successfully executed at the Tyr92 and Tyr115 sites of *TtAlaDH*. The effective incorporation of these alterations was validated using PCR and agarose gel electrophoresis analysis. The colonies acquired through transformation facilitated the establishment of a diverse mutant library. Sequencing results confirmed the precision and efficacy of the mutations.

Figure 4.25 illustrates the acquisition of multiple mutants at the Tyr92 location. Based on the activity study, it was shown that all mutants exhibited an enhanced activity against ketocaproate compared to the wild type. The Ser92 and Ala92 mutants exhibit the highest level of enhanced activity towards ketocaproate. The only mutant that exhibited enhanced activity against ketovalerate was the ser92 mutant. It was noted that the activity of all acquired mutants against ketobutyrate diminished.

The Tyr92Ser mutation increases the volume/area of the enzyme's active site. This change alters the positioning of substrate side chains, particularly affecting α -ketobutyrate with its small side chain. The enlarged active site becomes preoccupied with water molecules, creating a barrier for the substrate. This situation prolongs the enzyme-substrate transition complex's lifetime. Consequently, the mutation leads to a significant delay or prevention in the conversion of the substrate into the product. Contrary to what was predicted for α -ketobutyrate, the side chains of α -ketovalerate and α -ketocaproate easily fit into the additional volume created by the Tyr92Ser mutation, allowing them to interact with the catalytic amino acids to form products. The changes observed in the Michaelis-Menten kinetic constants (as shown in **Table 4.8**) reinforce these predictions. The Tyr92Ser mutation increases the K_M value for all three substrates. Catalytic efficiencies against α -ketocaproate and α -ketovalerate improve significantly due to much higher increases in k_{cat} . For α -ketocaproate, k_{cat} increases 99-fold and K_M increases 26-fold, resulting in a six-fold increase in catalytic efficiency. For α -ketovalerate, k_{cat} increases 2.2-fold and K_M increases 1.8-fold, leading to a two-fold increase in catalytic efficiency. However, for α -ketobutyrate, k_{cat} decreases 18-fold and K_M increases eight-fold, causing a 150-fold decrease in catalytic efficiency. It was noted that the Tyr115Met mutant showed increased activity against ketocaproate **Table 4. 9**.

Table 4.10 highlights the effects of the Tyr115Met mutation on the catalytic activity of the enzyme toward three different substrates: for α -ketocaproate: the catalytic activity increased significantly compared to the wild-type enzyme and the Tyr92Ser mutant. The k_{cat} increased 155-fold relative to the wild type, while the K_M increased 37-fold, resulting in an overall 4-fold improvement in catalytic efficiency. Compared to Tyr92Ser, the k_{cat} increased 1.5-fold and the K_M 1.4-fold, leading to a 1.2-fold improvement. As for α -ketovalerate: The catalytic activity decreased substantially compared to both the wild type and Tyr92Ser. The k_{cat} decreased 4-fold, and the K_M increased 5-fold relative to the wild type, causing a 20-fold reduction in catalytic efficiency. When compared to Tyr92Ser, the k_{cat} decreased 8.2-fold and the K_M increased 4-fold, resulting in a 33.5-fold reduction. When we look at α -ketobutyrate: A significant decrease in catalytic activity was observed. The k_{cat} decreased 6.5-fold, and the K_M increased 49-fold compared to the wild type, leading to a 375-fold reduction in efficiency. Compared to Tyr92Ser, the k_{cat} increased 2.8-fold, but the K_M increased 6.2-fold, resulting in an overall 2.5-fold reduction in activity. In summary, the Tyr115Met mutation enhanced catalytic activity toward α -ketocaproate but significantly reduced activity toward α -ketovalerate and α -ketobutyrate.

Since there are limited studies in the literature on the use of different substrates by L-AlaDH and the only mutation study on this subject is in the direction of oxidative deamination, we cannot compare the k_{cat}/K_M values of the mutants we obtained. However, Fernandes et.al. also observed that the mutations they made towards oxidative deamination caused an increase in volume and area in the active site, and as a result, substrates with larger side chains were used more effectively [Fernandes et al.,2015].

The current research on the production of different alanine derivatives utilizing various ketoacids is lacking in comprehensive data about K_M and k_{cat} values. Only a few reports have been published on this topic [Schröder et al., 2004; Giffin et al., 2012; Dave and Kadeppagari, 2019; Hu et al., 2020]. Therefore, our research aimed to assess the capability of recently discovered L-AlaDHs and previously examined L-AlaDHs (from existing literature) to produce alanine derivatives. Our main objective was to determine the corresponding kinetic values of these enzymes. The current work offers useful insights into the possible uses of L-AlaDH in several biotechnological fields, including the advancement of antimicrobial medications and biosensors. Moreover,

the results of this study suggest that *VI*AlaDH, *Met*AlaDH, *Acfh*AlaDH, *Af*AlaDH, and *Tt*AlaDH, which were previously investigated, could be viable candidates for future research on protein engineering. We have effectively obtained mutants that are capable of synthesizing unnatural amino acids and utilizing substrates with larger side chains as a result of our mutation studies. The applications of these advancements in the pharmaceutical chemistry and food industry are particularly noteworthy. The objective of this thesis is to improve the functional and catalytic properties of L-AlaDH for a variety of biotechnological applications.



6. CONCLUSION

The primary aim of this thesis was to investigate the properties and functionality of L-AlaDH enzymes with regard to their ability to catalyse the reductive amination of pyruvate and its derivatives. To achieve this goal, the enzymes were expressed through a heterologous system in *E. coli* BL21 (DE3) cells and subsequently purified to obtain a homogenous sample for analysis. The results here indicate that L-AlaDH enzymes can use pyruvate and its ketoacid derivatives as substrates in reductive amination reactions with good efficacy. The enzymes demonstrated efficacy in synthesizing optically active unnatural chiral amino acids.

This thesis emphasizes the high potential for using the L-AlaDH enzyme from *Thermus thermophilus* to enhance its reductive amination efficiency for ketoacids by introducing specific mutations at the Tyr92 and Tyr115 positions. The Tyr92Ser and Tyr115Met mutants exhibited significant improvements in catalytic activity against α -ketocaproate and α -ketovalerate. These improvements make them interesting biocatalysts for synthesis of useful chiral alanine derivatives including norvaline and norleucine. But the study also highlights the intricacy of enzyme-substrate interactions since the mutants showed lower activity toward α -ketobutyrate. This result underlines the complex character of enzyme-substrate interactions and the need of more structural and kinetic investigations. Overall, these engineered L-AlaDH variants offer a sustainable approach for green chemistry applications, particularly in the pharmaceutical and agricultural industries, by providing an environmentally friendly method for synthesizing enantiopure UAAs.

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BIOGRAPHY

Ğarip DEMİR earned a degree in Molecular Biology and Genetics from Gebze Technical University in 2016. In 2018, he completed a master's program in the same field at Gebze Technical University. He then began his doctoral studies in the same department, where he has since been actively engaged in research, contributing significantly to the field with his dedication and expertise.



PUBLICATIONS AND PRESENTATIONS FROM THESIS

Demir, Ğ., Valjakka, J., Turunen, O., Aktař, 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), e26899. <https://doi.org/10.1016/j.heliyon.2024.e26899>

Demir, Ğ., Valjakka, J., Turunen, O., Binay, B. 2024. “A Study on Reductive Amination of *Thermus Thermophilus* L-alanine Dehydrogenase Mutants”, V. International Enzyme and Bioprocess Days / EBDays, 27-29 August 2024, İzmir/ Turkey

Demir, Ğ., Valjakka, J., Turunen, O., Binay, B. 2023. “A study on Reductive Amination of Newly Characterized and Previously Studied L-alanine Dehydrogenases”, IV. International Enzyme and Bioprocess Days / EBDays, 07-09 September 2023, Kars/ Turkey

Demir, Ğ., Koca, K.K., Arslantař, C., Akdoĝan, K., Binay, B. 2022 “Synthesis of unnatural amino acids of L-alanine derivatives from L-alanine dehydrogenase” III. International Enzyme and Bioprocess Days / EBDays 2022 09- 11 September 2022, Tokat Gaziosmanpařa University, Tokat / Turkey

APPENDICES

APPENDIX-A: VECTOR INFORMATION OF PET-28A (+) VECTOR MAP USED IN THIS THESIS

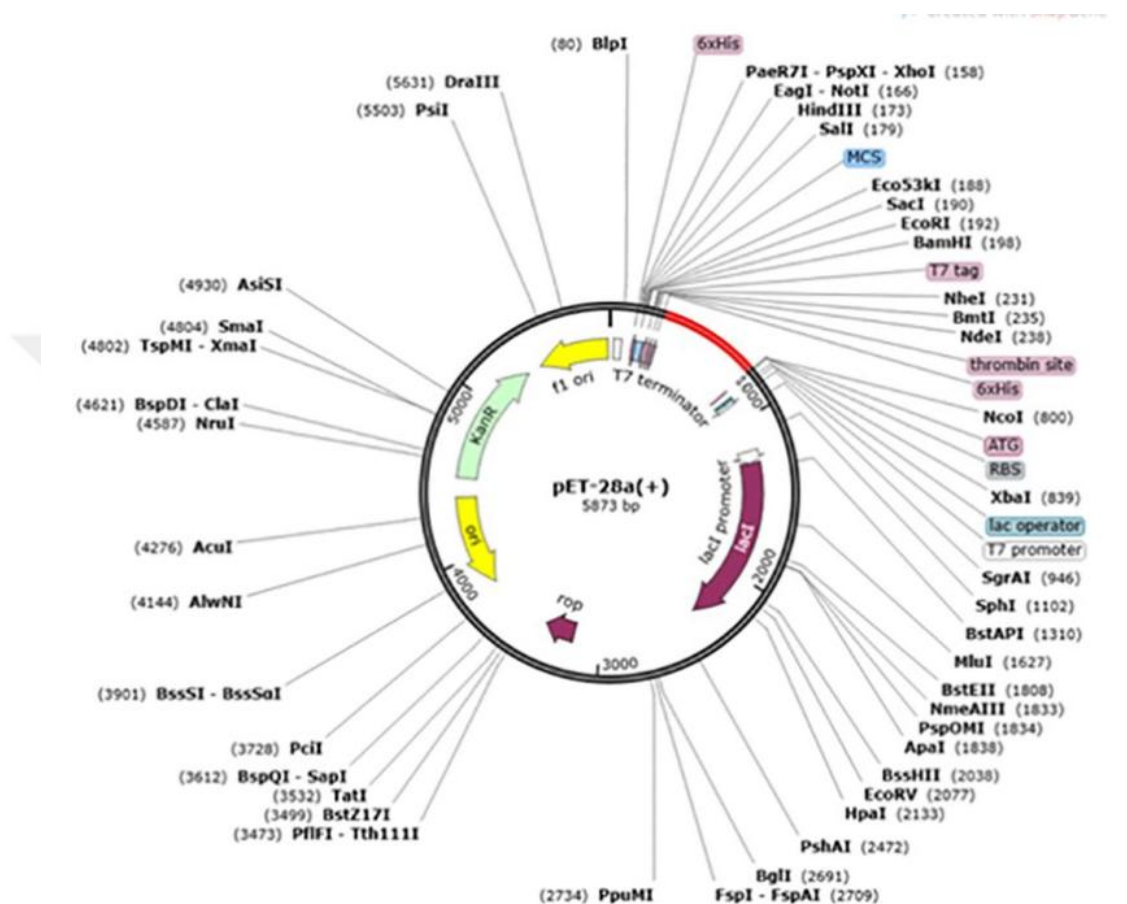


Figure A.1; pET-28a (+) vector map used in this thesis.

APPENDIX-B: NUCLEIC ACID SEQUENCES OF ALD GENES COMMERCIALY SYNTHESIZED AFTER CODON OPTIMIZATION FOR THE *E. COLI* EXPRESSION SYSTEM.

ATGAAGATTGGTGTTCGGAAGGAGATCAAAAACAACGAAAACCGTGTTGCGCTGACCCCGCGGGCGTGATG
ACCTGAAGAAAGCGGGTCACGAGGTTTTTCATTGAAACCGGTGCGGGTCTGGGCAGCGGTTTTACCGACGAG
CAGTATACCGAAGCGGGCGCGCAAATCGTGACCACCGCGAAAGAGGCGTGAGCCAGGAAATGGTTATGAAG
GTGAAAGAGCCGCTGCCGAGGAATACGATTATTTCTACGAAGGTCTGATTCTGTTTACCTATCTGCACCTG
GCGGCGGAGCCGGAACAGCAAAAGCGCTGATTGAGAAGAAAGTGGTTGGCATCGCGTACGAAACCGTTCAA
CTGGACAACGGTAGCCTGCCGCTGCTGACCCCGATGAGCGAGGTGGCGGGTCTGATGGCGACCCAGATTGGT
GCGCAATTCCTGGAAGACCGCACGGTGGCAAAGGCATCCTGCTGGGTGGCATTCGCGGGCGTTCTGCTGGC
AAGGTGACCATCATTTGGTGGCGGTGTGGTTGGCACCAACGCGGCGAAAATTGCGGTTGGCCTGGGTGCGGAT
GTTACCATCGTGGACCTGAACCCGGATCGTCTGCGTGAGCTGGACGACATCTTTGGTAGCAGCATTAAACACC
CTGATGAGCAACAGCCTGAACATTGCGGAGGCGGTGAAGGAAAGCGACCTGGTTATTGGTGGCGGTGCTGATT
CCGGGTGCGAAGGCGCCGAAACTGGTTACCGAGGATATGGTGAAAGAAATGAAGAAAGGACGCGTGATCGTT
GACGTGGCGATTGATCAGGGCGGTATCGTGGAGACCAGCGACCGTATTACCACCCACGATAACCCGACCTAT
GAAAAGCACGGTGTGGTTCACTATGCGGTTGCGAACATGCCGGGTGCGGTGCCGCGTACCAGCACCATTGGT
CTGACCAACGTTACCGTGCCGTATGCGGTTGAGCTGGCGAACAAGGGCTACAAACAAGCGTGCTGGACCA
GCGGCGCTGAAGAAAGGCATCAACACCTGAACGGTTATGTTACCTACCAAGCGGTGGCGGAAGCGCACGGT
CTGGAGTACGAAAACGTGGATCTGCTGCTGAAATAA

Figure B.1; Sequence of *metaladh* gene.

ATGATCATTGGTGTTCGGAAGGAGATTAAAAACAACGAAAACCGTGTTGGGTCTGACCCCGCGGGCGTGACC
GCGTTCAAGAACAACGGTCACGACGTGTGGGTTGAGACCAACGCGGGCATGGGTAGCGGCTTTACCGACGAA
GATTACACCGCGCGGGCGCGAAAATCGTTGATAGCGCGAGCGTGGTTTGGAGCGCGGAGATGGTGGTTAAG
GTGAAAGAACCCTGCCGAGGAATACACCTATTTCCGTGAGGGTCTGATTCTGTACACCTATCTGCACCTG
GCGCCGGAGCCGGAACAGCAAAAGCGCTGGTTGAGAAGAAAGTGATCGCGATTGCGTATGAAACCATTCAA
GCGGACAACGGCAGCCTGCCGCTGCTGACCCCGATGAGCGAGGTTGCGGGTCTGATGAGCATCCAGATTGGC
GCGCAATTTCTGGAAGAGCAAGGTGGCAAGGGTGTGCTGCTGGGTGGCGTTCCGGGTGTGGCGCCGGGC
GAAGTGGTTATTGTTGGTGGCGGTATTGTGGGCACCAACGCGGCGAAAATGGCGCTGGGTCTGGGTGCGAGC
GTGACCCTGCTGGACATTAGCGCGGATCGTCTGCGTCAGCTGGACGATATGTTCCAAGGTCTGCTGAAAACC
CTGATGAGCAACAGCTACAACATTGCGGAGGCGGTTAAGAAAGCGGATCTGGTGGTTGGTGGCGGTGCTGATC
CCGGGCGCGCTGCGCCGCTCTGGTTACCGAGGAAATGGTGAAGACCATGCAGCCGGGCAGCGTGATTGTT
GACGTGGCGATCGATCAAGGCGGTAGCATCGAAACCATGACCGTATCACCACCCACGATAACCCGACCTAC
GTTAAACACGGTGTGCTGCACTATGCGGTTGCGAACATGCCGGGTGCGGTGGCGCGTACCAGCACCATTGCG
CTGACCAACGTTACCGTGCCGTATGGTGTTCAGATCGCGAACAAGGGCTGAAACAAGCGGCGCTGGACAAC
AAGGCGCTGGCGAAAGGTATCAACGTGGTTGATGGCAAGGTTACCTACAAAGCGGTGGCGGAGGCGCACGGT
TATACCCCGGTTGCGGTGGAAACCGTGCTGACCGGTCAAAACGTTGGCGTGTA

Figure B.2; Sequence of *acfhaladh* gene.

ATGATCATCGGCATTCCGAAAGAAATTCTGGATAATGAAAACCGCGTGGCCCTGACCCCGGTTGGTGTGGAA
GCATTTCTGAAACAGGGCCATGAAGTTCGTGTGGAAAAGAAATGCAGGTGAAGGTAGCGGCTTTACCGATGAT
GCCTATGAAGCAGCCGGTGCAATTCTGAGCGATGATCCGGCCTTTGTTTGGCAGGCAGATATGGTTATGAAA
GTGAAAGAACCAGAACCGAGCGAATATCATTATTTTCGTGAAAATCTGATCCTGTTTACCTATCTGCATCTG
GCACCGGCAACCGAACTGACCCAGGCACTGATTGATGCAAAAAGTTACCGCAATTGCATACGAAACCATTACAG
CTGGAAGATCGTAGTCTGCCGCTGCTGATTCCGATGAGTGAAATTGCAGGCCGTATGAGTGTGCAGATTGGT
GCACAGCTGCTGGAAAAAGTAAAGGTGGCAAAGGCATTCTGCTGGCCGGTATTCCGGGTGTTAAAAAAGGT
AATGTGGTTATTATCGGTGGCGGCAATGTGGGTACCAATGCCGCAAAAATTGCAATTGGCCTGGGCGCCAAT
GTTACCATTCTGGATGTTAATCAGAAACGCCTGGCCGAACCTGGATGCCCTGTTTGGCAATGAAATTCAGACC
CTGATTAGCAATGCCCATAATATTGCCGAAACCGTGAAAACCGCCGATGTGGTTATTGGTACCGTTCTGATT
CCGGGCGCCAAAGCCCCGACCCTGGTTACCGAAGAAATGGTGGCCAGCATGGGTGAAGGCAGCGTTATTGTG
GATGTGGCCGTTGATCAGGGTGGCATTTTTGAACCATTGATCATGTTACCAGCCATAGCAATCCGACCTAT
GTTTCGTATGGTGTGATTCAATTATGCAGTGCCGAATATGCCGGGCAATGTGGCACGTACCAGCACCATTGCA
CTGACCAATGCCACCCTGCCGATGCCATGCAGATGGCAACCCATGATCTGAATGATGTTCTGCGTCAGACC
CCGAGCCTGCAGAAAGGTGTGAATATCTATCAGGGTGTGCTGACCAATGAACAGATTGCAGCCGCCAGAAT
CGCAAAATTCAGCAGCTGGCAAACTGATTGGTGAA

Figure B.3; Sequence of *cbaladh* gene

ATGATCATCGGCATTCCGAAAGAAATTCTGGATAATGAAAACCGCGTGGCCCTGACCCCGGTTGGTGTGGAA
GCATTTCTGAAACAGGGCCATGAAGTTCGTGTGGAAAAGAAATGCAGGTGAAGGTAGCGGCTTTACCGATGAT
GCCTATGAAGCAGCCGGTGCAATTCTGAGCGATGATCCGGCCTTTGTTTGGCAGGCAGATATGGTTATGAAA
GTGAAAGAACCAGAACCGAGCGAATATCATTATTTTCGTGAAAATCTGATCCTGTTTACCTATCTGCATCTG
GCACCGGCAACCGAACTGACCCAGGCACTGATTGATGCAAAAAGTTACCGCAATTGCATACGAAACCATTACAG
CTGGAAGATCGTAGTCTGCCGCTGCTGATTCCGATGAGTGAAATTGCAGGCCGTATGAGTGTGCAGATTGGT
GCACAGCTGCTGGAAAAAGTAAAGGTGGCAAAGGCATTCTGCTGGCCGGTATTCCGGGTGTTAAAAAAGGT
AATGTGGTTATTATCGGTGGCGGCAATGTGGGTACCAATGCCGCAAAAATTGCAATTGGCCTGGGCGCCAAT
GTTACCATTCTGGATGTTAATCAGAAACGCCTGGCCGAACCTGGATGCCCTGTTTGGCAATGAAATTCAGACC
CTGATTAGCAATGCCCATAATATTGCCGAAACCGTGAAAACCGCCGATGTGGTTATTGGTACCGTTCTGATT
CCGGGCGCCAAAGCCCCGACCCTGGTTACCGAAGAAATGGTGGCCAGCATGGGTGAAGGCAGCGTTATTGTG
GATGTGGCCGTTGATCAGGGTGGCATTTTTGAACCATTGATCATGTTACCAGCCATAGCAATCCGACCTAT
GTTTCGTATGGTGTGATTCAATTATGCAGTGCCGAATATGCCGGGCAATGTGGCACGTACCAGCACCATTGCA
CTGACCAATGCCACCCTGCCGATGCCATGCAGATGGCAACCCATGATCTGAATGATGTTCTGCGTCAGACC
CCGAGCCTGCAGAAAGGTGTGAATATCTATCAGGGTGTGCTGACCAATGAACAGATTGCAGCCGCCAGAAT
CGCAAAATTCAGCAGCTGGCAAACTGATTGGTGAA

Figure B.4; Sequence of *vlaladh* gene

ATGGTTATTGGTGTGCCGAAAGAAATTAAGACCTTGGAAAAATCGTGTGCGCTGACCCCGGCGCGGTGAA
AGTCTGGTGGCGCGTGGCCATACCGTGCTGGTTGAACGCGCGCAGGTGAAGGTAGCGGTCTGAGCGATGCA
GAATATGCACGTGCAGGCGCAGAACTGGTGGGTCTGTAAGAAGCATGGGGCGCAGAAATGGTGGTTAAAGTT
AAAGAACCCTGCCGGAAGAATATGGCTTTCTGCGTGAAGGCTGATTCTGTTTACCTATCTGCATCTGGCC
GCCGATCGCGGTCTGACCGAAGCCATGCTGCGTAGCGGTGTGACCGGTATTCCTATGAAACCGTGCAGCTG
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CAGTTTCTGGAAAAACCGAAAGGCGGCGCGGTGTGCTGCTGGGTGGCGTGCCTGGTGTGGCACCAGGCTAGC
GTGGTTATTCTGGGCGGCGGTACCGTTGGTACCAATGCCGCAAAAATTGCACTGGGTATGGGTGCCAGGTG
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ACCGCCACCGAAGCCAATATTAAGAAAAGCGTGCAGCATGCCGATCTGCTGATTGGCGCAGTGTGGTGCCG
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GTTGCCGTTGATCAGGGTGGTTGCGTTGAAACCATTGCCCGACACCCATGCAGAACCAGCCTATGTGGTT
GATGGCGTTGTTCAATTATGGTGTGCAAAATATGCCGGGTGCAGTTCGCGGTACCAGCACCTTTGCACTGACC
AATCAGACCCTGCCGATGTTCTGAAACTGGCAGAAAAAGGCTGGATGCACTGCTGGAAGATGCCGCACTG
CTGAAAGGTCTGAATACCCATAAAGGTCTGCTGACCCATCCGGGTGTGGCCGAAGCCTTTGGTCTGCCGTAT
ACCCCGCCGGAAGAAGCCTGCGCGGC

Figure B.5; Sequence of *tlaladh* gene

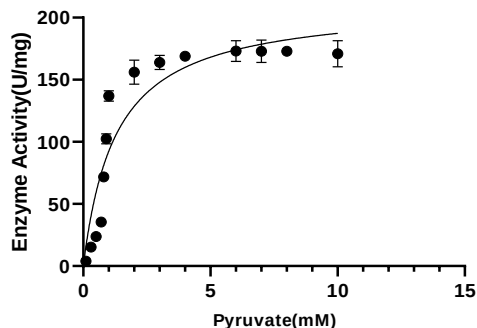
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 GGCGAAGTGATTGCGGGCCTGAAAGATGGCCGCGAAAGCGATGAAGAAATCACCATTTTCGATAGCACGGGC
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Figure B.6; Sequence of *afaladh* gene

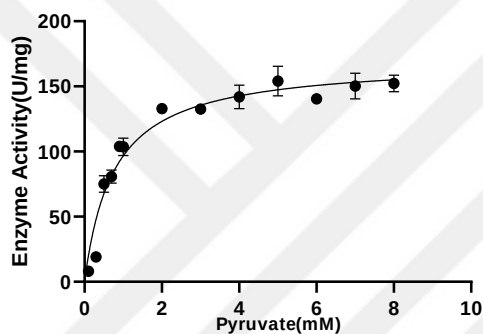


APPENDIX-C: MICHAELIS-MENTEN PLOTS OF L-ALADH ENZYMES AGAINST SUBSTRATES USED IN THIS THESIS.

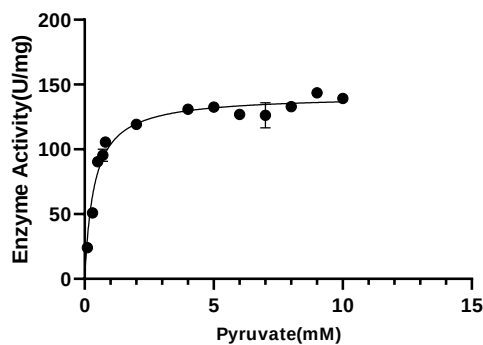
*Met*AlaDH Pyruvate Michaelis-Menten Graph



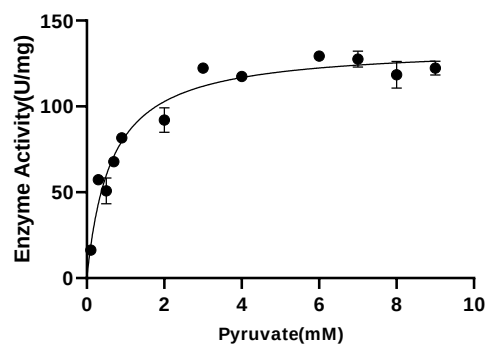
*Acfh*AlaDH Pyruvate Michaelis-Menten Graph



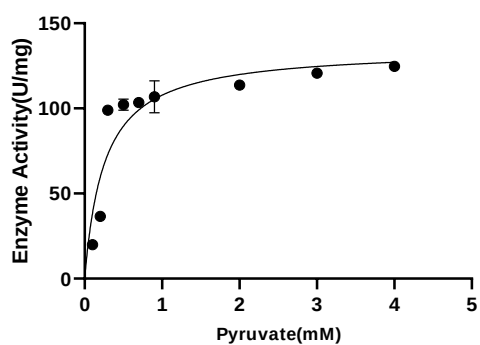
*VI*AlaDH Pyruvate Michaelis-Menten Graph



*Tt*AlaDH Pyruvate Michaelis-Menten Graph



*Af*AlaDH Pyruvate Michaelis-Menten Graph



*Cb*AlaDH Pyruvate Michaelis-Menten Graph

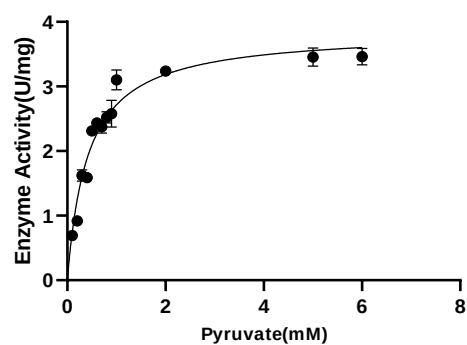


Figure C.1; Michaelis-Menten graphs of L-AlaDH enzymes against pyruvate as a substrate

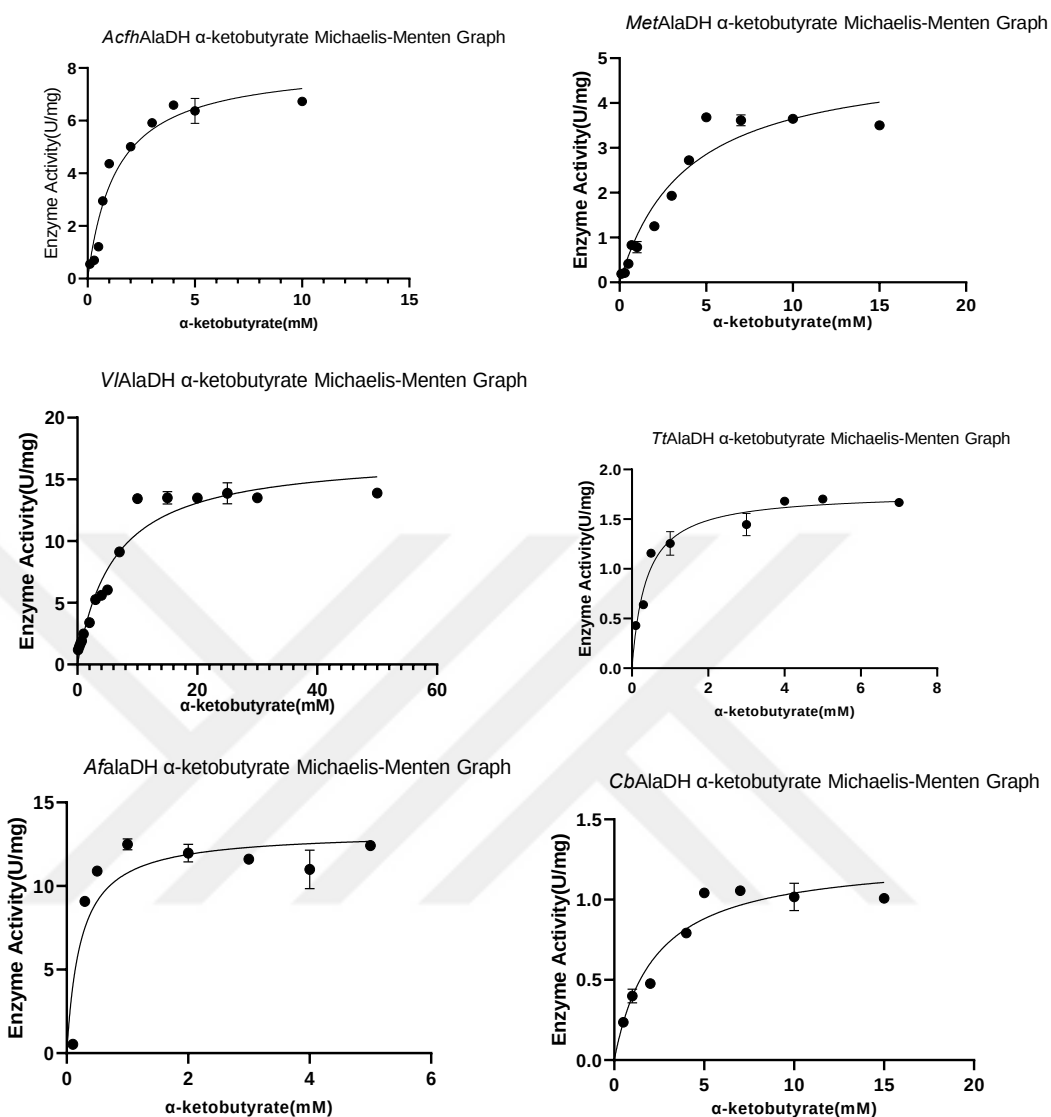


Figure C.2; Michaelis-Menten plots of L-AlaDH enzymes against α -ketobutyrate as substrate.

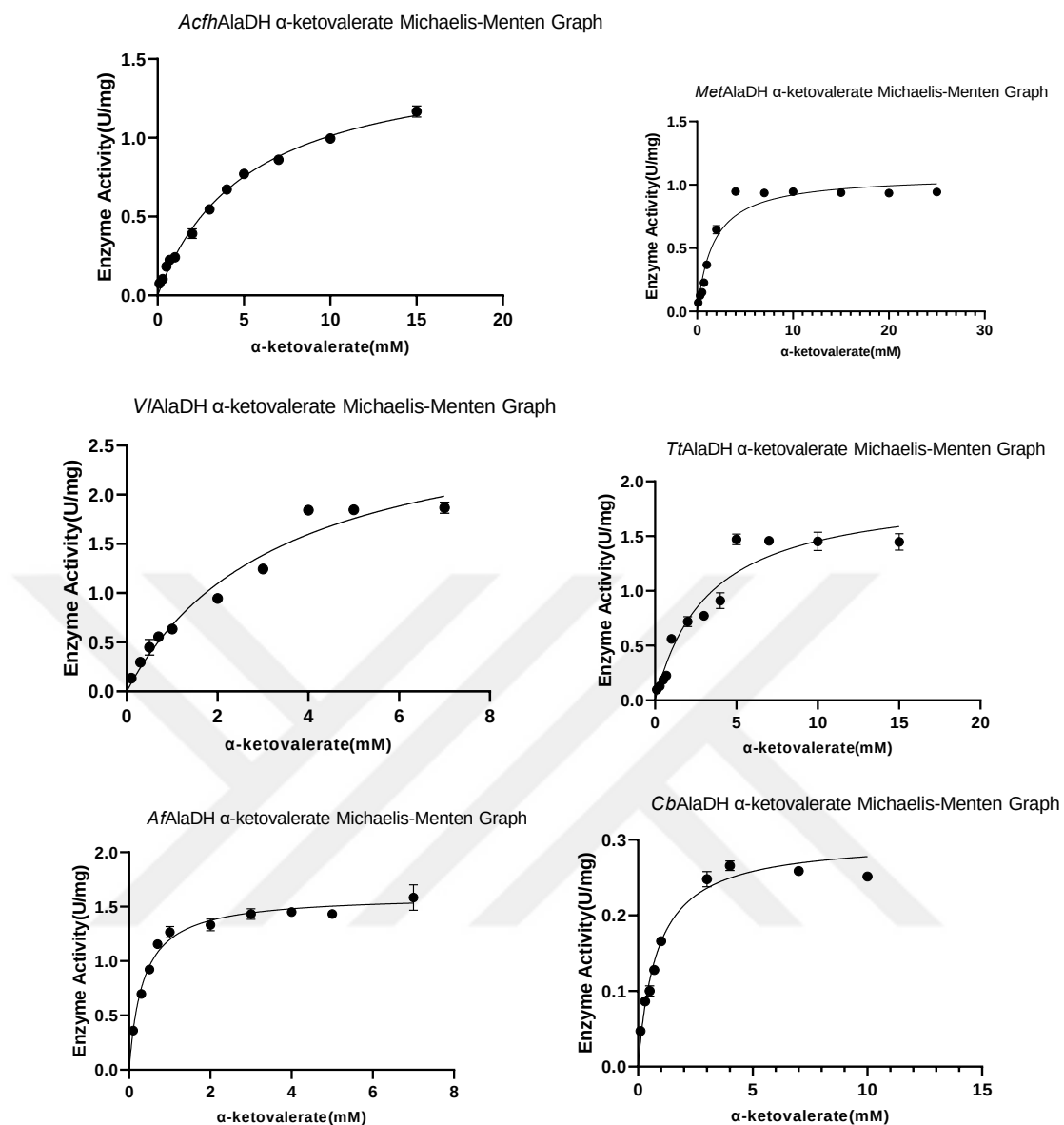


Figure C.3; Michaelis-Menten plots of L-AlaDH enzymes against α -ketovalerate as substrate.

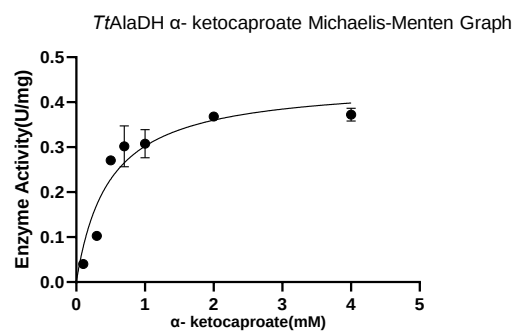


Figure C.4; Michaelis-Menten plots of L-AlaDH enzymes against α --ketocaproate as substrate.

APPENDIX-D: MICHAELIS-MENTEN PLOTS OF THE TYR92SER MUTANT AGAINST SUBSTRATES.

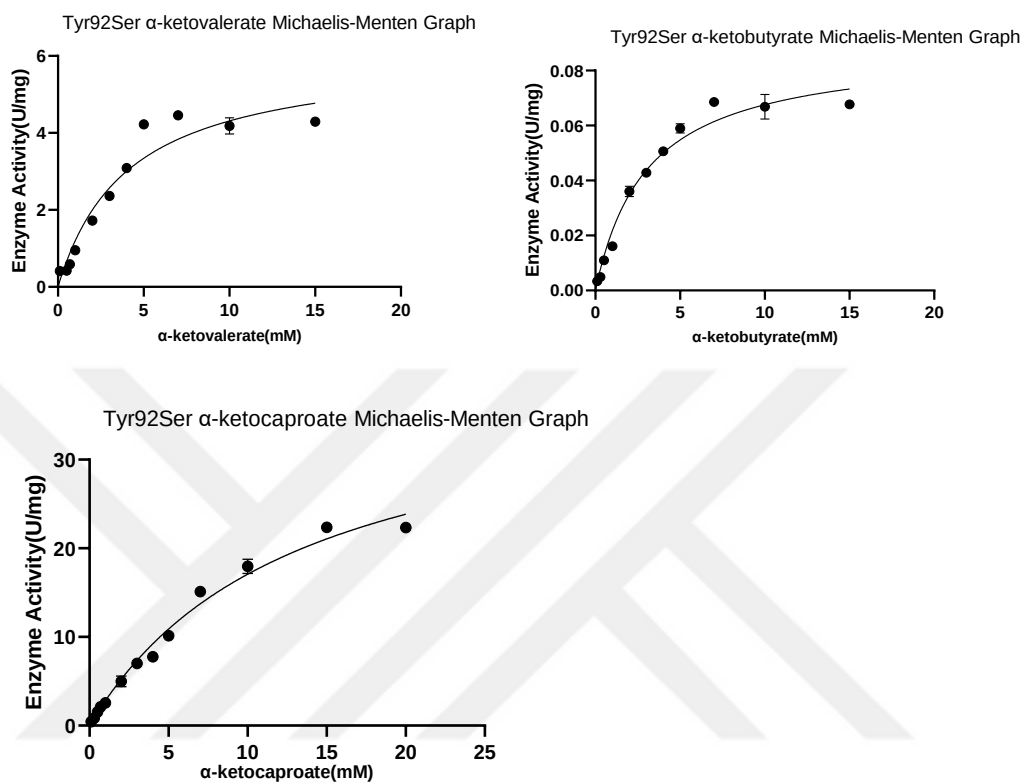


Figure D.1; Michaelis-Menten plots of Tyr92Ser mutant against substrates.

APPENDIX-E: MICHAELIS-MENTEN PLOTS OF THE TYR115MET MUTANT AGAINST SUBSTRATES.

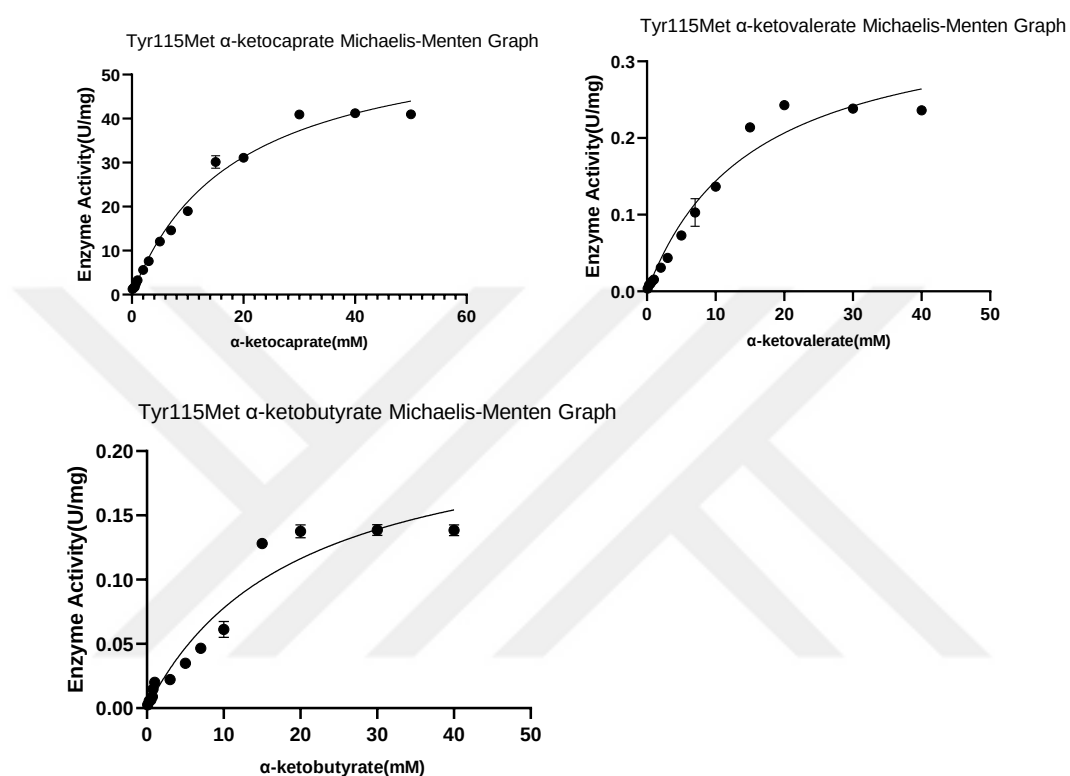


Figure E.1; Michaelis-Menten plots of Tyr115Met mutant against substrates