

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**EFFECT OF AMINO ACID POSITIONS LOCATED ON LINKED
REGION ON CHAETOMIUM THERMOPHILIUM FORMATE
DEHYDROGENASE (CTFDH) ACTIVITY**

ELİF GÜNAY
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
ASSOC. PROF. DR. BARIŞ BİNAY

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2021

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CHAETOMIUM THERMOPHILIUM
FORMAT DEHİDROGENAZ (CTFDH)
AKTİVİTESİNE BAĞLANTI
BÖLGESİNDEKİ AMİNO ASİT
POZİSYONLARININ ETKİSİ

ELİF GÜNAY
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

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2021



YÜKSEK LİSANS JÜRİ ONAY FORMU

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JÜRİ

ÜYE

(TEZ DANIŞMANI) : Doç. Dr. Barış BİNAY

ÜYE

: Prof. Dr. Bilge Hilal ÇADIRCI EFELİ

ÜYE

: Dr. Öğr. Üyesi Saliha İŞSEVER ÖZTÜRK

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

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SUMMARY

Dehydrogenase enzymes, which are in the oxidoreductase enzyme group, are responsible for the synthesis of optically active substances. Nicotinamide Adenine Dinucleotide-dependent Formate Dehydrogenase (NAD^+ -dependent FDH) uses formate as a substrate to catalyze its conversion to CO_2 while reducing from NAD^+ to NADH. FDH enzymes have become an enzyme used in industrial and chemical fields in terms of NADH regeneration. Recently, studies have been carried out by discovering that the enzyme *Chaetomium thermophilum* FDH (CtFDH) also has a high potential in the reduction from CO_2 to formate.

Our aim in this thesis is to increase the enzyme activity and thermostability of the NAD^+ -dependent CtFDH by site directed mutagenesis method which is one of the protein engineering technics.

In the study, primers containing codons of Glu164Ala and Asp188Arg CtFDH mutations were designed. Polymerase chain reaction (PCR) was performed with these degenerate primers. In order to measure the enzyme activity and thermostability of the mutant Glu164Ala and Asp188Arg CtFDHs, were purified at molecular level with Ni-NTA HisTrap columns using affinity chromatography method.

Studies conducted for the characterization of pure enzymes have shown that Glu164Ala mutation increases specific activity and thermostability towards sodium formate, and that the Asp188Arg mutation decreases specific activity towards sodium formate, increases thermostability and increases specific activity and thermostability towards sodium bicarbonate compared to wild type.

This thesis study is important in terms of increasing the activity of NAD^+ -dependent FDH enzyme, which is used in the academic field and industrial processes, and providing data for its production on large scale.

Key Words: NAD^+ -dependent CtFDH, protein engineering, specific activity, thermostability.

ÖZET

Oksidoredüktaz enzim grubu içerisinde yer alan dehidrogenaz enzimleri optikçe aktif maddelerin sentezlenmesinde görevlidirler. Nikotinamid adenin dinükleotit Format Dehidrogenaz (NAD^+)-bağımlı FDH) formatı substrat olarak kullanarak CO_2 'e dönüşmesini katalizlerken, NAD^+ 'i NADH formuna indirger. FDH enzimleri NADH rejenerasyonu açısından da endüstriyel ve kimyasal alanda kullanılan bir enzim haline gelmiştir. Son zamanlarda, *Chaetomium thermophilum* FDH (CtFDH) enziminin de CO_2 'nin formata indirgenmesinde yüksek potansiyele sahip olduğu keşfedilerek çalışmalar yapılmıştır.

Bu tez çalışması ile amacımız, NAD^+ -bağımlı CtFDH enziminin protein mühendisliği yöntemlerinden birisi olan yönlendirilmiş bölge mutagenез yöntemi ile üretilmesi, enzim aktivitesi ve termostabilitesinin artırılmasıdır.

Çalışmada, CtFDH protein geni üzerinde Glu164Ala ve Asp188Arg mutasyonlarını içeren primerler tasarlanmıştır. Bu dejenere primerler ile polimeraz zincir reaksiyonu (PCR) yapıldı. Mutant Glu164Ala ve Asp188Arg CtFDH'lerin enzim aktivite ve termostabilite ölçümleri yapılmak üzere, elde edilen proteinler afinite kromatografisi yöntemi kullanılarak Ni-NTA HisTrap kolonları ile moleküler düzeyde saflaştırıldı.

Saf enzimlerin karakterizasyonu için yapılan çalışmalarda yabancı tip ile karşılaştırıldığında Glu164Ala mutasyonunun sodyum formata karşı spesifik aktiviteyi ve termostabiliteyi artırdığı ve Asp188Arg mutasyonunun sodyum formata karşı spesifik aktiviteyi düşürdüğü termostabiliteyi artırdığı ve sodyum bicarbonata karşı spesifik aktiviteyi ve termostabiliteyi artırdığını göstermiştir.

Bu tez çalışması, akademik alanda ve endüstriyel proseslerde kullanım alanı olan NAD^+ -bağımlı FDH enziminin aktivitesinin artırılması ve büyük ölçekte üretilmesinde veri sağlanması açısından önemlidir.

Anahtar Kelimeler: NAD^+ -bağımlı CtFDH, protein mühendisliği, spesifik aktivite, termostabilite.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
Å	: Angstrom
°C	: Celsius Degree
μ	: Micro
3-D	: Three-Dimensional
Ala	: Alanine
Arg	: Arginine
Asp	: Aspartic acid
CO ₂	: Carbon Dioxide
CtFDH	: <i>Chaetomium thermophilum</i> Formate Dehydrogenase
DNA	: Deoxyribonucleic acid
Glu	: Glutamic acid
HCl	: Hydrochloric Acid
L	: Liter
LB	: Luria-Bertani
mg	: Milligram
min	: Minute
mM	: Millimolar
NAD ⁺	: Nicotinamide Adenine Dinucleotide
NAD(P)	: Nicotinamide Adenine Dinucleotide Phosphate
NaPi	: Sodium Phosphate
PCR	: Polymerase Chain Reaction
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Super
SOC	: Super Optimal Broth with Catabolite Repression
Val	: Valine
Wt	: Wild type

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1.INTRODUCTION

1.1. Aim and Contribution

Enzyme-catalyzed processing has been employed industrially for centuries. Fermentation processing is well-recognized, for instance, making cheese, bread, wine, and beer. The biocatalysis process is a purifier, easier and more rapid to work compared with the microbial process [Scibioh and Viswanathan, 2018].

The largest users of industrial enzymes are Europe and North America; but, a request is quickly augmentation in China, Japan, and India [Patel et al., 2016]. Food and drinks, which are an integral part of human life, create a very large area for enzyme use. This area is likely to grow further as the industrial enzyme market is projected to grow to \$ 17.2 billion by 2027 [Miguel et al., 2013].

To increase the yield from the protein, it would be useful to know well the enzyme structure and its function and modifications. The engineering of genetics contains a few methods bear upon the manipulation of molecules to modify novel gene with beneficial properties. Any change that can be made on the gene sequence can directly affect the protein and create changes in its structure. [Sharma et al., 2019]. The rational design method can be used to explain how properties of the enzyme are affected by residues known to be functional in proteins.

FDH is an enzyme frequently encountered in nature in order to obtain energy in methylotrophic organisms and as a stress response developed by plants against negative conditions in their environment [Tishkov and Popov, 2004].

FDHs are cheap and existing, and the cost of generation of the enzyme is low. The content of FDH in the beginning strains of methylotrophic bacteria and yeasts may be up to 10-18% of all protein, and recombinant DNA technology can use for acquiring of higher expression of the requested product.

The carbon-hydrogen bond between the substrate and the FDH is broken in reaction and a new carbon-hydrogen bond the transfer of the hydride ion is formed between NADH and the enzyme [Schiott et al., 1998]. The simple nature of the substrate of the FDH enzyme used in the reaction and the absence of proton transfer in the reaction increased the number of studies on FDH [Tishkov and Popov, 2004]. The efficiency in the synthesis of bulk drug materials and agricultural yields, chiral

intermediates, and fine chemicals is in high request from the agrochemical industry and pharmaceutical [Patel, 2003].

The FDH enzyme is a good candidate for cofactor regeneration and catalyzes an irreversible reaction with high catalytic efficiency even in ambient conditions. Formate and CO₂ molecules in NADH regeneration studies are unlikely to affect the enzyme used in the paired reaction. The ability of FDHs to catalyze between pH 5.5 and pH 11 allows a coupled reaction to being established with other dehydrogenases [Schirwitz et al., 2007].

The rapid increase of CO₂, CH₄ and NO_x gases in the atmosphere has been followed for years. The increase of CO₂ is the result of all kinds of fossil fuel consumed by humans, it is estimated that the CO₂ in the atmosphere has increased by 30% after the industrial revolution. When all this information is considered, taking various precautions and technological developments in order to reduce the CO₂ concentration is necessary. Steps to reduce the concentration of CO₂ in the atmosphere were taken in the Kyoto Climate Change Conference (COP3). Besides, according to the Paris climate agreement, the limitation of the release of greenhouse gases to nature, which is valid since 2020, has been taken and the United Nations Framework Convention on Climate Change (UNFCCC) has been shown as funding [Amao, 2018].

For CO₂ fixation, the idea of reducing the amount of CO₂ by using it as a substrate in biocatalytic reactions, thus using the products formed as a renewable fuel as a result of the reaction is interesting [Baskaya et al., 2010; Crable et al., 2011; Tong et al., 2011]. The studies involving FDH enzymes have been carried out to reduce CO₂ and obtain chemicals such as formate or methanol [El-Zahab et al., 2008; Le et al., 2011]. Formic acid is noted to be a hopeful substitution for methanol in miniature fuel cells [Jung et al., 2007].

Especially for NADH regeneration and CO₂ fixation, FDH obtained from *Chaetomium thermophilum*, a thermophilic microorganism, has been found to be a good candidate. In this study, strengthen the interaction between subunits was aimed at transforming the Glu164 residue, located at the CtFDH interface, to alanine. To increasing the enzyme activity of the mutations designed by the closeness of the active site of CtFDH to this subunit interaction region is aimed. By converting the Asp188 residue to arginine was aimed to increase the thermostability and enzyme activity by increasing the pKa of the Glu164 residue.

2. LITERATURE REVIEW

2.1. *Chaetomium thermophilum*

Fungi are the largest eukaryotic diverse organism in our environment, with a conjectural of 1.5 million species and can be said to be the main decomposers [Capote et al., 2012]. Many species of fungi are used in medical and commercial areas, and it is known that they are beneficial for living creatures and the environment in the ecological [Abdel-Azeem, 2020].

Within the eukaryotic organisms, just several varieties of fungi can improve between 45 °C and 55 °C. Suchlike types of fungi are called thermophilic and thermotolerant based on their maximum growth temperature [Cooney et al., 1964]. Thermophilic fungi must have an optimum temperature between 20 °C and 50 °C for growth. Thermophiles in fungi are not resistant to extreme temperatures like archaea that can survive at 100 °C [Blöchl et al., 1997; Brock et al., 1995].

The thermophilic fungus has been recommended as a model organism for chemical and possible structural analyses macromolecular building and biotechnological usage of thermostable eukaryotic proteins [Li et al., 2010; Amlacher et al., 2011].

Among eukaryotic, *Chaetomium thermophilum* (Ct), a thermophilic type of ascomycete that can grow between 50 °C and 55 °C or even 60 °C, has been accepted as thermophilic. The thermal stability characteristic of Ct proteins has been formerly shown and immediately crosschecked to mesophilic homologs [van Noort et al., 2013].

After the genome of Ct, essentially isolated by La Touche [La Touche, 1948], its genome has been sequenced by Amlacher [Amlacher et al., 2011]. Ct gives hope perfect pledge for structural biology, it has the extraordinary capability to grow even at 60 °C. The nuclear genome of Ct has sequenced 28.3 Mb comprised of eight chromosomes. 7227 protein-coding genes of Ct were identified and genome-wide proteome and transcriptome analysis have continued [Bock et al. 2014]. The specific purification of the gene construction by transcriptomics and proteomics approach provides special gene expression works and following empirical characterizations.

Ct has assisted as a wealthy source for thermostable eukaryotic proteins. On a few assays have been utilized the Ct proteome for biotechnological practice [Li et al.,

2010] or high-resolution three-dimensional (3-D) characterization of proteins or combined proteins [Stuwe et al., 2015]. Studies have been conducted to completely employ the potential of this thermophilic eukaryote as a resource for the structural and biochemical examination of proteins. A conversion tract was developed for *Ct*, enabling the strong integration of the structure into the genome which offers this thermophile reachable for isolation of natural thermal stable proteins and protein complexes gathered nature [Abdel-Azeem, 2020].

Studies displayed that *Chaetomium* enzymes like formate dehydrogenase, cellulases, L-methioninase, laccase, polysaccharide monooxygenase (PMO), β -1,3-glucanase, dextranase, pectinolytic, lipolytic, amylolytic, proteolytic, and chitinolytic represent an important agent in different areas. Today, in the investigates, the perfect comprehension of the mechanisms, structure, function, and substrate characteristics aiming effective enzyme generation to promote the improvement of an applicable bio-refinery industry is very important [Abdel-Azeem, 2020].

2.2 Enzyme

Enzymes are proteins that act as biological catalysts. Catalysts accelerate reactions. While the substances on which enzymes act are called substrates, the enzyme transforms substrates into different molecules called products.

The usage of word "enzyme", first used by Wilhelm Kuhne in 1877, is present admitted to define a biological catalyst. The enzyme names nowadays contain the suffix “-ase” proposed for all enzyme names by Émile Duclaux in 1898. Some proteolytic enzymes are an important exclusion to this ordinary rule.

Each protein is formed by the amino acid sequence called the primary structure assigned by the deoxyribonucleotide sequence that makes up the genetic structure. The deoxyribonucleic acid (DNA) sequence is initially copied into an mRNA molecule, and when the mRNA sequence reaches the ribosome, it is translated into an amino acid sequence, and the synthesized polypeptide chain folds a three-dimensional structure. This conversion may involve different post-translational steps that can be quite suitable for its functionality.

Many properties of enzymes as catalysts derive from the molecular structure. Enzymes are proteins containing multiple amino acid residues that are covalently bonded by the peptide bond formed between the carbon atom of the carboxyl group of

amino acid and the nitrogen atom of the α -amino group of the adjacent amino acid (Figure 2.1) [O'Connor and Adams, 2014].

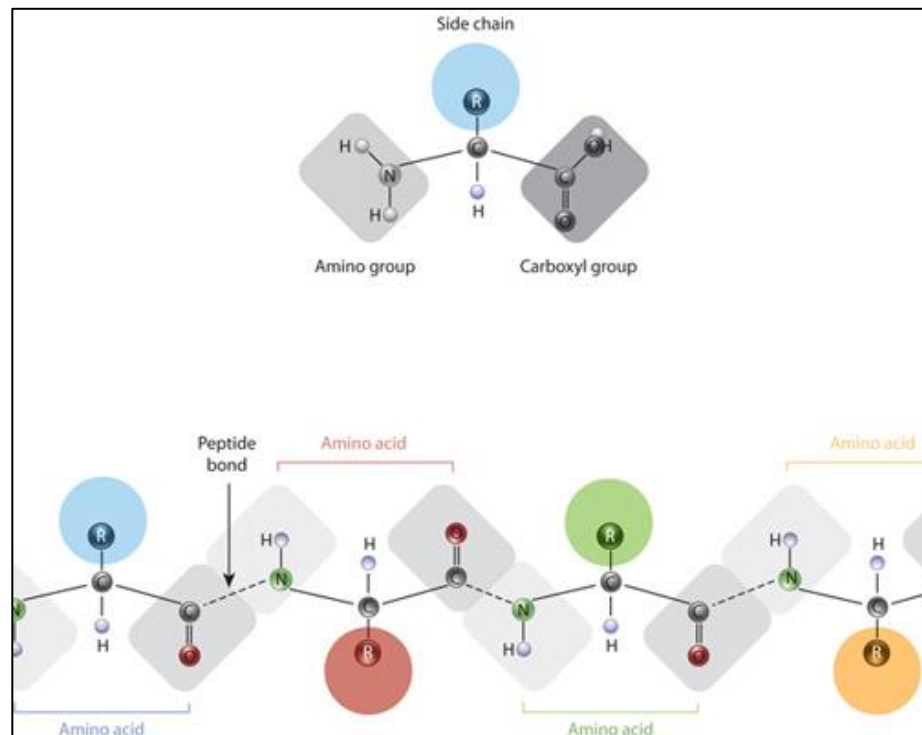


Figure 2.1: Peptide bond formation.

For the inherent nature of the R group, amino acids may be polar or non-polar and are the basis for building the 3-D structure of the protein [Lehninger, 1970].

The 3-D structure of a protein formed from a single polypeptide chain by various interactions and bonds is known as its tertiary structure. Tertiary structure is generally obtained by the interactions of the R groups of amino acids on the protein surface and between the water molecules and other amino acids on the protein surface. The stabilizing and non-covalent interactions of the enzyme are divided into ionic bonds, hydrogen bonds and Van der Waals forces [Matthews, 1993].

Small proteins can consist of only one domain, while larger enzymes can contain more domains. Essentially, most enzymes can contain dimers, tetramers or polymers of several polypeptide chains. Each polypeptide chain is called a "subunit" and can be the same or different from the others. The entire system of subunits is known as the quaternary structure and hence the quaternary structure is a known adhesion of enzymes with multiple subunits. The four order enzyme structure is shown in Figure 2.2 [Simpson et al., 2012].

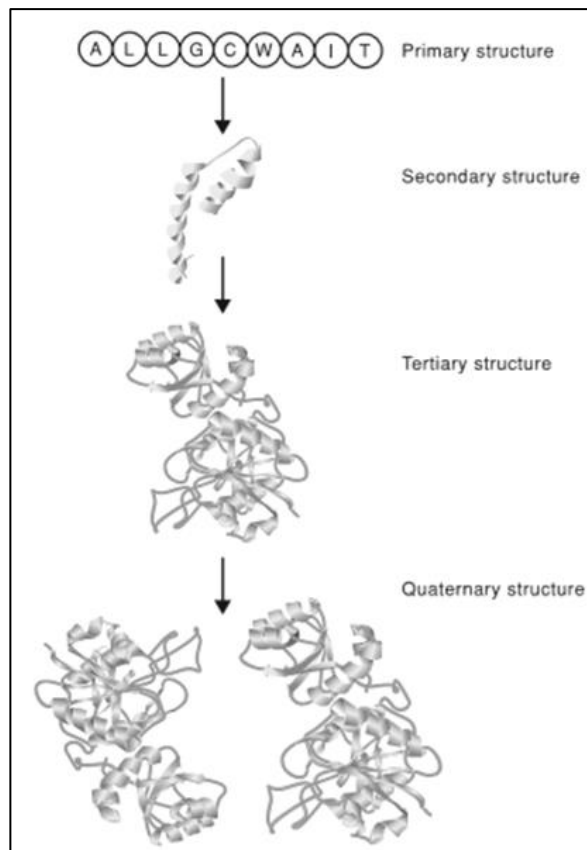


Figure 2.2: Schematic representation of the four levels of protein structure.

Enzyme activity, the ability of an enzyme to catalyze a chemical reaction, is known to be linked to the 3-D structure of the enzyme. The enzyme activity depends on the presence of a suitable structure of the active site formed by a small number of amino acid residues, together with the 3-D structure of the protein [Simpson et al., 2012].

Proteins are rich in nucleophilic groups but insufficient for electrophiles. In most cases, the enzyme alone is not capable of catalyzing a reaction. The non-protein molecules, the cofactor, are important for the catalysis of the reaction. In such enzymes, the non-cofactor enzyme is called inactive, and when in combination with an apoenzyme or cofactor it is called an active enzyme. The cofactors may be metal ions (e.g., Mn[II] in arginase, Ni[II] in urease, and Ca[II] in DNase I) or coenzymes (NAD⁺ and its phosphorylated form (NAD(P)⁺), Flavin Adenine Dinucleotide (FAD), Pyridoxal Phosphate (PLP), Thiamine Pyrophosphate (TPP), biotin, cobamide, and heme). The covalent bond of prosthetic groups to apoenzyme is very common [Punekar, 2018].

2.2.1. NAD⁺ Coenzyme

The coenzymes are the low molecular mass molecules that is a substrate for an important number of enzymes and that transferences a chemical group, electrons, or hydrogen. Those molecules increase the chemical reaction rate. Coenzymes are molecules that take or give away chemical groups, hydrogen, or electrons in the enzyme active region.

Many enzymatic redox reactions necessitate one or more cofactors that are used up along the reaction. In enzymatic reductions, a coenzyme (or cofactor) such as NAD⁺ and NAD(P)⁺ is required as the hydrogen source (hydride donor).

NAD⁺ and NAD(P)⁺ carry hydride ions which have a central role in the metabolism of entire cells [Berg et al., 2012; Voet and Voet, 2010]. The alone distinction in the construction of these two molecules is the phosphate on the number 2' carbon of the ribose that is linked to adenine. They are prevalently meant as pyridine nucleotides for the nicotinamide include the ring of a pyridine. The abridgment NAD(P)⁺ refers to either form. These pyridine nucleotides, by acceptance or granting the formal equivalent of a hydride ion, achieve the oxidation-reduction of other substrates. They can also conduce to the saving of energy when the oxidized form, NAD⁺ or NAD(P)⁺, wins two electrons to become NADH or NAD(P)H (Figure 2.3) [Wang et al., 2017], which are the respective reduced forms. They liberate the stocked energy when the reverse reaction begins and finally, they become oxidized by oxygen [Lehninger et al., 2008].

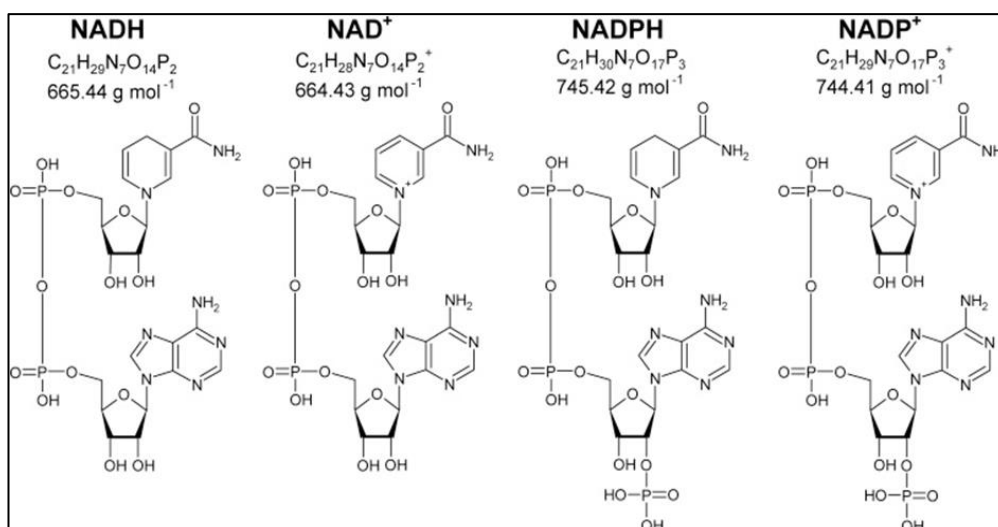


Figure 2.3: Molecular structure of NAD⁺ cofactors.

The most obvious difference between NAD(P)H and NAD(P)⁺ molecules is the hydride ion, for instance, H⁻ (H⁺ + 2e⁻). Renewal of NAD(P)H requires two electrons to NAD(P)⁺ (for instance reduction of NAD(P)⁺) and a (catalytic or non-catalytic) transference of a proton. Although some produce excess by-products, formate, phosphite, glucose, TEOA, propanol and H₂ gas are just a few of the hydride donors [Wang et al. 2017].

NAD(P)H can take a hydride (H⁻) ion from an oxidized substrate in the reactions called the oxidation reactions, or can give a hydride ion to the reduced substrate in the reduction reaction. The sites on the NAD(P)⁺ for the removal or adding of hydrides are indicated in Figure 2.4 [Taberman et al., 2016].

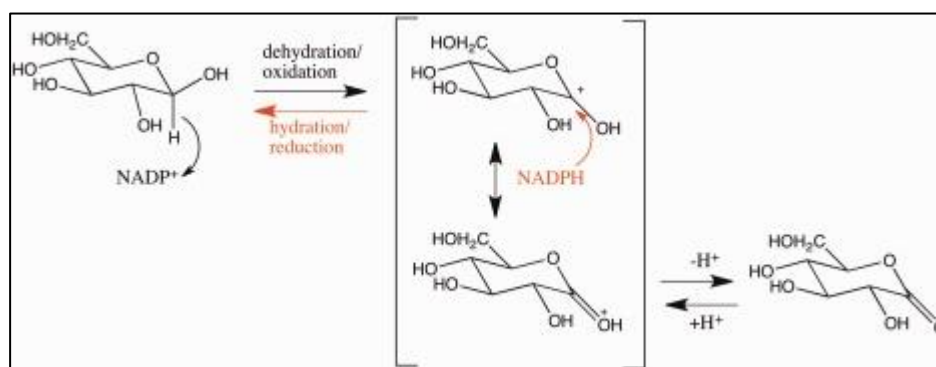


Figure 2.4: The reaction mechanism for oxidation and reduction with NAD(P)⁺ coenzymes.

Considering NAD(P)H (mass price per mol \$ 3.000 for NADH and \$ 215.000 for NAD(P)H), stoichiometric procure is not cheap and it is a necessity to find a much more efficient regeneration system if its applications exist in the industry. For this reason, NAD(P)H-dependent enzymes such as dehydrogenases have been overshadowed by enzymes that are not dependent on such cofactors in large scale applications [Wang et al., 2017].

The reduced coenzymes NADH or NAD(P)H have a strong absorbance peak at 340 nm, while the reduced forms of these enzymes NAD⁺ and NAD(P)⁺ do not give an absorbance peak at 340 nm. Thus, in a reaction that produces or depletes the reduced coenzymes NADH or NAD(P)H, an increase or decrease in activity can be detected by taking measurements at 340 nm by spectral measurements. This study can be repeated many times, which can be done with equipment found in every laboratory [Illanes, 2008].

2.2.2. Enzyme Classification

Enzymes are classified into six families according to the rules of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) [Anonymous, 1984] depending on the kind of chemical reaction catalyzed.

The six families are:

- **Oxidoreductases:** Enzymes catalyzing oxidation and reduction reactions that include the convection of electrons, hydrogen or oxygen atoms. There are 22 subclasses of oxidoreductases and there are several technological importance, for example, the dehydrogenases that oxidize a substrate by transporting hydrogen atoms to a coenzyme (NAD^+ , NAD(P)^+ , FAD^+ , FMN) that acts as an acceptor. Oxidoreductases produced and secreted inside the cell are involved in many reactions in the living cell.
- **Transferases:** Enzymes catalyzing the transport of a functional group from a donor to an appropriate acceptor. It has been understood that these enzymes, which are the head of enzymes such as acyltransferases, methyltransferases, transaminases, glycosyltransferases, have important roles in various cell metabolisms.
- **Hydrolases:** Enzymes catalyzing reactions of hydrolysis, this is, the separation of a chemical bond by the effect of water. Some of the enzymes used in biotechnology relate to hydrolases for instance glycosidases, esterases and proteases.
- **Lyases:** Enzymes catalyzing reactions of non-hydrolytic and non-oxidative disconnection of bonds. Significant among lyases are aldolases, generally playing in opposite reactions of C-C bond formation, dehydratases, decarboxylases, hydratases and carboxylases.
- **Isomerases:** Enzymes catalyzing reactions of the transformation of a substrate into an isomer, this is, a matter with the identical number and types of atoms. Too few isomerases are being utilized biotechnologically, but the situation of glucose isomerase (indeed xylose isomerase) is an exception.
- **Ligases:** Enzymes that catalyze the covalent binding reactions of two molecules. Despite their metabolic suitability, ligases, which have a complex and unstable structure, have no application in the industry.

Oxidoreductases, which catalyze the reduction and oxidation reactions, are essential for the continuity of life in living cells. Such enzymes can also catalyze reactions on natural or non-natural substrates. There are distinct oxidoreductases that can be obtained from organisms, such as oxidases that utilize molecular oxygen as oxidizing agent however generally display a fairly limited substrate specificity, and oxygenases, monooxygenases and dehydrogenases that need cofactors and can catalyze unnatural substrates [Illanes, 2008]. Although they use complex coenzymes, they are important enzymes in converting substrates into products. These enzymes are particularly involved in synthetic pathways in drug production, by obtaining optically active compounds and efficiently producing chiral building blocks.

Enzymes are indispensable for all biotechnological processes, especially molecular biology and genetics. Biotechnology aims to further evaluate the value of the product and develop reactions involving enzymes. Thousands of studies are carried out aiming to develop catalysts in the cell or enzymes, which are of great importance for biotechnology [Roberts, 2000; Sugai, 1999; Drauz and Waldmann, 2002].

2.2.3. Dehydrogenases

Oxidoreductases, usually, catalyze oxidation and reduction reactions. These enzymes divide into six classes: oxygenases, hydroxylases, reductases, peroxidases, oxidases, and dehydrogenases. Most oxidoreductase enzymes are dehydrogenases.

Among microorganisms, thermophilic is a very effective resource to use in industrial processes. Dehydrogenases are enzymes found in all organisms and have active roles in important pathways. For this, it has recently become important to isolate dehydrogenase enzymes from thermophilic organisms and to work in the laboratory. In the case of using unnatural substrates to obtain high value products, it is desirable to preserve the selectivity and activity [Illanes, 2008]. Dehydrogenases can be categorized according to the substrates they catalyze [Hummel, 1999].

Dehydrogenases can catalyze redox reactions using NAD^+ or NAD(P)^+ coenzymes as reducing and oxidizing agents.

Dehydrogenases can take part in a reaction carried out at a high concentration of the auxiliary enzyme and a low concentration of its coenzyme. A redox reaction planned using two enzymes would be much more effective [Illanes, 2008].

2.2.3.1 Formate Dehydrogenase

The title formate dehydrogenase assembles different categories of enzymes robustly varied in quaternary structure, existence and kind of coenzyme group, additionally in substrate selection [Tishkov and Popov, 2004]. NAD^+ -dependent FDH (EC 1.2.1.2) in the dehydrogenase group oxidizes formate to carbon dioxide in the redox reaction simultaneously reduces cofactor NAD^+ to NADH (Figure 2. 5) [Castillo et al., 2008].

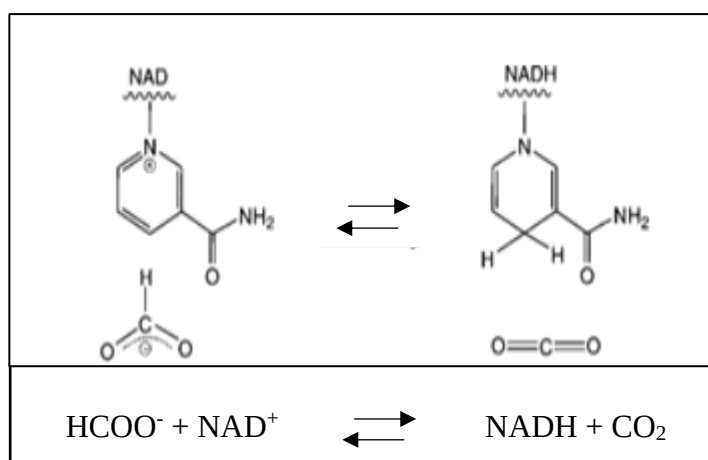


Figure 2.5: FDH catalyzed oxidation of formate ion to CO_2 .

FDHs can be categorized into two groups. In contrast to the first group containing FDHs obtained from bacteria and plants, FDHs in the second group are obtained from yeast and fungi. Despite a conserved ternary glycine amino acid group in FDHs, FDHs from yeast and fungi have an additional aspartic acid residue that appears to interact with NAD^+ [Gül-Karagüler et al., 2001; Serov et al., 2002].

It is possible that FDH can be isolated in large quantities from a variety of organisms. It is known that FDH activity depends on the microorganism it is isolated from, especially when grown in media with non-rich content, and more than one form is formed in the environment depending on them [Niks and Hille, 2018].

NAD(P)H regeneration is very useful in that the reactions carried out are simple. The basis of NAD(P)H regeneration is a reaction that enables the reduction of NAD(P)^+ . While the NAD(P)^+ molecule is reduced to NAD(P)H, this redox reaction requires an enzyme and a reducing agent. The known H_2 gas can be used in this redox reaction and protons can be used comfortably in bioconversions, thus achieving 100%

atom efficiency. Figure 2.6 illustrates the enzymatic CO₂ reduction as a sample [Wang et al. 2017].

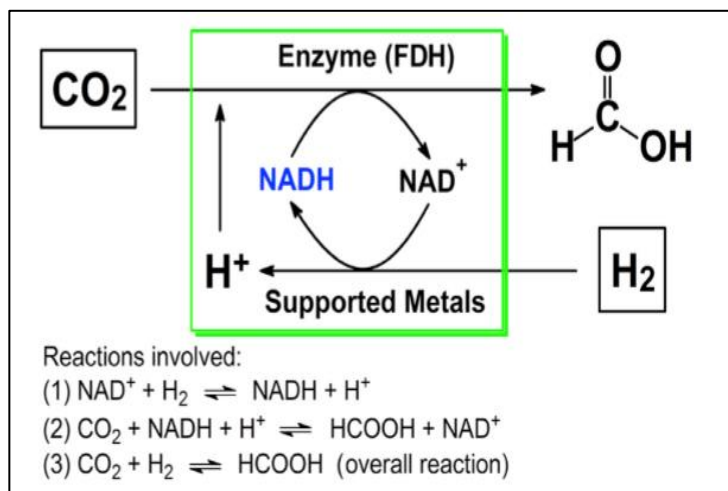


Figure 2.6: Reactions demonstrating the use of CO₂. Simultaneous NADH regeneration occurs as CO₂ is reduced to formic acid.

FDH may be separated into two categories, Metal-Dependent and Metal-Independent. Metal-independent FDH has only NAD⁺ as the cofactor and the metal-dependent FDH uses both NAD⁺ and a metal cofactor. Metal-dependent FDHs have a high CO₂-reducing activity however contain extremely oxygen-labile catalytic components, like metal ions (tungsten or molybdenum), iron-sulfur stacks, and selenocysteine, making these FDHs unadaptable for industrial practices [Almendra et al., 1999; Graentzdoerffer et al., 2003; Reda et al., 2008]. Metal-independent FDHs are dependent on NAD⁺ and form complex with formate and NAD⁺ for hydride transfer. Recently, NAD⁺-dependent FDHs are used as an alternative to NAD⁺ independent FDHs in studies to reduce CO₂.

2.2.3.1.1. Metal-Dependent FDH

Metal-dependent FDH enzymes obtained from prokaryotic belongs to the dimethyl sulfoxide reductase group, which have molybdenum (Mo) and tungsten (W) in their active sites. FDHs transfer two electrons in the substrate to the active site of the enzyme, while the formic acid is oxidized to CO₂. The hexoordinate W or Mo in a crooked triangular prismatic geometry structure contributes to the oxidation of the active area [Mota et al., 2011]. The oxidized form of the active sites involves W(VI)

or Mo(VI) ions in a warped trigonal prismatic geometry with four sulfur atoms promoted by two pyranopterin ligands, a sulfur atom from a cysteine residue or a selenium atom from a selenocysteine residue and is either sulfur or an oxygen atom in the sixth coordination site [Dobbek, 2011]. The fact that the metal is very close to the center suggests that it is close to arginine and histidine, the conserved sequences that will participate in the reaction. A square pyranopterin oriented from two pyranopterin ligands to four sulfur atoms contains another ligand that centers Mo or W of a pyramidal-like structure and is likely to be the sulfur atom in the apical position. All these described bond formation and breakdown between carbon and hydrogen, structural definitions describe the mechanism shown in Figure 2.7 [Scibioh and Viswanathan, 2018]. The formation and breakdown of this bond is a reaction involving the transfer of paired two electrons and a proton [Scibioh and Viswanathan, 2018].

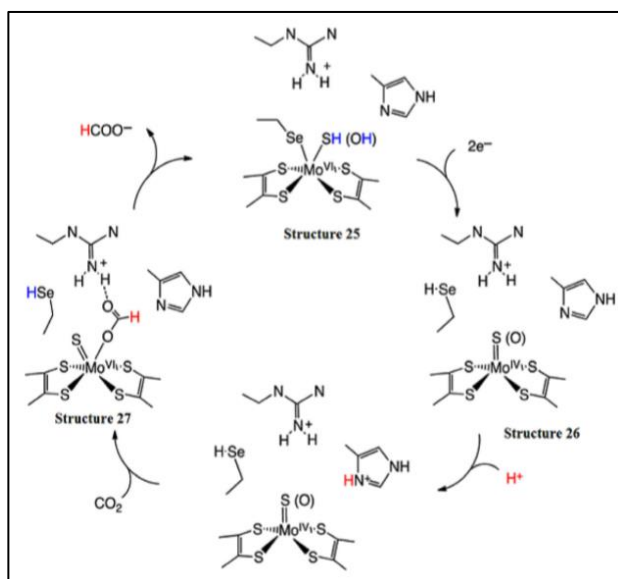


Figure 2.7: A suggested mechanism for the reduction of CO_2 to HCOO_2 by metal-dependent FDHs.

Metal-dependent enzymes containing Mo or W, which are continuous components of extremely large combinations and work through a ping-pong mechanism where the oxidized metal center reacts with the formate, are reduced by equivalents after transfer to other redox-active substances, and thereby the centers are regenerated the oxidized metal center [Niks and Hille, 2019].

2.2.3.1.2. NAD⁺-Dependent FDH

The most common class of formate dehydrogenases is NAD⁺-dependent FDH. NAD⁺-dependent FDH (EC 1.2.1.2) (Figure 2.8) [Yilmazer et al., 2020] is found in a lot of yeast and methylotrophic bacteria.

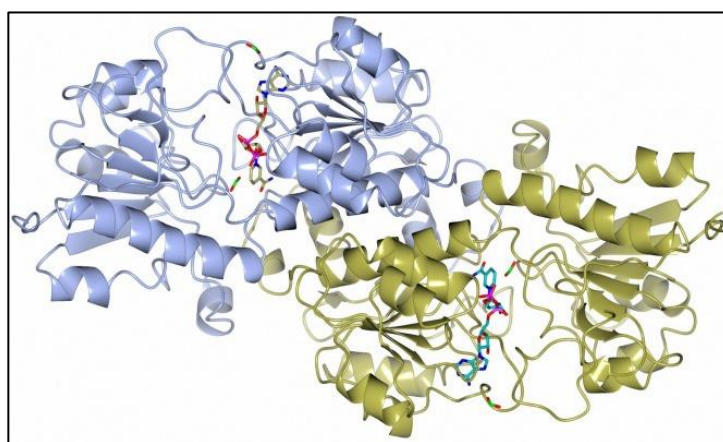


Figure 2.8: Ribbon representation of NAD⁺-dependent FDH.

While the FDH enzyme oxidizes the formate to CO₂, NAD⁺ is reduced to NADH, these two reduction and oxidation reactions occur simultaneously. Regeneration of NADH can also be achieved during the reaction carried out in the unsymmetrical production of chiral compounds for reaction thermodynamic [Slusarczyk et al., 1998].

In FDH enzymes, the reaction is catalyzed by the hydride transfer from the carbon atom of the formate to the C4 atom of the pyridine ring of the NAD⁺, while this transfer decreases the rate of the reaction [Blanchard et al., 1980; Rotberg et al., 1991]. The formate and NAD⁺ are likely located close to each other to facilitate hydride transfer between atoms. It has been found that NAD⁺ has a bipolar structure that increases the partial positive charge and electrophilicity, thus simplifying the hydride transfer between NAD⁺ and formate [Shabalin et al., 2009]. There is no reversal in this reaction that takes place in living organisms. FDHs are known to transfer two electrons in the structure of the proton and hydride ions from molecule to other at the same time.

Oxidation of the formate or reduction of CO₂ using NAD⁺-dependent FDHs is known to be reversible processes. At the beginning of the formate oxidation reaction of the formate molecule, a hydrogen bond is established between the two hydrogens

and the carbonyl oxygen of the amide group of NAD^+ and residues of threonine, aspartic acid, histidine (Figure 2.9) [Amao, 2018]. While the negatively charged formate is held electrostatically by the positively charged arginine residue, a hydrogen bond occurs between the oxygen atom of the formate and the histidine residue. All these bond formations in the active site continue until the formate is oxidized to CO_2 and the NAD^+ molecule reduced to NADH . Finally, as the products of the reaction, CO_2 and NADH leave the active site and the active site returns to its initial state for the next reaction [Amao, 2018].

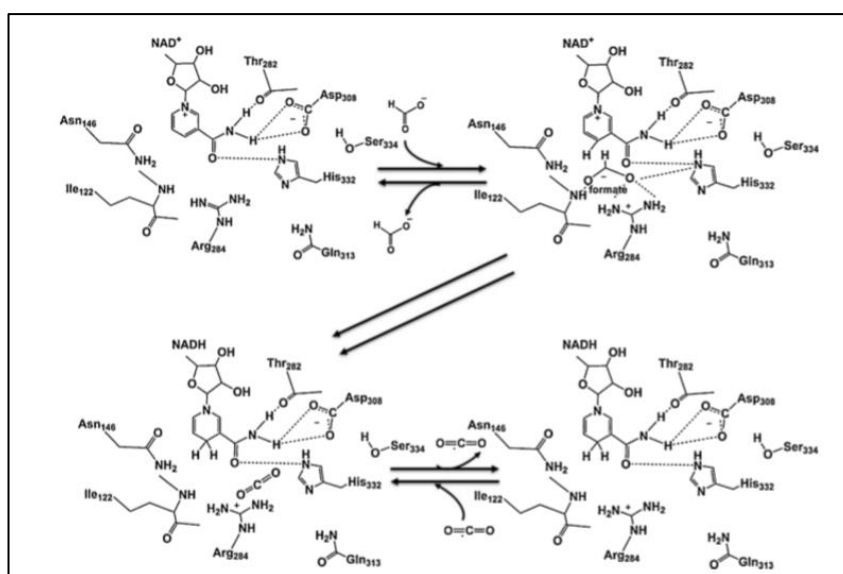


Figure 2.9: Probable mechanisms of formate oxidation to CO_2 and CO_2 reduction to formate with FDH.

2.3. Protein Engineering

Enzymes manufactured by several bacteria and yeasts have been utilized for centuries in food-producing (bakery, cheese), purification, filtration, and different fermentation processes like beer or wine making [Leisola et al., 2001; Patel et al., 2016].

The first commercially available enzyme was protease enzyme isolated from a *Bacillus* species. Large-scale applications for enzymes started in the 60s and thus microbial production started. The first enzyme produced in the laboratory is "ribonuclease" produced by Ralph Hirschmann and his team [Hirschmann et al., 1969].

One of the aspects of genetic engineering is that the change to be made on the gene also has an effect on the enzyme and allowing the opportunity to perform enzyme engineering. This allows nucleotides to be added, deleted, or altered into the gene sequence of an enzyme that is isolated and whose structure is known. The planned mutations on the enzyme are carried out with the help of degenerate primers designed in accordance with the desired mutation on the gene of the enzyme. To lose the function and activity of the enzyme is easy and to obtain a new function and to increase the activity of the enzyme in mutations made to the region is difficult. Even as such, many enzymes with the desired properties have been obtained. A mutation can be created in the desired region of the 3-D structure of the enzyme with the techniques of recombinant DNA technology. Genetic engineering techniques (Figure 2.10) used today are powerful and getting stronger [Punekar et al., 2018].

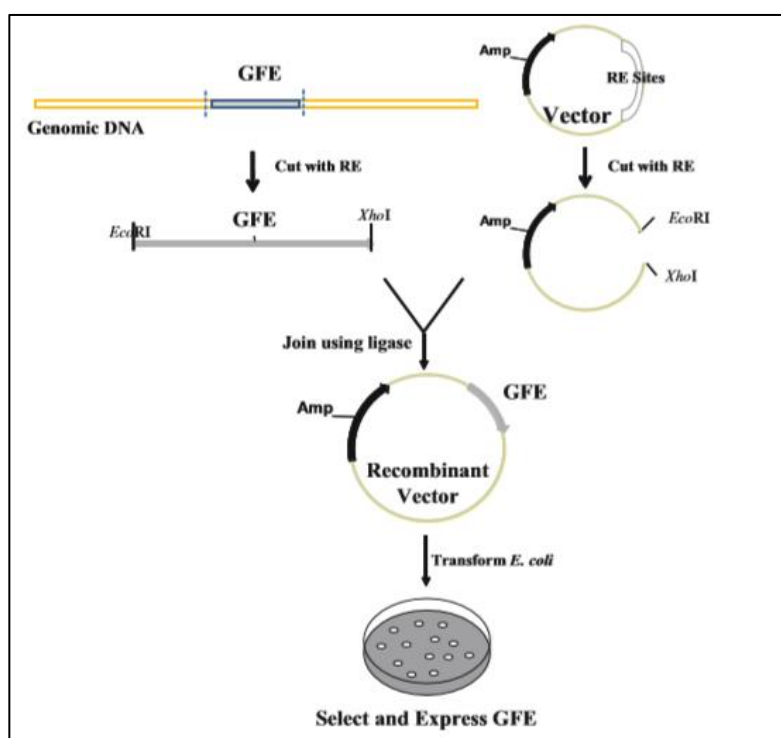


Figure 2.10: A general genetic engineering strategy for enzyme expression.

Today, there are various tools and algorithms used to increase the activity and thermostability of the enzyme using protein engineering [Wijma et al., 2013]. Protein engineering is a strategy used to give the protein a desired property. It is usually categorized into 3 parts: directed evolution, semi-rational approach and rational design.

2.3.1 Directed Evolution

The directed evolution method often enables random mutations in the gene sequence of the protein [Dashtban et al., 2010]. Directed enzyme evolution is an effective answer to the question of how to change the properties of enzymes. This method, which is used to change the properties of the enzyme, enables the change that can occur in thousands of years to be created in a shorter time such as days. No enzyme found in nature is evolution-proof. But this method shortens the duration of evolution and all kinds of processes that could be created using enzymes were revealed [Bornscheuer et al., 2012].

In studies conducted on large scale enzymes, the inability to optimize enzymes brought along many problems. Frances Arnold and coworker Pim Stemmer helped solve these problems thanks to the random mutation techniques. Arnold started the development of the enzyme by creating random mutations on the gene using error-prone PCR (epPCR) [Leung et al., 1989; Cadwell and Joyce, 1992]. Stammer brought the studies of randomly breaking down genes and recombining them, DNA shuffle studies, to science. A gene library was created for the mutant enzymes obtained as a result of these two techniques. The starting point of this method is Darwin's principles of evolution. While random mutant genes generated using epPCR mimic asexual replication in nature, DNA shuffling is the accelerated acquisition of various variants that occur spontaneously in nature, inspired by sexual reproduction [Arnold, 1998].

It is not necessary to know the 3-D structure of the enzyme for preliminary work in directed evolution applications. In such a case, interactions such as the formation or breaking of bonds between the enzyme and the substrate during the reaction are also unknown [Punekar et al., 2018].

Directed evolution studies create gene libraries by collecting randomly generated mutations. The enzyme expressed in the host is obtained by selection from this gene library according to the purpose of the study [Punekar et al., 2018].

Since gene libraries contain enzymes with desired properties created by random mutations, it is not necessary to know even its sequence of the enzyme [Tao and Cornish, 2002; Jestin and Kaminski, 2004; Williams et al., 2004]. Although it has advantageous features, screening and selection processes can be completed in a very long time to create a gene library.

2.3.2. Semi-Rational Design

Semi-rational design is a novel and effective approach for functional enzymes. Using degenerate primers, desired mutations can be made at one or more amino acid positions on the enzyme gene. Libraries are created using screening and selection techniques on obtained mutant enzymes. It includes various variants of a mutation resulting from random mutation [Zheng et al., 2004]. In order to obtain various variants of a gene in semi-rational design, it is necessary to know the where desired mutation is the position in the 3-D structure of the enzyme [Schwede, 2013].

Semi-rational design based on a 3-D structure reduces the time and effort required for mutation studies. Variants are generated by saturation mutagenesis technique at triple or more codons selected on an enzyme gene. The selection of these amino acids includes positions for increasing the catalytic property of the enzyme, substrate binding sites, and thermostability based on the enzyme structure information [Reetz and Carballera, 2007].

The site saturation mutagenesis technique requires the determination of the amino acid sequence and 3-D structure of an enzyme. This method is a mixture of directed evolution and rational design, and it is a method commonly used to overcome the various limitations of directed evolution and rational design and to obtain improved enzymes [Bommarius et al., 2006].

2.3.3. Rational Design

The rational design approach is the most important method of protein engineering predicated on “site-directed mutagenesis” of proteins [Heinzelman et al., 2009]. Site-directed mutagenesis is based on the replacement of the desired amino acids based on the obtained sequence information as a result of bioinformatics analysis. Overlap extension and all plasmid single round PCR are two techniques used in site-directed mutagenesis studies. Each of the overlap extension mutant pair contains mutant codons. Primers containing mutant codons allow mutation on the gene. Owing to the denaturation and annealing of the gene sequences obtained, heteroduplex products are formed. Then, the 3' and 5' ends of the heteroduplexes are completed by DNA polymerase. In this technique, primers containing mutations complete a double-stranded plasmid running in opposite directions. In the double-stranded plasmid DNA

sequence, the breaks do not overlap and thus the double strands of the plasmid are duplicated by DNA polymerase. To select the gene sequences that do not contain mutations from the plasmids obtained, wild type, the property of the sequence being methylated or hemimethylated is used. The DpnI enzyme creates nicks on these genes. In the last stage, circular plasmids that can replicate themselves are transferred to suitable cells [Li et al., 1997].

Although the sequence of amino acids and even its 3-D structure are known, today technology does not yet have the opportunity to design an excellent properties protein. For effective rational design it should contain the following items [Simpson et al., 2012]:

- Knowing the amino acid sequence of the enzyme whose properties are to be developed and the existence of an expression system that enables enzyme production.
- Structure study of the protein sequence and definition of the desired residues mutations
- Existing functional analyses for the definition of alters in the features of the protein.
- Knowing the 3-D structure of the protein or obtaining the most similar to the real 3-D structure of the enzyme with current technology.
- Mutations made on the gene sequence of the enzyme desired to be improved. It is necessary to examine the works done to screen all residues in the protein that can be functional according to the purpose and to create a template using the 3-D structure of the enzyme with sequence analysis.

Generally, the residues to be mutated are selected by examining into two or ternary amino acid groups that interact with the R groups. In this case, the changes in interactions between amino acids can be traced. The gene library has been demarcated by scanning the region where the mutation is desired, using classic screening equipment [Vogel, 2019].

Bioinformatics, which is used in various fields, seems to be more important and will continue to increase the importance. Nowadays to analyze big data and obtain advanced enzymes with technological advances is easier [Badenhorst and

Bornscheuer, 2018; Saito et al., 2018]. The big data obtained from various studies are closely related to the enzyme study and the processing of the obtained data. Mutation studies created using the 3-D structure are the biggest advantage used in improving the properties of the enzyme [Vogel, 2019].

If the function of the enzyme and the folding of the amino acid sequence are found most accurately by computer calculations, it enables the use of enzymes in bioprocesses without the need for additional studies. This is still not possible with today's technology [Vogel, 2019].

2.4. Application of FDH

The high level of enzyme selectivity, high efficiency, low by-product wastes are the advantages of studies using enzymes, they have disadvantages such as expensive cofactors. The applicability of the reaction conditions and the continuation of the reaction for a longer time compared to conventional studies can be shown among these advantages. The low reaction efficiency obtained as a result of the studies to reduce CO₂ in biocatalytic processes makes the studies difficult. Thus the necessity of more effective and more applicable enzymes was understood. Nowadays an engineering branch including reaction systems is needed to isolate new enzymes and to increase catalytic efficiency [Scibioh and Viswanathan, 2018].

The coenzyme is used as a hydrogen source in enzymatic reduction reactions. NADH, one of the best-known coenzymes, is used by 80% of oxidoreductases, while 10% uses NAD(P)H. In an enzymatic reaction, the substrate of the enzyme is reduced while NAD(P)H is oxidized to NAD(P)⁺ (Figure 2.11) [Burnett et al., 2020].

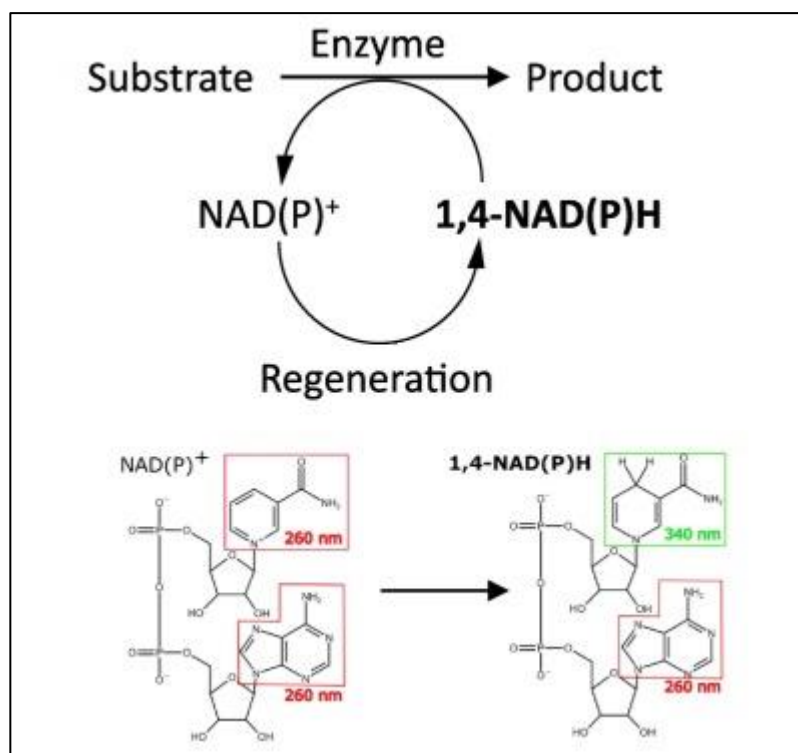


Figure 2.11: Schematic representation of enzymatic reaction using cofactor 1,4-NAD(P)H with in situ regeneration.

Given the high price of NAD(P)H (cost per mol: \$215.000 for NAD(P)H) which limits its use in bioprocesses, it is not economical to obtain it chemically. Regeneration systems are needed to obtain cheaper and more NAD(P)H. Enzymes that use NAD(P)H as a coenzyme, such as hydrolases, are not preferred in industry, while enzymes that are not dependent on coenzymes are more trendy [Wang et al.,2017].

In recent days, the increase in global warming and its effects and fossil fuel resources are depleted that are used in every field have attracted much attention to reduce the CO₂ concentration. Although scientists are aware of the problem, they know that the solution is not easy. For CO₂ fixation, the idea of reducing the amount of CO₂ by using it as a substrate in biocatalytic reactions, thus using the products formed as a renewable fuel as a result of the reaction is interesting [Baskaya et al., 2010; Crable et al., 2011; Tong et al., 2011]. The studies involving FDH enzymes have been carried out to reduce CO₂ and obtain chemicals such as formate or methanol [El-Zahab et al., 2008; Le et al., 2011]. Formic acid is noted to be a hopeful substitution for methanol in miniature fuel cells [Jung et al., 2007].

CO₂ reduction reactions, which provide the production of CO and formic acid, are one of the strategies to reduce the amount of CO₂ [Lee et al., 2007] and catalytic synthesis utilization CO₂ as a raw material [Tamura et al., 2016].

FDH can catalyze CO₂ to form an organic chemical, and the formate by pairing the with alcohol dehydrogenase and aldehyde dehydrogenase reactions, efforts to reduce it to formaldehyde or methanol, which are widely used, have recently been adopted as a double gain, and it has been extensively studied [El-Zahab et al., 2008].

2.5. NAD⁺-Dependent FDH Studies

Various studies have been carried out on FDHs from microorganisms to investigate the catalytic mechanism of the high-value FDH enzyme, increase thermal stability or change coenzyme and substrate specificity.

Optically active molecules are known to be one of the most important regulators for the survival of the cell. While 58% of the current drugs sold are composed of chiral compounds, this number is expected to increase to 70% in the coming years, considering the current studies. The use of dehydrogenases for the production of optically active substances increases its importance. Furthermore, combining this catalysis reaction with NADH regeneration increases the yield from the reaction [Tishkov and Popov, 2004].

For the high cost of NADH and particularly of NAD(P)H, processings including solely dehydrogenases are economically profitless. Transformation of NAD(P)⁺ to NAD(P)H has been proposed in many studies to reduce the cost of the coenzyme used in the reaction [Wichmann et al., 1981; Hummel and Kula, 1989; Shaked and Whitesides, 1980]. These catalysis reactions can be performed by the major enzyme as well as the other enzyme in the conjugated reaction. But the reaction catalyzed by FDH is encouraging for NADH regeneration [Tishkov, 2002]. The reaction of formate oxidation to carbon dioxide is irreversible, and this permits the utilization of thermodynamic pressure on the essential processings and achieves 99-100% yield of the requested product.

The FDH activity does not alter in the pH range from 5.5 to 11.0 and, values of Michaelis constants for NAD⁺ and formate are unaltered in the pH range from 6.0 to 9.5 [Mesentsev et al., 1997]. Therefore, FDH the conjugated every dehydrogenase that has efficiency optimum in this pH range may be used.

Formate salts are low-cost and easily available substances. The formate does not have an inhibitory property for the enzymes used in the reaction, the exception is xylitol reductase [Neuhauser et al., 1998]. In the catalysis reactions of FDH enzymes, CO₂, which is a product, does not inhibit dehydrogenases, does not limit the production of the desired product as a result of the reaction, and can be easily separated from the reaction. FDHs from yeasts and bacteria are extremely stable enzymes and can study in systems for days or months.

The utilization of FDHs for the regeneration of NADH was recommended even 41 years ago [Wichmann et al., 1981; Shaked and Whitesides, 1980], yet the high cost of the enzyme manufacture from native strains delayed their active practice. Actually, native enzymes leucine dehydrogenase and FDH from *Candida boidinii* (CbFDH) [Bommarius et al., 1995] were used for the industrial manufacture of tert-L leucine, and this is the largest-scale process containing dehydrogenases. FDH has some limitations such as low stability and low activity during their industrial using under different conditions. Protein engineering techniques is applied to overcome these limitations.

Cys255Ser and Cys255Met mutations were applied in the FDH enzyme obtained from *Pseudomonas* sp. 101 [Tishkov et al., 1993]. These mutants increased thermostability 2-fold compared to wild type while eliminating affinity in NAD⁺.

In *Pseudomonas* sp. 101, to investigate the stability of the Cys54 and Cys145 residues and their contribution to the reaction mechanism, Cys145 is replaced by alanine and serine, and Cys54 is replaced by serine and arginine [Odintseva et al., 2002]. With the best results among the mutations was combined with the Cys255Ala mutations to obtain an enzyme 1000 times more resistant to chemical deactivation.

CbFDH has the cysteine amino acid in residues 23 and 262 of each subunit. When the 3-D structure of this enzyme is examined, it is determined that it does not bond with water [Slusarczyk et al., 2000]. Cys23Ser, Cys262Val, Cys23Ser/Cys262Val and Cys23Ser/Cys262Ala mutations on CbFDH were designed and their activities, half-life of the enzyme, inactivation with metals were investigated. It was determined that none of mutations caused a change in activity, but its stability against Cu⁺² was increased and the half-life increased 7.7 times compared to wild type [Slusarczyk et al., 2000].

The *azoA* gene from *Enterococcus* sp. L2 was cloned into *E.coli* BL21 and *P. fluorescens* PfO-1. The azoreductase enzyme used in degradation of recalcitrant azo

dyes increased approximately 2-fold was determined. In the expression studies performed in *E.coli* BL21 and *P. fluorescens* PfO-1, the expression of NAD⁺-Dependent FDH enzyme obtained from *Mycobacterium vaccae* N10 was increased and NADH was obtained. Thus, approximately 4-fold improvement in azo dye biodegradation was observed with overexpression of *azoA* and *fdh* genes in buffer containing formate [Rathod et al., 2017].

Several mutations for position 290 on the NAD⁺-dependent SoyFDH enzyme have been designed. All mutations were determined to increase the catalytic constant up to 4.7 s⁻¹. The substitution Phe290Ala increased affinity for NAD⁺ while NAD⁺ affinity of the substitutions Phe290Tyr and Phe290Glu increased and decreased, respectively. The best improvement was detected for Phe290Ala SoyFDH. When compared with wild type, all mutations were observed to increase thermostability at various rates. As a result of the study, all mutants are good candidates for use in coenzyme regeneration was understood [Kargov et al., 2015].

The disadvantages of most FDHs are their low enzyme activity and stability. In a study to obtain an enzyme with higher enzyme activity and better enzyme stability, mutations were designed for the substrate and NAD⁺ binding sites at the active site. k_{cat} and k_{cat}/K_M of Val120Ser increase 3.48 and 1.60 times against COONH₄, respectively. Val120Ser/Asn187Asp double mutant was determined to have high catalytic efficiency against the NAD⁺ [Jiang et al., 2016].

FDH is a multimeric protein and in multimeric proteins interactions between subunits affect the thermostability of the enzyme. It has been suggested that glutamic acid at the interfaces of FDH enzymes has high pKa. *Pseudomonas* sp 101 with the highest thermostability, there is an aspartic acid residue at the interface, close to glutamic acid. *Candida boinidii* has Asn (position 187) instead of Asp at this position in FDH, and the Asn187Asp mutation appeared to raise the pKa of Glu163 and also increased thermostability [Özgün et al., 2015].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Equipments

Table 3.1 shows the laboratory equipment used.

Table 3.1: Laboratory Equipments.

Device	Trademark
Autoclave	N-Biotek
Balance	And
Benchtop Shaking Incubator	N-Biotek
Biosafety Cabin	Faster
Centrifuge	Hettich
Electrophoresis power supply	Bio-rad
Etuve	Nüve
Gel Screening	Serva
Magnetic Stirrer	Ika
Microplate Spectrophotometer	Biotek
Microwave Oven	Arçelik
pH Meter Instruments	Hanna
SDS-PAGE Electrophoresis System	Bio-Rad
Sonicator	Bandelin
Thermal Cyclers	Techne Prime
Ultrahigh freezer	Arctika
Vortex Mixer	Vwr
Water Bath	Stuart
Water Purification System	Milli-Q

3.1.2. Chemical Components and Kits

Table 3.2 shows the laboratory equipment used.

Table 3.2: Chemicals and kits.

Material	Trademark
Plasmid DNA Purification Kit	Macherey-Nagel
DpnI	Thermo Fisher Scientific
Tryptone	Multicell
Yeast Extract	Biolife
KH ₂ PO ₄	Multicell
MgSO ₄	Merck
Dextrose	LAB M
Alpha-D-Lactose	Acros Organics
NaCl	Merck
5-alpha Competent <i>E. coli</i>	NEB
BL21 (DE3)	NEB
Peptone	Multicell
Agar	Multicell
Ampicillin	Serva
Na ₂ HPO ₄	Merck
NaH ₂ PO ₄	Merck
KH ₂ PO ₄	Multicell
Glycerol	Merck
Imidazole	Serva
NaOH	Sigma-Aldrich
HCOONa	Sigma-Aldrich
NaHCO ₃	Sigma-Aldrich
Lysozyme	Serva
Acrylamide	Thermo Fisher Scientific
Bis-Acrylamide	Thermo Fisher Scientific
Tris Base	Thermo Fisher Scientific
Sodium Dodecyl Sulfate (SDS)	Multicell
H ₃ BO ₃	Sigma-Aldrich
Ethylene Diamine Tetraacetic Acid (EDTA)	Sigma-Aldrich
Amoryum Peroksidisülfit (APS)	Thermo Fisher Scientific
TEMED	Thermo Fisher Scientific
6X Tritrack DNA Loading Dye	Thermo Fisher Scientific
Agarose	Isolab
β-mercaptoethanol	Chemcruz
DNA Ladder	Thermo Fisher Scientific
DNA Jel Yükleme Boyası	Thermo Fisher Scientific
Safe DNA Gel Stain	Invitrogen
Nikotinamid adenin dinükleotit (NAD ⁺)	Sigma-Aldrich
Nikotinamid adenin dinükleotit (NADH)	Prozomix
HisTrap Column	GE

3.1.3. Vector System

Bacterial transformation is the processing of horizontal gene transfer by which some bacteria get external genetic material (naked DNA) from the out of the cell. The presupposition for bacteria to carry transformation is their ability to get independent, extracellular genetic material. Such bacteria are termed as competent cells. All bacteria are not capable of getting exogenous DNA from their environment. The practice

approach to getting competent cells is to do the bacterial cells artificially competent using chemicals or electrical pulses.

The pET System is the most robust system but improved for the expression and cloning of recombinant proteins in *E. coli*. Target genes are cloned in pET vectors under the check of robust bacteriophage T7 transcription and (optionally) translation signs; expression is induced by supply a resource of T7 RNA polymerase in the host cell.

The pET-45b(+) (Figure 3.1) [Novagen®, 2009] plasmid can express fusion proteins with an N-terminal His-Tag coding sequence that results in native protein after purification and cleavage.

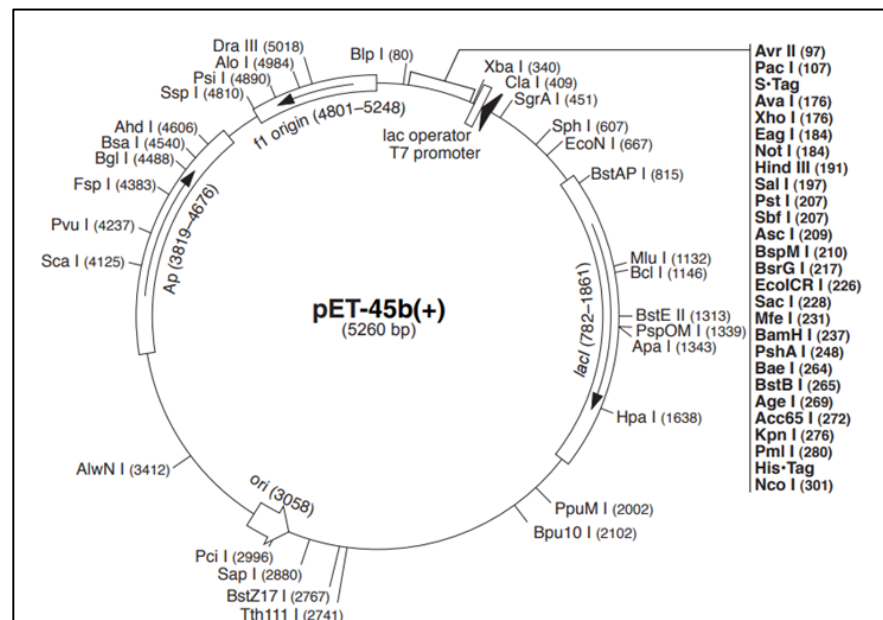


Figure 3.1: pET-45b(+) vector is used for protein purification.

The plasmid involves a robust T7 promoter, an amino-terminal His-Tag coding sequence, and multiple cloning site (MCS) regions designed to permit the generation of target proteins with minimal vector-encoded fusion. The Pml I cloning site permits direct fusion to the His-Tag sequence for involves that are blunt and in the suitable reading frame. For practices that request a removable amino-terminal His-Tag sequence, the MCS contains a PshA I cloning site (GACNNNGTC). The PshA I site overlaps the cleavage site for the enterokinase (EK) protease (AspAspAspAspLys). Cloning accordingly designed inserts into this site recreates the full EK site and permits of whole amino-terminal vector-encoded sequences to be removed by EK

digestion. The remainder of the MCS encodes restriction enzyme sites to ease insert transfer. An unprompted C-terminal S-Tag coding sequence is coherent with purification, detection, and quantification [Kim and Raines, 1993].

In this thesis, the pET-45b(+) plasmid containing the *Chaetomium thermophilum* formate dehydrogenase (*Ctfdh*) gene, which was previously created by the BERC laboratory group, was used.

3.1.4. Mediums

3.1.4.1. Luria-Bertani (LB)

5 g yeast extract, 10 g tripton and 10 g NaCl were weighed and the final volume was completed to 1000 mL with distilled water (dH₂O). This medium was sterilized at 121 °C for 15 minutes by autoclave.

3.1.4.2. Luria-Bertani (LB) Agar

5 g yeast extract, 10 g tripton, 10 g NaCl and 15 g agar were weighed and the final volume was completed to 1000 mL with dH₂O. This medium was sterilized at 121 °C for 15 minutes by autoclave. After decrease of temperature to 55 °C, 1 µL/1 mL ampicillin (100 mg/mL) was added.

3.1.4.3. Autoinduction Studier Medium

5 g of yeast extract, 20 g of tryptone, 0.5 g of glucose, 3 g of KH₂PO₄, 6.67 g of Na₂HPO₄, 5 g of NaCl, 6 mL of glycerol (100%) and 3.11 g of lactose were dissolved in distilled water and pH was fixed 7.0 and so the medium was completed 1000 mL. This medium was autoclaved at 121 °C to ensure sterility.

3.1.4.4. Super Optimal Broth with Catabolite Repression (SOC)

5 g Yeast Extract, 20 g tryptone, 4.8 g MgSO₄, 3.603 g dextrose, 0.186 g KCl 0.5g NaCl were weighed and pH was adjusted to 7.0. The final volume was completed

to 1000 mL with distilled water dH₂O. This medium was autoclaved at 121 °C to confirm sterility.

3.1.5. Buffers and Solutions

3.1.5.1. Ampicillin Solution

1 g ampicillin was dissolved in 10 mL dH₂O and this solution filtrated a 0.45 mm sterile filter. After the ampicillin solution was separated into 1 mL and stored at - 20 °C.

3.1.5.2 Sodium Dodecyl Sulphate Polyacrylamide Gel (SDS-PAGE) and Buffers

3.1.5.2.1. 4X Sample Loading Buffer

4X sample buffer (loading buffer) was used to denature protein samples. This buffer was mixed with a protein sample and mixtures were loaded onto the SDS-polyacrylamide gel. The contents and amount of 4X sample buffer are given in Table 3.3.

Table 3.3: The contents and amount of SDS-PAGE 4X sample buffer.

Contents	Amount
SDS (10% (w/v))	3.0 mL
Beta-Mercapto-Ethanol (10 mM)	2.5 mL
Glycerol (100 % (v/v))	2.5 mL
Tris-HCl (pH 6.8) (1 M)	0.6 mL
Bromophenolblue (0.05% (w/v))	4.0 mg
dH ₂ O	Up to 10.0 mL

3.1.5.2.2. Separating Gel and Stacking Gel

The SDS-PAGE gel in a single electrophoresis run contains separating gel and stacking gel. Stacking gel is added to the surface of the separating gel (12%) (following solidification) and a gel comb is placed in the stacking gel. The acrylamide ratio in

SDS-PAGE gel hinges on the largeness of the target protein. The contents and amount of separating gel (Table 3.4) and stacking gel (Table 3.5) are given below.

Table 3.4: The contents and amount of SDS-PAGE 12 % Acylamide separating gel.

Contents	Amount
dH ₂ O	2.45 mL
Acrylamide/Bis-acrylamide (30% (w/v))	3.0 mL
Tris (pH 8.8) (1.5 M)	1.9 mL
SDS (10% (w/v))	0.075 mL
Ammonium Persulfate (APS) (10% (w/v))	0.075 mL
TEMED	0.003 mL
Total	7.5 mL

Table 3.5: The contents and amount of SDS-PAGE stacking gel.

Contents	Amount
dH ₂ O	3.40 mL
Acrylamide/Bis-acrylamide (30% (w/v))	0.83 mL
Tris (pH 6.8) (1.0 M)	0.63 mL
SDS (10% (w/v))	0.5 mL
APS (10% (w/v))	0.5 mL
TEMED	0.005 mL
Total	5.0 mL

3.1.5.2.3. 1X Running Buffer

Running buffer was used as a buffer in the electrophoresis stage in SDS-PAGE processes. The content and amounts of 1X running buffer are given in Table 3.6.

Table 3.6. The content and amounts of SDS-PAGE 1X running buffer.

Content	Amount
Tris	3.03 g
Glycine	14.04 g
SDS (0.1% (w/v))	1.0 g
dH ₂ O	Up to 1L

3.1.5.3. Protein Purification Buffers

Proteins purification from bacterial lysates was performed by used HisTrap purification columns. Purification buffers are explained below;

- Buffer A: 3.12 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (20 mM), 29.22 g NaCl (500 mM) and 2.04 g Imidazole (30 mM) were dissolved in dH_2O and the pH was adjusted to 7.4 using NaOH. Then buffer was completed to 1000 mL dH_2O .
- Buffer B: 3.12 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (20 mM), 29.22 g NaCl (500 mM) and 34.04 g Imidazole (500 mM) were dissolved in dH_2O and the pH was adjusted to 7.4 using NaOH. Then buffer was completed to 1000 mL dH_2O .
- Buffer A with 100 mM Imidazole (50 mL): 42.55 mL Buffer A and 7.45 mL Buffer B were combined.
- Buffer A with 200 mM Imidazole (50 mL): 31.9 mL Buffer A and 18.1 mL Buffer B were combined.
- Buffer A with 400 mM Imidazole (50 mL): 10.60 mL Buffer A and 39.40 mL Buffer B were combined.

Buffer A and Buffer B were filtered before use.

3.1.5.4. Enzyme Activity Assay Buffers and Solutions

Enzyme activity assay buffer are explained below;

- Sodium Phosphate Buffer Solution (NaPi) (100 mM): 8.20 g Na_2HPO_4 and 5.82 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were dissolved in dH_2O and NaPi buffer were adjusted to pH 7.0 by using Na_2HPO_4 as a base and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ as an acid. The final volumes were completed to 1000 mL.
- Sodium Phosphate Buffer Solution (NaPi) (100 mM): 10.90 g Na_2HPO_4 and 3.10 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were dissolved in dH_2O and NaPi buffer were adjusted to pH 8.0 by using Na_2HPO_4 as a base and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ as an acid. The final volumes were completed to 1000 mL.

- Citrate Buffer (100 mM): 1.47 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ and 0.96 g $\text{C}_6\text{H}_8\text{O}_7$ were dissolved in dH_2O and citrate buffer were adjusted to pH 4, 4.5, 5, and 5.5 by using $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ as a base and $\text{C}_6\text{H}_8\text{O}_7$ as an acid. The final volumes were completed to 50 mL.
- Tris-HCl Buffer (100 mM): 0.73 g $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3\text{-HCl}$ was dissolved in dH_2O and Tris-HCl buffer were adjusted to pH 8.5, and 9 by using HCl. The final volumes were completed to 60 mL.
- Glycine Buffer (100 mM): 0.38 g $\text{C}_2\text{H}_5\text{NO}_2$ was dissolved in dH_2O and Glycine buffer were adjusted to pH 9, 9.5 and 10 by using HCl. The final volumes were completed to 50 mL.
- Sodium Formate Solution (200 mM): 0.20 g HCOONa were weighed and dissolved in NaPi (100 mM, pH 7). The final volumes were completed to 15 mL.
- Sodium Bicarbonate Solution (200 mM): 0.25 g NaHCO_3 were weighed and dissolved in NaPi (100 mM, pH 8). The final volumes were completed to 15 mL.
- Enzyme activity solution are explained below;
- 4 mM NAD^+ Solution: 13.0 mg NAD^+ was weighed and dissolved in 5 mL NaPi buffer (100 mM, pH 7).
- 4mM NADH Solution: 14.0 mg NADH was weighed and dissolved in 5 mL NaPi buffer (100 mM, pH 8).
- 20 mM NAD^+ Solution: 68.0 mg NAD^+ was weighed and dissolved in 5 mL NaPi buffer (100 mM, pH 7).
- 20 mM NADH Solution: 70.0 mg NADH was weighed and dissolved in 5 mL NaPi buffer (100 mM, pH 8).

3.2 Methods

3.2.1. Bioinformatic Analysis

In this study, bioinformatic analyzes were performed to select residues for enzyme improvement based on the crystal structure of *Ct*FDH (PDB structure 6T8Y). FDH is a multimeric protein and the interactions between subunits affect the thermostability and activity of the enzyme. There is an interaction between glutamic acid at position 164 and valine122 in the other subunit of *Ct*FDH, that regulates the interactions between the subunits.

In this study, increasing hydrophobic interactions with valine by converting Glu164 to alanine located at the *CtFDH* interface was aimed. The mutation of residue 188 which has a hydrophobic interaction with 164 was decided. By converting the Asp188 residue to arginine, it was aimed to increase the thermostability and activity by increasing the pKa of the Glu164 residue.

3.2.2. Primer Design

Gene sequence of *Chaetomium thermophilus* NAD⁺-dependent FDH (G0SGU4) protein was obtained from Uniprot Data Bank. Primers were designed in accordance with the site-directed mutagenesis for conversion from Glutamic acid to Alanine for position 164 and from Aspartic acid to Arginine for position 188. These primers were analyzed with the Oligo-Evaluator program. Accordingly, primers are given in Table 3.7. The dark gray site shows directed mutagenesis codons.

Table 3.7: Primer sequences for Glu164Ala and Asp188Arg.

Primer	Sequence (5'-3')
Wt <i>Ctfdh</i>	5' GATGAATATGACCTGGAAGGCCAAAGTTGTCGGC 3'
Glu164Ala Forward	5' GATGAATATGACCTGGCGGGCAAAGTTGTCGGC 3'
Glu164Ala Reverse	5' GCCGACAACCTTGCCCGCCAGGTCATATTCATC 3'
Wt <i>Ctfdh</i>	5' CTGAAAGGCTTCGATTGCAAAGAAGCTGCTGTATTAC 3'
Asp188Arg Forward	5' CTGAAAGGCTTCGCTGCAAAGAAGCTGCTGTATTAC 3'
Asp188Arg Reverse	5' GTAATACAGCAGTTCTTTGCAGCGGAAGCCTTTCAG 3'

The properties of Glu164Ala and Asp188Arg *Ctfdh* primers are given in Table 3.8.

Table 3.8: The properties of Glu164Ala and Asp188Arg *Ctfdh* primers.

Primer	Length (bp)	Tm (°C)	GC%
Glu164Ala <i>Ctfdh</i> Primer	33	77.4	54.5
Asp188Arg <i>Ctfdh</i> Primer	36	73.7	47.2

3.2.3. PCR

Site-directed mutagenesis was created on the *Ctfdh* gene using Glu164Ala and Asp188Arg degenerate primers. Cycling parameters of PCR for Glu164Ala and Asp188Arg are given in Table 3.9.

Table 3.9: Cycling parameters of PCR for Glu164Ala and Asp188Arg *CtFDH*.

Stage	Temperature(°C)	Time	Number of cycles
Initial Denaturation	98 °C	30 s	1 cycle
Denaturation	98 °C	10s	30 cycles
Annealing	65 °C	20 s	
Extension	72 °C	150 s	
Final Extension	72 °C	8 min	1 cycle

Reactant and amount used in PCR suitable for site-directed mutagenesis for Glu164A and Asp188Arg are given in Table 3.10.

Table 3.10: Reactant and amount used in PCR for Glu164Ala and Asp188Arg *CtFDH*.

Reactant	Amount
ddH ₂ O	14.0 µL
GC Buffer	4.0 µL
DNA Template (120 ng/µL)	0.4 µL
dNTPs (10 mM)	0.4 µL
Primer Forward (100 ng/µL)	0.4 µL
Primer Reverse (100 ng/µL)	0.4 µL
DMSO (1%)	0.2 µL
Phusion High Fidelity DNA Polymerase	0.2 µL
Total Volume	20.0 µL

3.2.4. DpnI Digestion of the PCR Products

DpnI is used for choosing the methylated and hemi-methylated copy of plasmids for the acquisition of a vector containing the mutant gene. As a result of PCR, 15 µL of the product was incubated with 0.5 µL of DpnI for 1 hour at 37 °C.

3.2.5. Agarose Gel Electrophoresis

0.8% agarose gel was prepared. 0.4 g agarose was weighed, dissolved in 50 mL 1X TBE and heated. When the gel was at the proper temperature poured into the cassette without creating bubbles, and a comb was placed. After the gel solidified, the pre-DpnI PCR product and the post-DpnI PCR product were mixed with 1.0 µL 6X

Tritrack Thermo DNA loading dye and loaded into the wells. Agarose gel was run at 20 min 80 V and then at 40 min 100 V. The image was acquired under UV light.

3.2.6. Transformation of *Ctfdh* to *E. coli* DH5 α

In this study, NEB-High Efficiency Transformation Protocol (C2987I) was applied using the competent *E.coli* DH5 α strain. The step of the protocol is given below:

- *E. coli* cells were thawed in a tube on ice until the ice crystals disappeared.
- 1.5 μ L post-DpnI PCR product was added to the cell mixture. The mixture was placed on ice for 30 min.
- Heat shock was applied at 42 °C for 30 s.
- It was placed on ice for 5 minutes.
- 300 μ L SOC at room temperature was pipetted into the mixture.
- The mixture was shaken at 37 °C, 225 rpm for 60 min.
- It was spread on LB-agar plate containing ampicillin (100 μ g/mL) and incubated overnight at 37 °C.

3.2.7. Plasmids Purification

A single colony was selected from each plate and inoculated into 10 mL of LB containing ampicillin (100 μ g/mL). Samples were incubated overnight at 225 rpm at 37 °C.

The Macherey-Nagel NucleoSpin® Plasmid (NoLid)-Plasmid DNA Purification Kit protocol including the following steps was used.

- **Cell Lysis:** 10 mL of a saturated *E. coli* LB culture was used, pellet cells in a microcentrifuge (Sigma, Germany) for 10 min at 4.500 x g. The supernatant was discarded. 250 μ L Lysis Buffer A1 was added and the cell pellet was resuspended by pipetting up and down. 250 μ L Lysis Buffer A2 was added and tube and tube were inverted 6-8 times. It was incubated at room temperature for 5 min. 300 μ L Lysis Buffer A3 was added and a tube was inverted 6-8 times until blue samples turn colorless exactly.

- Clarification of lysate: The sample was centrifuged for 5 min at 11.000 g at room temperature for clarification of lysate.
- Bind DNA: A Column in a Collection tube was placed and the supernatant was pipetted onto the column. It was centrifuged for 1 min at 11.000 g to bind DNA to the column. The flowthrough was discarded and the Column was placed back into the collection tube.
- Wash silica membrane: 600 µL Wash Buffer was added onto column and tube was centrifuged for 1 min at 11.000 x g. Flowthrough was discarded and the column was placed back into the empty collection tube.
- Dry silica membrane: It was centrifuged for 2 min at 11.000 x g and the collection tube was discarded.
- Elute DNA: The column was placed in a microcentrifuge tube and 50 µL Elution Buffer was added. It was incubated for 1 min at room temperature and was centrifuged for 1 min at 11,000 x g. The eluted plasmids were stored at -20 °C.

3.2.8. DNA Sequence Analysis

15 µL of the isolated DNA sample (100 ng/µl) was sent for DNA sequence analysis to Eurofins Genomics (Germany). The T7 promoter (5' TAATACGACTCACTATAGGG 3') and terminator (5' GCTAGTTATTGCTCAGCGG 3') nucleotide sequence was used for sequence analysis.

3.2.9. Transformation of *Ctfdh* to *E. coli* BL21(DE3)

Cells were transformed for protein expression of FDHs. Plasmids containing the mutant gene and wt gene were transformed into *E. coli* BL21 (DE3) cells by applying NEB-High Efficiency Transformation Protocol (C2987I) as previously described in Section 2.2.6. The samples were spread onto LB-agar plate containing ampicillin (100 µg/mL) and incubated overnight at 37 °C. After incubation, one colony was selected for each sample and the colonies were inoculated into 10 mL LB medium containing ampicillin (100 µg/mL). Pre-culture was prepared by incubating for 16 hours at orbital shaker at 225 rpm at 37 °C.

3.2.10. Purification of Wild Type and Mutant CtFDHs

The 4 mL pre-culture was added to the 400 mL Autoinduction Studier Medium containing ampicillin (100 µg / mL) which was incubated at 30 °C at 225 rpm for 16 hours.

After cultivation, cells were centrifuged at +4 °C at 4.000 rpm for 40 min and pellets of all mutants and wt proteins were obtained. The FDH enzyme is an intracellular enzyme and the protein in the cells can be removed. For this, lysozyme (100 µL/g) was added in proportion to the weights of the pellets. The pellets were resuspended in Buffer A and incubated on the ice at orbital shaker for 30 min. Homogenates were sonicated (Bandelin, Germany) 10 s x 10 times in 10 s intervals in ice. The sonicated cells were clarified by centrifugation at +4 °C at 4.000 rpm for 20 min. The lysate was filtered through a 0.45 µm filter thereafter applied to a HisTrap column which was washed and balanced with the Buffer A. Since the recombinant and wt proteins obtained had His-Tags at the N- and C-terminal, they were purified by HisTrap column (GE, Sweden). The column was washed 25 mL of Buffer A and the CtFDH was eluted with 15 mL Buffer A containing 100 mM Imidazole, 25 mL Buffer A containing 200 mM Imidazole and 15 mL Buffer A containing 400 mM Imidazole concentrations and washed Buffer B. All methods were performed on ice. The expression efficiency was determined by SDS-PAGE.

3.2.11. SDS-PAGE Analysis

After purification, an SDS-PAGE study was done to see the expression of FDH protein. Proteins were incubated at 95 °C for 15 min then centrifuged at 13.500 rpm for 10 min for denaturation before loading the samples into the gel. Samples were prepared by mixing 40 µL sample and 13.5 µL sample loading buffer.

For SDS-PAGE gel; 12% separating gel and stacking gel were prepared according to given in Table 2.4 and Table 2.5. After the SDS gel was placed in the electrophoresis tank, the tank was filled with 1X running buffer. 5 µL protein standard was loaded into the initial well and 15 µL of samples are loaded into the next wells. Later the running was began with 80 V for 10 min and it was continued at 100 V for 1 h.

3.2.12. Ultrafiltration

The fractions of wt, Glu164Ala, and Asp188Arg CtFDHs, which were determined to have CtFDH protein expression with SDS-PAGE images, were mixed in a new falcon tube and completed up to 50 mL with NaPi buffer (pH 7.0). Later, these protein mixtures of wt and mutants were centrifuged at +4 °C in 30.000 MW ultrafiltration tubes (Merck, Germany) at 3.500 rpm for 15 minutes. Centrifugation was continued until 50 mL of protein mixes became 2.5 mL. In this way, both imidazole and salts are removed from the structure and the concentration of mutant proteins is increased.

3.2.13. Enzyme Activity Assay

The activity measurements of the enzyme were determined against sodium formate in the concentration range between 1-200 mM containing 100 mM NaPi buffer. 4 mM NAD⁺ was added to the reaction last. For the reverse reaction, kinetic measurements of the enzyme were determined against sodium bicarbonate in a concentration range between 1-200 mM containing 100 mM NaPi buffer. The reaction was initiated by adding the 4 mM NADH. Reactants and quantities of reactions in enzyme activity studies are shown in Table 3.11.

Table 3.11: Reactants and amount used in the oxidation and reduction reactions to determine the enzyme activity.

Oxidation of Sodium Formate		Reduction of Sodium Bicarbonate	
Reactant	Amount	Reactant	Amount
Sodium Formate (1-200 mM)	100 µL	Sodium Bicarbonate (1-200 mM)	100 µL
Enzyme (0.25 mg/mL)	50 µL	Enzyme (0.25 mg/mL)	50 µL
NAD ⁺ (4 mM)	50 µL	NADH (4 mM)	50 µL
Total Volume	200 µL	Total Volume	200 µL

NAD⁺-dependent FDH catalyzes the conversion of the formate to CO₂ in conjunction with NAD⁺ reduction to NADH. NADH is a molecule that gives a peak at 340 nm in spectrophotometric measurements. Absorbance values were obtained by monitoring the change in absorbance at 340 nm per twenty-second.

3.2.14 Effect of pH

The effect of changing pH in various buffers was examined for wt and mutant CtFDHs. Sodium citrate buffer (pH 4, 4.5, 5, 5.5), NaPi buffer (pH 6, 6.5, 7, 7.5, 8), Tris-HCl buffer (pH 8, 8.5) and Glycine buffer (pH 9, 9.5, 10) was used. 20 mM NAD⁺ or NADH according to reaction was added last. In Table 3.12, the reactants and their amounts in the reactions established to determine the effect of different pH on the enzyme activity are given.

Table 3.12: Reactants and amount used in the forward and reverse reactions to determine the effect of pH.

Forward Reaction		Reverse Reaction	
Reactant	Amount	Reactant	Amount
Buffer (100 mM)	160 μ L	Buffer (100 mM)	160 μ L
Enzyme (0.25 mg/mL)	20 μ L	Enzyme (0.25 mg/mL)	20 μ L
Sodium Formate (400 mM)	10 μ L	Sodium Bicarbonate (400 mM)	10 μ L
NAD ⁺ (20 mM)	10 μ L	NADH (20 mM)	10 μ L
Total Volume	200 μL	Total Volume	200 μL

3.2.15. Effect of Temperature

The thermostability was determined for wt and mutant CtFDHs. Enzymes were pre-heated in the temperature range 25-60 °C for 20 minutes. In Table 3.13, the reactants and their amounts in the reactions established to determine the effect of different temperatures on the enzyme activity are given.

Table 3.13: Reactants and amount used in the forward and reverse reactions to determine the thermostability.

Forward Reaction		Reverse Reaction	
Reactant	Amount	Reactant	Amount
Sodium Formate (40 mM)	100 μ L	Sodium Bicarbonate (40 mM)	100 μ L
Enzyme (0.25 mg/mL)	50 μ L	Enzyme (0.25 mg/mL)	50 μ L
NAD ⁺ (4 mM)	50 μ L	NADH (4 mM)	50 μ L
Total Volume	200 μL	Total Volume	200 μL

3.2.16. Kinetic Analysis

The activity of an enzyme can be measured by observing the consumption of the substrate or the production of the product. To follow the product gives more accurate results, as small changes in product formation are easier to detect than substrate reduction. While NAD⁺-dependent CtFDH activity is measured, the reduction of NAD⁺ to NADH was observed. The ratio of NADH can be easily determined since NADH gives an absorbance peak at 340 nm.

Using spectrophotometric measurements, the concentration of a solution can be calculated with the Equation (3.1) according to Beer-Lambert Law. "A" indicates absorbance and does not contain units. The unit " ϵ " represents the molar absorptivity of $M^{-1}.cm^{-1}$. It is specific and fixed for each compound. The molar absorptivity of NADH is $6.22.10^3 M^{-1}.cm^{-1}$. "d" is the path length of the sample, which is the path length of the cuvette containing the sample, expressed in centimeters. "C" means the concentration of the sample; its unit is expressed as mM.

$$A = \epsilon dC \quad (3.1)$$

Specific activity (U/mg) can be obtained using Equation (3.1). Equation (3.2) was used to obtain the specific activity from the absorbance values obtained as a result of spectral measurements.

$$Specific\ activity = \frac{(\Delta A / \min) \cdot Volume\ of\ assay}{(\epsilon \cdot Volume\ of\ enzyme \cdot d \cdot C)} \quad (3.2)$$

Using Equation (3.3), Michaelis-Menten kinetic values are obtained. The equation that describes the enzyme reaction rate (V), expressed in terms of the concentration of a substrate (S) is. V_{max} is the highest reaction rate that can be obtained from the reaction, the Michaelis constant K_M is the substrate concentration at which the reaction rate is half of V_{max} .

$$V = \frac{V_{max} [S]}{K_M + [S]} \quad (3.3)$$

After V_{max} and K_M values were calculated for each variable, the k_{cat} value of the enzyme was calculated according to Equation (3.4).

$$k_{cat} = V_{max} \cdot Mw_{Enzyme} \quad (3.4)$$

The reaction involved 4 mM NAD^+ or 4 mM NADH according to reaction and the concentration range was used for measuring the kinetic parameters was 200-1 mM for sodium formate and 200-1 mM for sodium bicarbonate. According to the results obtained, enzyme kinetics calculations including K_M , k_{cat} , k_{cat}/K_M concepts were made and Michaelis-Menten graph was created by GraphPad Prism 9.

3.2.17. Molecular Modelling

PDB construct 6T8Y was used to model the 3-D structure of CtFDH. The interface that enhances the dimer-dimer interaction was demonstrated using the PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC. Theoretical pKa calculations were made at <http://biophysics.cs.vt.edu> H+++ (calculation at pH 8). Mutations were made in all four subunits in the 6T8Y.

Glu164 in CtFDH (PDB structure 6T8Y), Glu190 in *Pseudomonas* sp. 101 (PDB structure 2NAD) and Glu163 in *Candida boinidii* FDH (PDB structure 2FSS) located in the interface between the FDH dimer molecules. In the crystal structure 2NAD, there doesn't exist a hydrogen bond between the aspartic and glutamic acids (over 4.0 Å distance). In 2FSS Asp187 was in 3.9Å distance from the Glu163 in the mutation Asn187Asp. In 2NAD, the Asn side chain was rotated into hydrogen bonding distance in mutation Asp214Asn [Özgün et al., 2015]. The effect of the mutation on possible

interactions on the enzyme has been studied using the structure of CtFDH PDB Structure 6T8Y.

Mutation of Glu164Ala and mutation of Asp188Arg (Figure 3.2) were modeled on 6T8Y using the PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC.

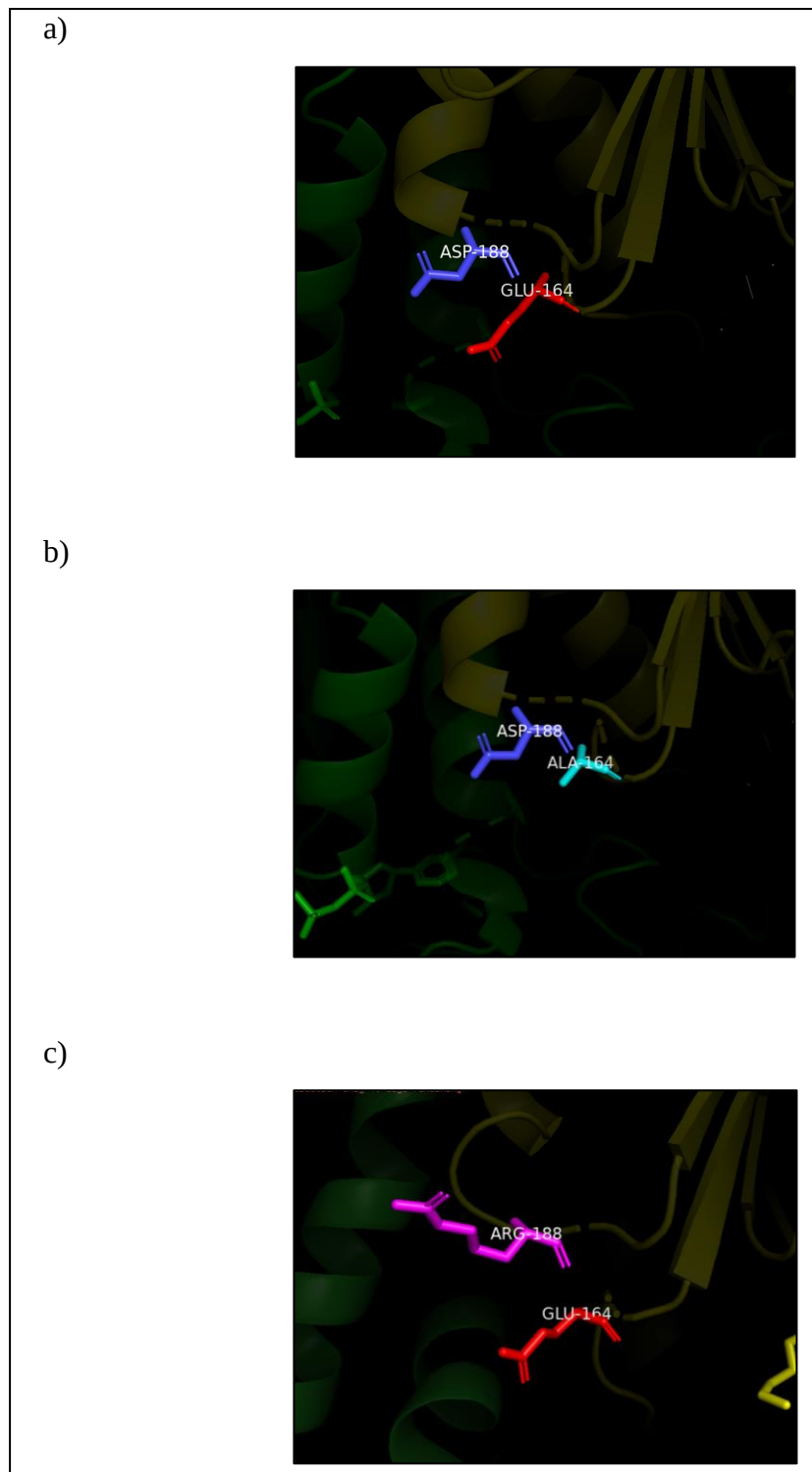


Figure 3.2: 3-D structure of wt, Glu164Ala and Asp188Arg CtFDH based on PDB 6T8Y (a) wt, (b) Glu164Ala, (c) Asp188Arg. Red: Glu164; blue: Asp188; magenta: Ala164; purple: Arg188.

4. RESULTS

4.1. Post-PCR Control of Site-Directed Mutagenesis

Mutations were applied on the pET-45b(+) vector containing the *Ctfdh* gene using the designed degenerate primers. It was converted to alanine for Glu164 position and to arginine for Asp188 position in PCR. The PCR products were visualized on agarose gel electrophoresis. According to the gel images obtained, the expected PCR product bands are shown in Figure 4.1, with the pET-45b(+) vector containing the wt and mutant *Ctfdh* gene of approximately 6403 bp size.

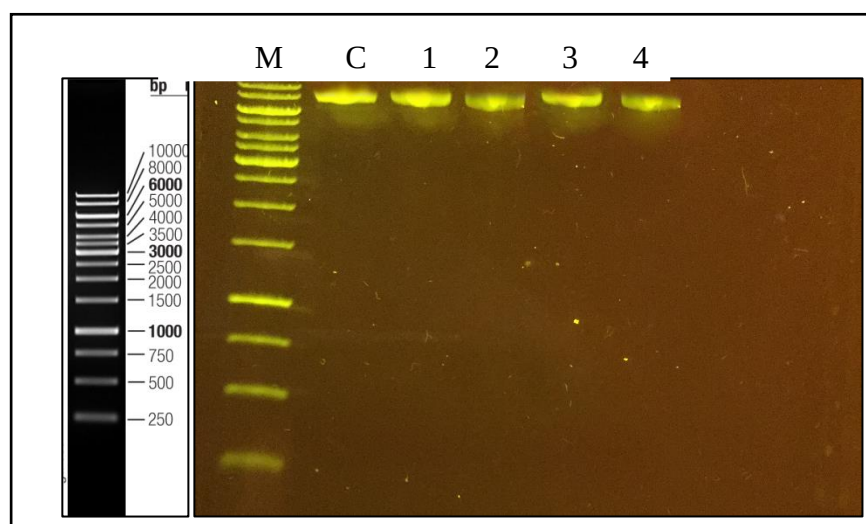


Figure 4.1: CtFHDs Electrophoresis agarose gel image of FHDs after PCR and after DpnI. M: marker; C: control wt *Ctfdh*; 1: Glu164Ala *Ctfdh* after PCR; 2: Asp188Arg *Ctfdh* after PCR; 3: Glu164Ala *Ctfdh* after DpnI; 4: Asp188Arg *Ctfdh* after DpnI.

4.2. Transformation of *Ctfdh* to *E. coli* DH5- α

Transformation of plasmids containing mutation into NEB® 5 α competent *E. coli* cells was performed. Bacterial colonies on LB-agar containing ampicillin (100 μ g/mL) are shown in Figure 4.2.

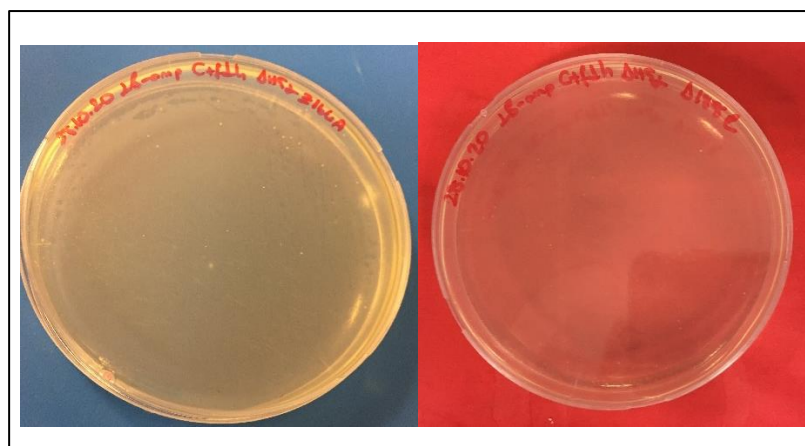


Figure 4.2: Transformation plates of CtFDHs to *E.coli* DH5α

4.3. DNA Sequence Analysis Results of Mutations

After PCR product transformation, plasmids containing the *Ctfdh* genes were purified from NEB® 5a competent *E. coli* cells using the Macherey-Nagel NucleoSpin® Plasmid (NoLid)-Plasmid DNA Purification Kit according to the protocol described in section 2.2.7. The plasmids were sent to Eurofins (Germany) for sequence analysis. The T7 promoter (5' TAATACGACTCACTATAGGG 3') and terminator (5' GCTAGTTATTGCTCAGCGG 3') nucleotide sequence was used for sequence analysis. Sequencing results were compared to wt *Ctfdh* using the BioEdit Version 7.2 Sequence Alignment Editor program. Glu164Ala and Asp188Arg mutations were confirmed by sequencing results. In Residue 164, the AAG codon encoding glutamic acid was mutated to the CGG codon encoding alanine. In Residue 188, the GAT codon encoding aspartic acid was mutated to the CGC codon encoding arginine. Sequence analysis results of mutations for regions containing related positions are shown in Figure 4.3.

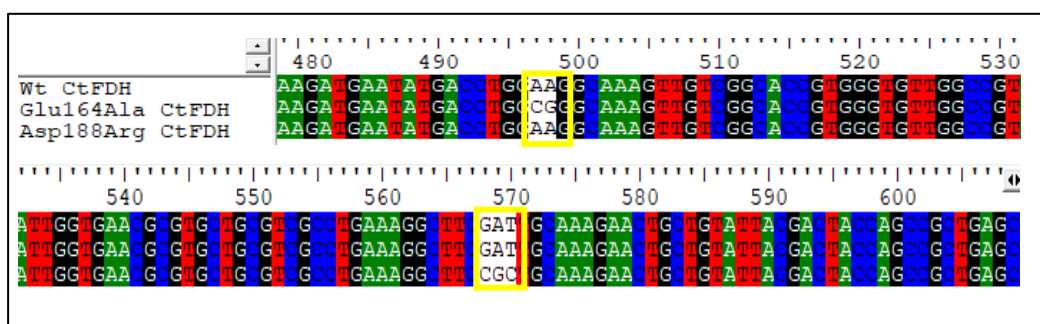


Figure 4.3: Sequencing results to compare to wt *Ctfdh* with Glu164Ala and Asp188Arg mutations. Yellow boxes indicate the sites where the mutation was made.

4.4. Results of Mutant CtFDH Protein Expression

pET-45b(+) vectors containing mutant *Ctfdh* genes were transformed into *E. coli* BL21 (DE3) cells according to the protocol described in section 2.2.9. Bacterial colonies on the LB-agar containing ampicillin (100 µg/mL) were shown in Figure 4.4.

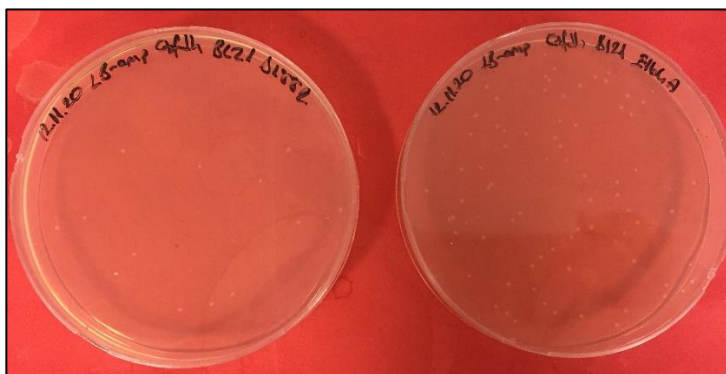


Figure 4.4. Transformation plates of CtFDHs to *E.coli* BL21 (DE3)

Protein expression and purification were performed as described in Sections 2.2.10. The SDS-PAGE gel images obtained by loading the collected appropriate fractions at the end of purification show in Figure 4.5. The gel image shows that CtFDH is ~45,6 kDa in size and pure fractions were obtained for kinetic analysis.

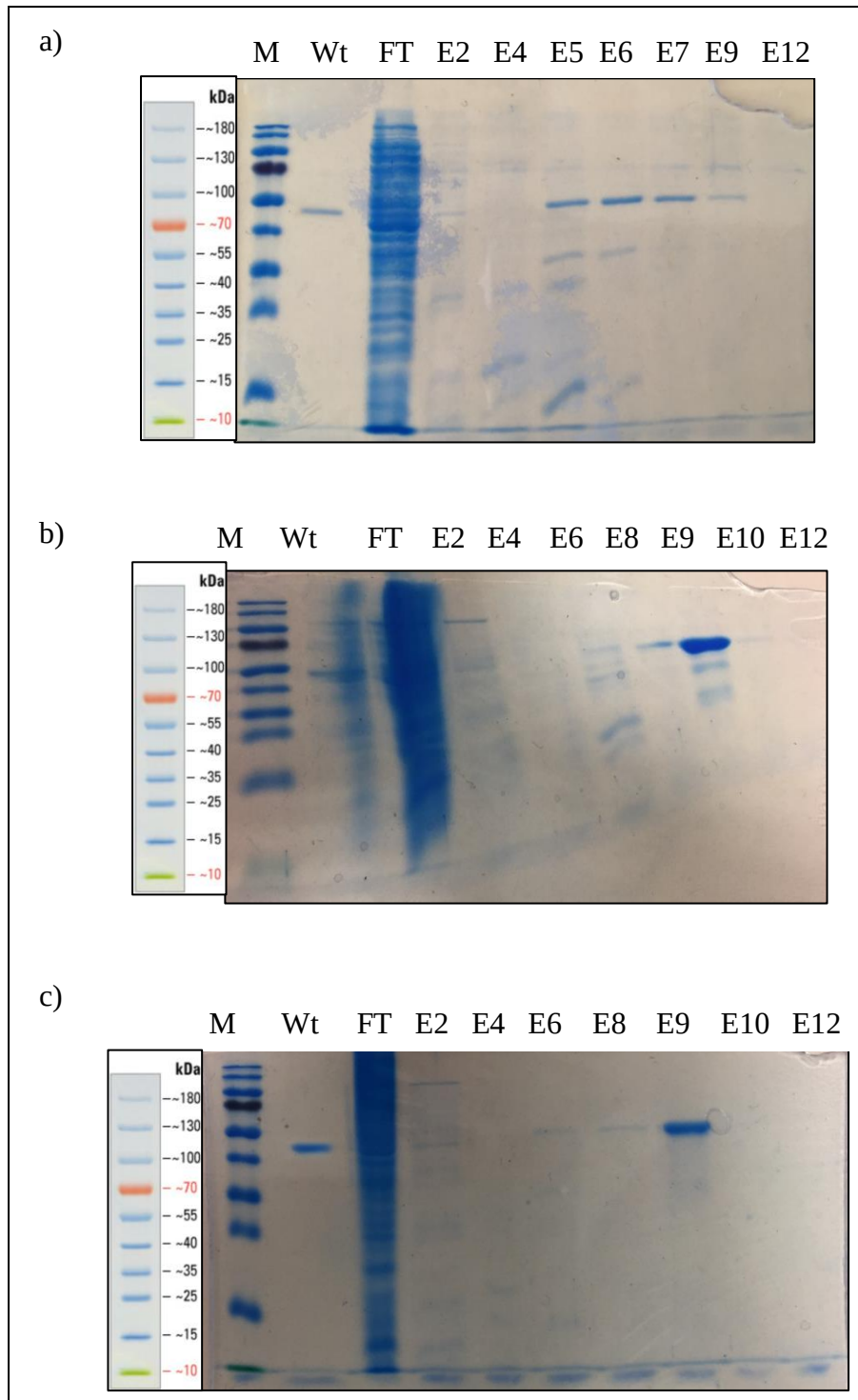


Figure 4.5: SDS-PAGE images of wt and mutants *CtFDHs*. (a) Wt *CtFDH*, (b) *CtFDH* Glu164Ala (c) *CtFDH* Asp188Arg. M: marker; Wt: wild type *CtFDH*; FT: flow through; E2, E4, E5, E6, E7, E8, E9, E10 and E12: eluted fractions.

4.5. Effect of pH on CtFDH Enzyme Activity

According to the relative activity graph created towards sodium formate (Figure 4.6.a.), pH for wt was pH 8, while it was pH 7 for Glu164Ala and Asp188Arg CtFDHs.

In the relative activity graph created according to the activity measurements against sodium bicarbonate (Figure 4.6.b.), the optimum pH for wt and Asp188Arg CtFDH is pH 8, while it is pH 7.5 for Glu164Ala.

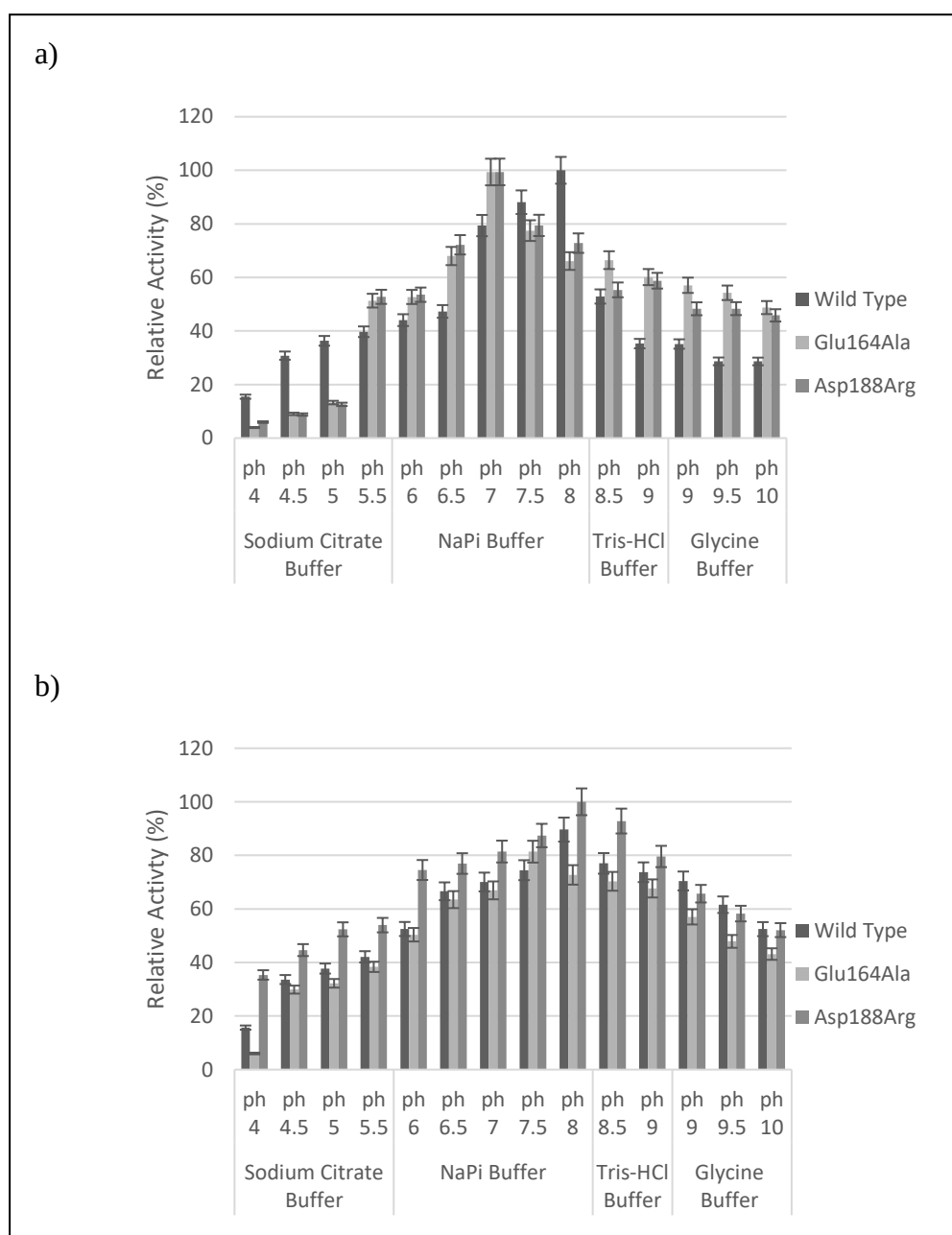


Figure 4.6: Effect of different pH values on wt, Glu164Ala, Asp188Arg CtFDHs towards (a) sodium formate, (b) sodium bicarbonate.

4.6. Effect of Temperature on *Ct*FDH Enzyme Activity

According to the relative activity graph created in the towards of Sodium format for enzyme thermostability determination (Figure 4.7.a.), it was determined as 40 °C for wt, Glu164Ala and Asp188Arg *Ct*FDHs. In the relative activity graph (Figure 4.7.b.) created according to the activity measurements obtained towards sodium bicarbonate, the thermostability for wt and Glu164Ala *Ct*FDHs is 45 °C, while it is 50 °C for Asp188Arg *Ct*FDH.

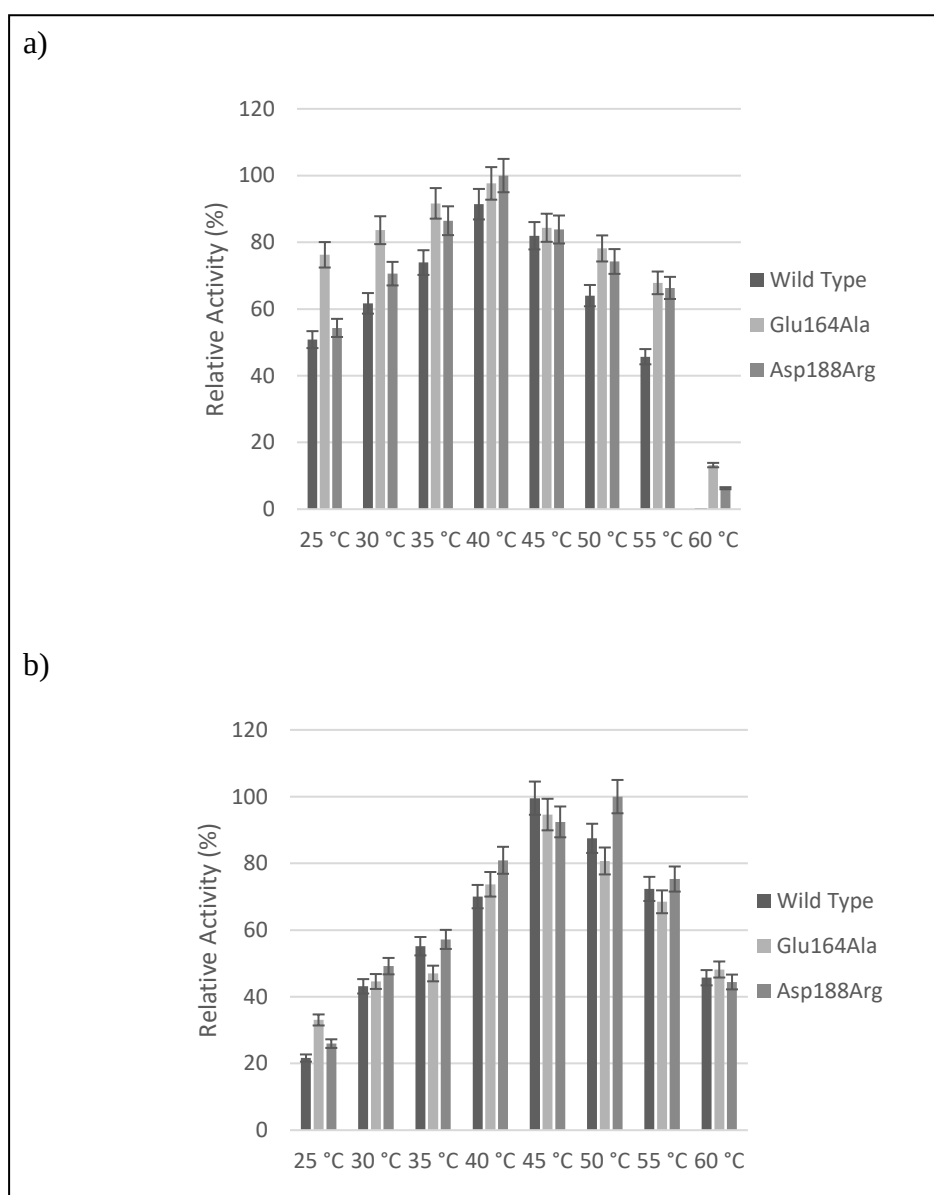


Figure 4.7: Effect of different temperatures on thermostability of wt, Glu164Ala, Asp188Arg *Ct*FDHs towards (a) sodium formate, (b) sodium bicarbonate.

4.7. Results of Kinetic Analysis for Wild Type and Mutant CtFDHs

Kinetic measurements were taken at room temperature as described in Section 2.2.14, 2.2.15. Michaelis-Menten kinetic values were determined. Michaelis-Menten graphs towards sodium bicarbonate and sodium formate for wt and mutants CtFDH were shown in Figures 4.8 and 4.9. K_M , k_{cat} and k_{cat}/K_M values for the wt and mutant CtFDHs are shown in Table 4.1.

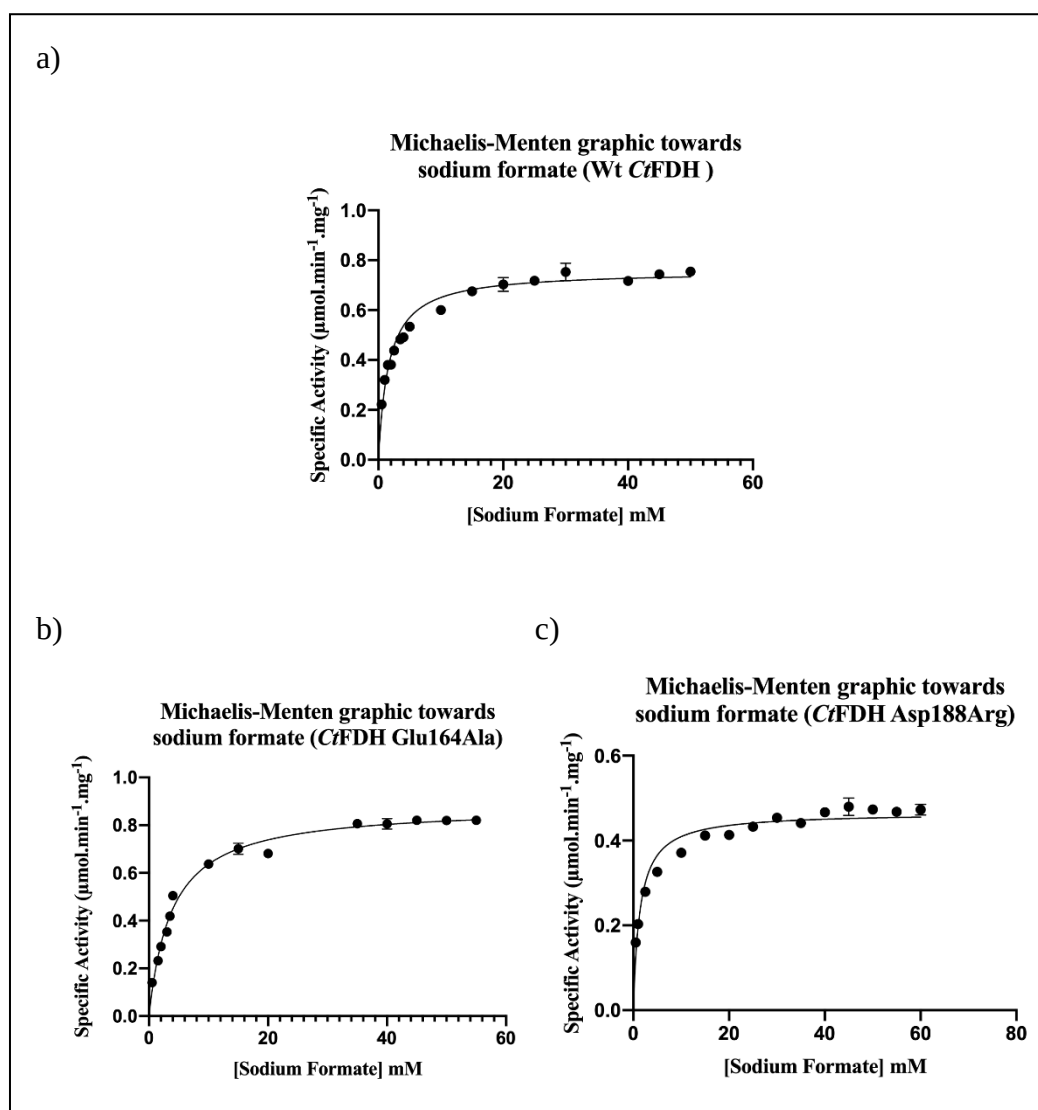


Figure 4.8: Michaelis-Menten graphs of sodium formate for (a) Wt CtFDH, (b) Glu164Ala CtFDH, (c) Asp188Arg CtFDH.

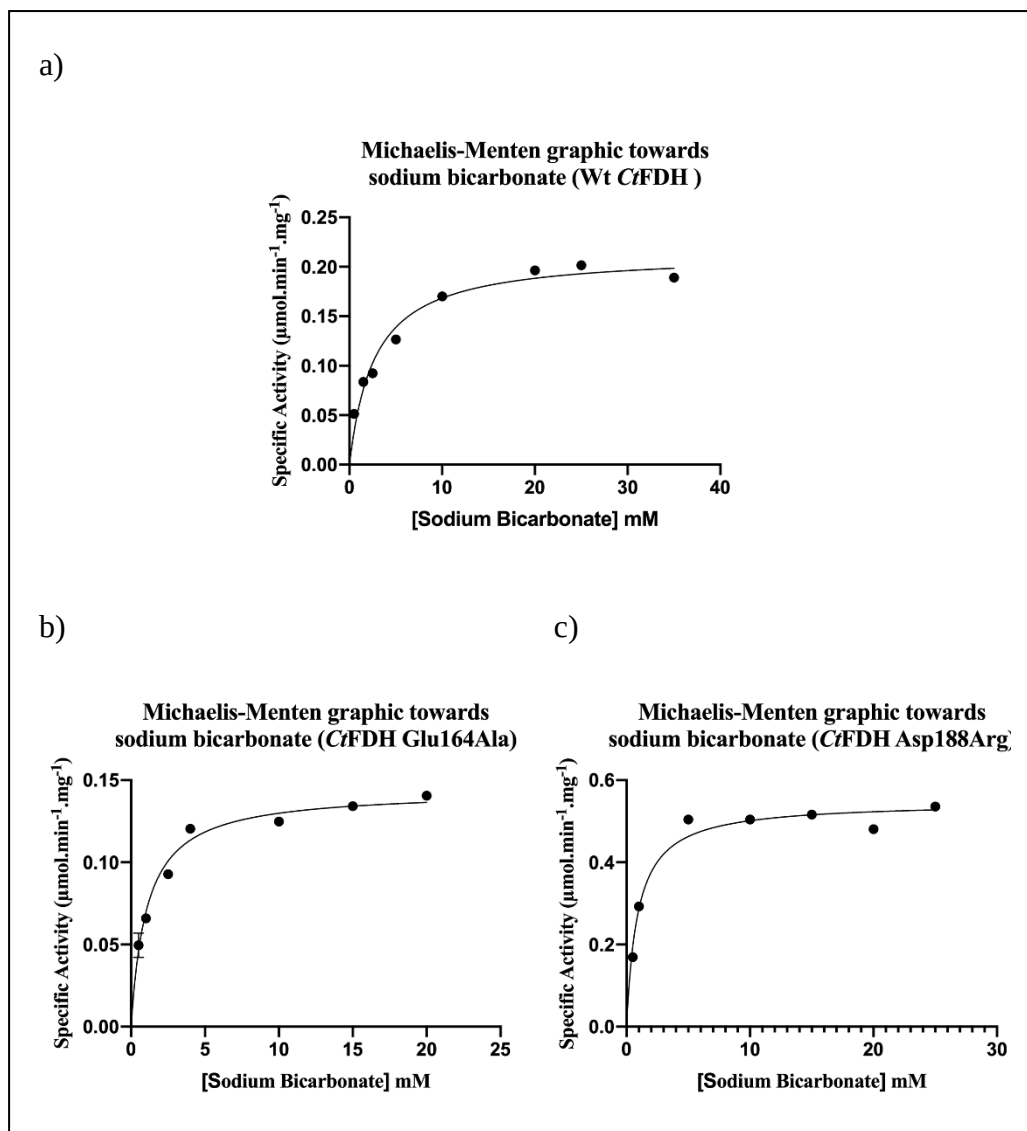


Figure 4.9: Michaelis-Menten graphs of sodium bicarbonate for (a) Wt *CtFDH*, (b) Glu164Ala *CtFDH*, (c) Asp188Arg *CtFDH*.

Tablo 4.1: K_M , k_{cat} and k_{cat}/K_M values of the wt and mutant CtFDHs towards (a) sodium formate and (b) sodium bicarbonate.

a)

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1} s^{-1}$)
Wt CtFDH	1.64 ± 1.27	0.58 ± 0.10	0.35
Glu164Ala CtFDH	3.84 ± 0.74	0.67 ± 0.06	0.17
Asp188Arg CtFDH	1.38 ± 0.15	0.35 ± 0.02	0.26

b)

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1} s^{-1}$)
Wt CtFDH	2.71 ± 0.84	0.16 ± 0.18	0.06
Glu164Ala CtFDH	1.07 ± 0.33	0.11 ± 0.00	0.10
Asp188Arg CtFDH	0.91 ± 0.04	0.42 ± 0.13	0.48

4.8. Molecular Modelling

The FDH enzyme has a homodimeric structure (Figure 4.10). There is an interaction between the side chain of Glu164 amino acid in each subunit and the side chain of Val122 in the other subunit that allow the enzyme to be packaged (Figure 4.11.) [Özgün et al., 2015]. Figure 4.12 shows the estimated distances among Glu164, Asp188 and Val122 for wt CtFDH. There is no H bond between Glu164 and Asp188. The Asp188Arg mutation increased the predicted distance between Glu164 and Asp188Arg compared to the wt (Figure 4.13.). The images were created by the PyMOL Molecular Graphics System, Version 2.3, Schrödinger, LLC.

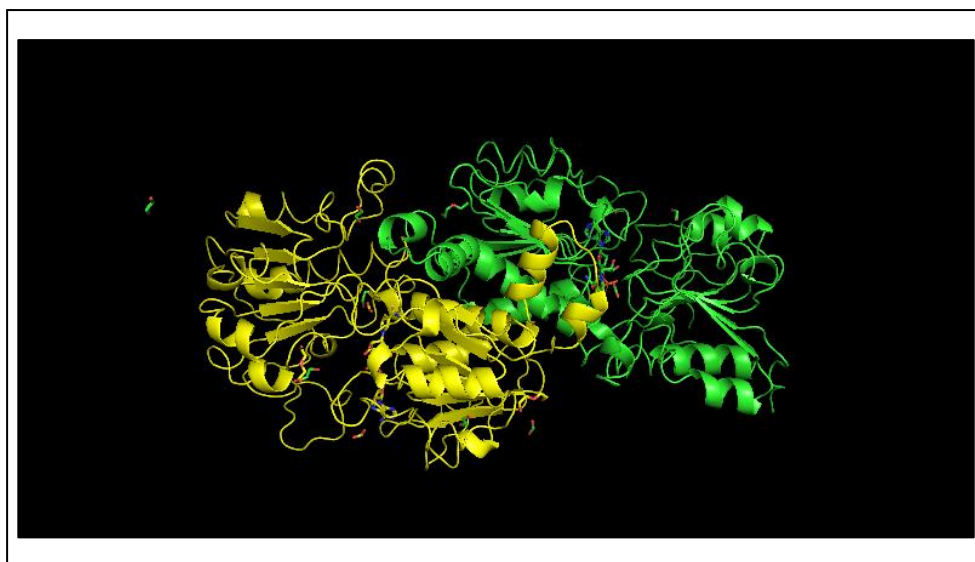


Figure 4.10: Ribbon presentation of the modeled structure of the dimer *CtFDH* enzyme. A chain is shown green, B chain is shown yellow.

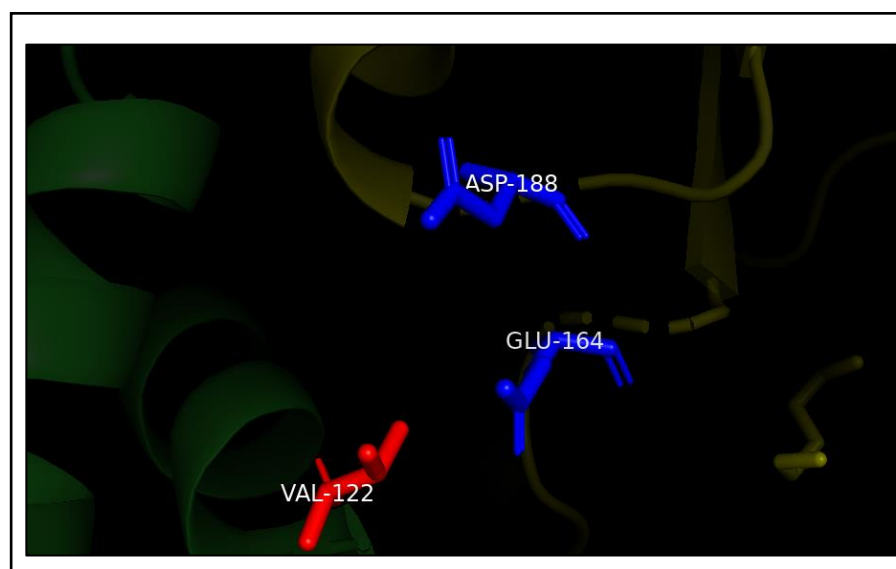


Figure 4.11: Glu164, Asp188 and Val122 presentation of the modeled structure of the dimer *CtFDH* enzyme. Glu164 and Asp188 in the A chain are shown blue, Val122 in the B chain are shown red.

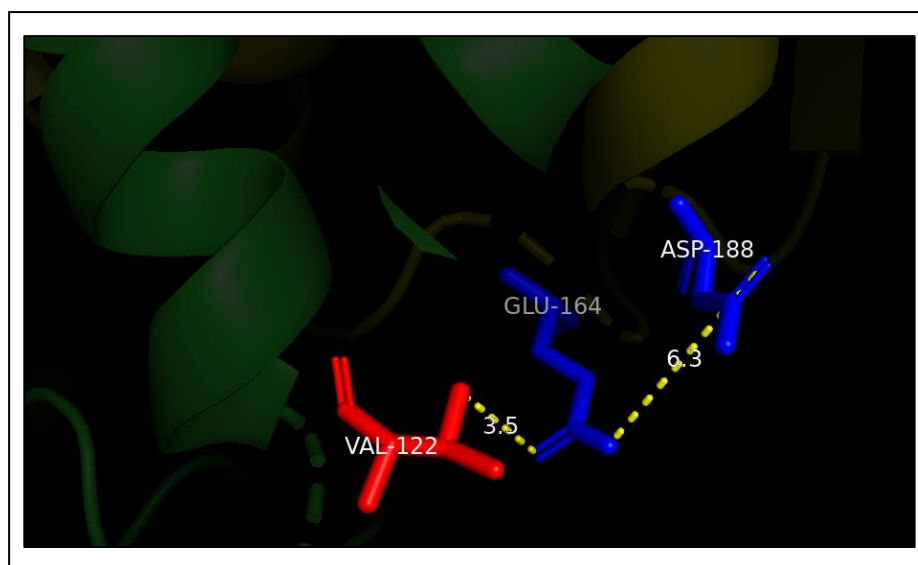


Figure 4.12: Estimated distance measure among Glu164, Asp188 and Val122 for wt CtFDH. Glu164 and Asp188 are shown blue, Val122 are shown red.

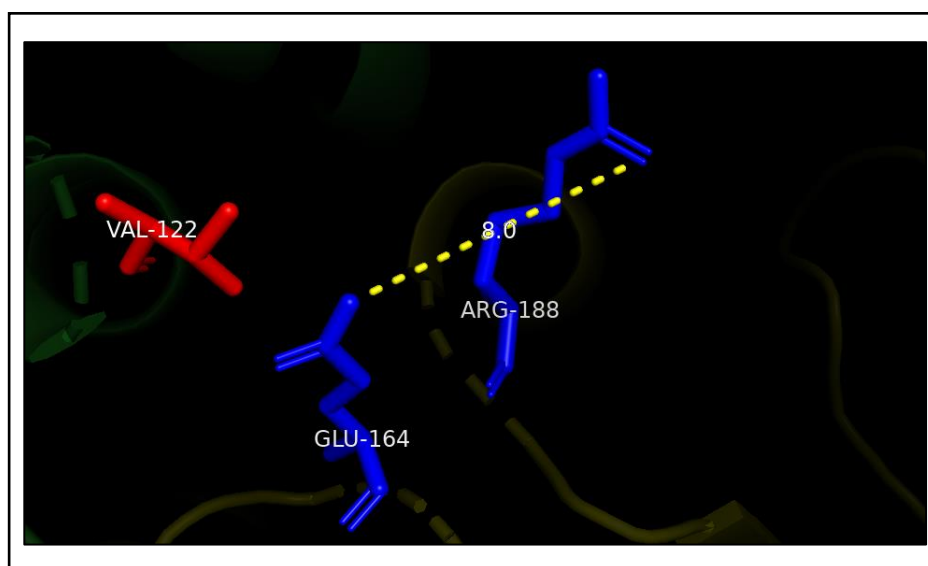


Figure 4.13: Estimated distance measure between Glu164 and Asp188Arg. Glu164 and Asp188Arg are shown blue, Val122 are shown red.

5. DISCUSSION

In this study, primers for Glu164Ala and Asp188Arg mutations were designed using the directed mutagenesis technique to increase the specific activity and the stability of the NAD⁺-dependent CtFDH enzyme proposed on Özgün et al., [Özgün et al., 2015]. PCR was performed to generate mutant genes at appropriate parameters. The amplified genes were transformed into competent cells after being imaged in agarose gel electrophoresis. Plasmid isolation was made from the obtained cells and samples were analyzed for DNA sequence in order to confirm the mutations. Cells containing the mutant gene were incubated in auto-induction medium to obtain protein from the confirmed mutations.

Spectral measurements were made to determine the optimum pH and thermostability in the oxidation reaction of the formate by equating wt and mutant proteins to the same concentrations. Optimum pH was determined as pH 8 for wt CtFDH, while it was determined as pH 7 for Glu164Ala and Asp188Arg CtFDH mutants. In the spectral measurements made to determine the optimum pH in the reduction reaction of sodium bicarbonate, it was determined that the pH was 8 for wt CtFDH. Optimum pH was determined as pH 7.5 for Glu164Ala CtFDH, while pH 8 Asp188Arg CtFDH.

According to the relative activity determined by spectral measurements, there was an increase in thermostability for Glu164Ala and Asp188Arg CtFDHs in the oxidation of the formate compared to wt, but it is not remarkable. In the reverse reaction, the thermostability was not increased for Glu164Ala CtFDH, while the thermostability for Asp188Arg CtFDH increased by 5 °C compared to the wild type.

Specific activity values were determined in spectrophotometer measurements at 340 nm based on the increase and decrease of NADH in various substrate concentrations. According to the values obtained against sodium bicarbonate, K_M value decreased 2.53 times for Glu164Ala while the affinity of the enzyme for the bicarbonate increased at the same rate. For the Asp188Arg, the K_M value decreases by 2.97 times, the affinity of the enzyme towards the bicarbonate increased at the same rate. The k_{cat} and k_{cat}/K_M values for Glu164Ala decreased by 0,67 times and increased 1.70 times, and for Asp188Arg, 2.80 times and 7.62 times increasing was determined,

respectively. The k_{cat}/K_M values indicate 1.7 times increase for Glu164Ala *CtFDH* and a 7.62 times increase for Asp188Arg *CtFDH* in catalytic efficiency.

For the Glu164Ala mutation, the glutamic acid amino acid residue was transformed into alanine. Glutamic acid is negative charged while alanine is non-polar. *CtFDH* enzyme has a homodimer structure containing A, B, C and D chains. There is an interaction between the Glu164 in the A chain and the Val122 residue in the B chain that regulates the dimer interactions. Studies have shown that the interactions between subunits affect the stability and activity of the enzyme. In the study of Hong et al., it was understood that Leu290Phe, Ala222Thr, Val262Leu mutations on Ribulose enzyme change stability and activity with interactions at the interface [Hong and Spreitzer, 1997]. Conversion from negative-charged glutamic acid to non-polar alanine in the residue at position 164 affected their interactions with valine, known as non-polar, on the interface. Our study suggests that it causes a negative change in specific activity and thermostability against sodium bicarbonate. However, the Glu164Ala mutation increased specific activity against sodium formate.

The proposed modeling of HCO_3^- oxidation in *CtFDH* was performed using PDB structure 2NAD for *PseFDH*. The residue numbers given belong to *PseFDH*.

In aqueous solution, firstly, HCO_3^- converts to electrophilic CO_2 and then moves forward as a reactive substrate. During the reduction of CO_2 , the hydride is pushed from the saturated carbon C4 of NADH to hydrogen carbonate. The conversion of NADH to NAD^+ occurs through the arrangement of the nicotinamide ring. The reaction proceeds to the intermediate state of the carbon-hydride bond with two negative oxygens. Such a structure is unstable and thus negative charges cause the leaving hydroxyl group to be removed resulting in the formation of a formate anion. Hydride transfer inside the active site can occur between the hydrogen in the C4 position and the HCO_3^- form [Aslan et al., 2017].

His332 (His312 in *CtFDH*) is in contact with O3 of HCO_3^- by a hydrogen bond, and O1 has hydrogen bonds with the amide proton in the backbone of Ile122 (Ile94 in *CtFDH*) and the side chain of Asn146 (Asn120 in *CtFDH*). These contacts on both sides of the HCO_3^- can help hold it in place until the hydride ion from NADH attacks the carbon atom. The proximity of the positively charged Arg284 (Arg259 in *CtFDH*) can assist the released hydroxide ion to exit the active site and enter the substrate channel. According to a model of the active site, the exit OH^- may move towards

Arg284 [Nilov et al., 2011]. CO₂ and formate in the active site do not have the same positions during catalysis [Aslan et al., 2017].

The fact that the residue 188 of CtFDH mutation has a specific activity enhancing role in the conversion of NADH to NAD⁺ may lead to the suggestion that the above-described NADH and HCO₃⁻ may have a role in their transformation. The strengthening of the interaction between Glu164 and Val122 by the mutation of Asp188Arg may have rearranged all the substrate and NADH binding positions in the active site to positively affect the reaction in order to bind NADH.

It was also transformed from negative charged aspartic acid to positive charged arginine on the CtFDH protein for the Asp188Arg mutation. As suggested in the study of Özgün et al., the Asp188Arg mutation was expected to decrease the thermostability and catalytic activity for Glu164 because the Asp188 position supports the pKa of Glu164 position [Özgün et al., 2015]. The capability to re-engineer enzymatic pH activity profiles is of significance for industrial applications of enzymes. Mutation in the enzyme can affect pKa values so that pH activity profiles can be redesigned. The pKa value of the residue of 164 decreases to 3.4 from 4.7 when the residue of 188 is mutated from aspartic acid to arginine. But the thermal stability and catalytic activity increased against sodium bicarbonate compared with the wt. It was suggested that the Asp188Arg mutation improves packaging by increasing the interaction on the protein-dimer interface between Glu164 and Val122 in the other subunit.

In the study of Jiang et al., residues 120 and 187 in CbFDH are located close to the active site of the enzyme. They suggested that these residues affect substrate and coenzyme binding. The Asn119 residue, which is the substrate-binding site, is close to the Val120 residue. In the Val120S mutation, k_{cat}/K_M increased 2.60 times, indicating that it may affect the efficiency of the enzyme. The k_{cat}/K_M increased 1.48 times in Asn187Asp mutation compared to the wild type. In 3-D studies based on 2FSS PDB structure, it has been shown that the Val120 residue studied affects the substrate affinity and Asn187 affects coenzyme affinity in particular [Jiang et al., 2016].

In the study of Souza et al., it was shown that Ala263Phe and Ser337Phe mutations located far from the active site affected more than mutations closer to the active site [Souza et al., 2018]. Some studies have been done about the effects of mutation on the enzyme activity of amino acids located away from the active site. As a result; It has been shown that mutations in the interface can affect the quaternary structure [Odokonyero et al., 2014], and mutations can cause various conformational

changes in the enzyme [Petrovic et al., 2018]. Studies have shown that mutations in amino acids located 14 Å away from the active site affect enzyme activity. [Chen et al, 1997; Souza et al., 2018]. The fact that the amino acid changes made in the residues create gaps or reduce the gaps on the enzyme, affecting 3-D and the amino acids having new positions, this effect spreads like a wave until the end. Such a wave effect indicates that even though it is a distant mutation to the active site, it may affect the active site [Mendonça and Marana, 2011]. The fact that the Glu164Ala and Asp188Arg mutations made in this study are at 8 and 12.3 Å to Asn120, respectively, and the CtFDH enzyme activity changes, it is thought that they affect the quaternary structure by causing conformational changes in the enzyme.

K_M values of Glu164Ala and Asp188Arg decreased against sodium bicarbonate. Both mutations appear to have increased affinity for Sodium bicarbonate. During the reaction, a hydrogen bond is formed between the oxygen atom of the bicarbonate and Asn120. This bond helps hold the bicarbonate in place until the hydride ion from NADH attacks the bicarbonate. At the same time, positions 121 and 124 interact with NADH. It suggests that mutations at positions 164 and 188, which are close to the active site, rearrange their interactions with Val122 with residues 120, 121 and 124, and increase affinity specifically for sodium bicarbonate.

According to the results, it was seen that residues of 164 and 188 affect the specific activity and thermostability for CtFDH. In the light of these data, alternative mutation studies and modeling studies can be done to examine these positions.

REFERENCES

- Abdel-Azeem A.M., (2020), "Recent Developments on Genus *Chaetomium*", Springer, 1th Edition, Switzerland.
- Almendra M.J., Brondino C.D., Gavel O., Pereira A.S., Tavares P., (1999), "Purification and characterization of a tungsten-containing formate dehydrogenase from *Desulfovibrio gigas*" *Biochemistry*, 38, 16366-16372.
- Amao Y., (2018), "Formate dehydrogenase for CO₂ utilization and its application", *Journal of CO₂ Utilization*, 26, 623-641.
- Amlacher S., Sarges P., Flemming D., van Noort V., Kunze R., Devos D.P., Arumugam M., Bork P., Hurt E., (2011), "Insight into structure and assembly of the nuclear pore complex by utilizing the genome of a eukaryotic thermophile", *Cell*, 146(2), 277-289.
- Arnold F.H., (1998), "Design by Directed Evolution", *Acc. Chem. Res.*, 31, 125-131.
- Aslan A.S., Valjakka J., Ruupunen J., Yildirim D., Turner N.J., Turunen O., Binay B., (2017), "Chaetomium thermophilum formate dehydrogenase has high activity in the reduction of hydrogen carbonate (HCO₃⁻) to formate", *Protein Engineering, Design & Selection*, 30(1), 47-55.
- Badenhorst C.P.S., Bornscheuer U.T., (2018), "Getting Momentum: From Biocatalysis to Advanced Synthetic Biology", *Trends Biochem. Sci.*, 43(3), 180-198.
- Baskaya F.S., Zhao X., Flickinger M.C., Wang P., (2010), "Thermodynamic feasibility of enzymatic reduction of carbon dioxide to methanol", *Appl. Biochem. Biotechnol.*, 162, 391-398.
- Berg J.M., Tymoczko J.L., Stryer L., (2012), "Biochemistry", W.H. Freeman, 7th Edition, New York.
- Blanchard J.S., Cleland W.W., (1980), "Kinetic and chemical mechanisms of yeast formate dehydrogenase", *Biochemistry*, 19, 3543-3550.
- Blöchl, E., Rachel R., Burggraf S., Hafenbradl D., Jannasch H. W., and Stetter K. O., (1997), "Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C.", *Extremophiles*, 1, 14-21.
- Bock T., Chen W.H., Ori A., Malik N., Silva-Martin N., Huerta-Cepas J., Powell S.T., Kastiris P.L., Smyshlyaev G., Vonkova I., Kirkpatrick J., Doerks T., Nesme L., Baßler J., Kos M., Hurt E., Carlomagno T., Gavin A.C., Barabas O., Müller C.W., van Noort V., Beck M., Bork P., (2014), "An integrated approach for genome annotation of the eukaryotic thermophile *Chaetomium thermophilum*", *Nucleic Acids Res.*, 42(22), 13525-13533.
- Bommarius A.S., Broering J.M., Chaparro-Riggers J.F., Polizzi K.M., (2006), "High-throughput screening for enhanced protein stability", *Curr. Opin. Biotechnol.*, 17(6), 606-610.

Bommarius, A. S., Schwarm M., Stingl K., Kottenhahn M., Huthmacher K., Drauz, K., (1995), "Synthesis and use of enantiomerically pure tert-leucine", *Tetrahedron Asymmetry*, 6, 2851-2888.

Bornscheuer U.T., Huisman G.W., Kazlauskas R.J., Lutz S., Moore J.C., Robins K., (2012), "Engineering the third wave of biocatalysis", *Nature*, 485, 185-194.

Brock T. D., (1995), "The road to Yellowstone and beyond.", *Annu. Rev. Microbiol*, 49, 1-28.

Burnett J.W.H., Howe R.F., Wang X., (2020), "Cofactor NAD(P)H regeneration: how selective are the reactions?", *Trends in Chemistry*, 2(6), 488-492.

Cadwell R.C., Joyce G.F., (1992), "Randomization of genes by PCR mutagenesis", *PCR Methods Appl.*, 2(1), 28-33.

Capote N., Aguado A., Pastrana A.M., Sánchez-Torres P., (2012), "Molecular tools for detection of plant pathogenic fungi and fungicide resistance." INTECH Open Access Publisher, 1st Edition.

Carter J.L.L., Bekhouche M., Noiriel A., Blum L.J., Doumèche B., (2014), "Directed Evolution of a Formate Dehydrogenase for Increased Tolerance to Ionic Liquids Reveals a New Site for Increasing the Stability", *Chembiochem*, 15, 2710-2718.

Castillo R., Oliva M., Martí S., Moliner V., (2008), "A theoretical study of the catalytic mechanism of formate dehydrogenase", *J. Phys. Chem.*, 112, 10012-10022.

Chen R., Greer A.F., Dean A.M., (1997), "Structural constraints in protein engineering the coenzyme specificity of *Escherichia coli* Isocitrate Dehydrogenase", *Eur. J. Biochem.*, 250, 578-582.

Cooney D. G., and Emerson R., (1964), "Thermophilic fungi: An account of their biology, activities and classification" W. H. Freeman & Co, 1st Edition.

Crable B.R., Plugge C.M., McInerney M.J., Stams A.J.M., (2011), "Formate formation and formate conversion in biological fuels production", *Enzyme Res.*, 532-536.

Dashtban M., Schraft H., Syed T.A., Qin W., (2010), "Fungal biodegradation and enzymatic modification of lignin", *Int. J. Biochem. Mol. Biol.*, 1(1), 36-50.

Dobbek H., (2011), "Structural aspects of mononuclear Mo/W-enzymes", *Coord. Chem. Rev.* 255, 1104-1116.

Drauz K., Waldmann H., (2002), "Enzyme catalysis in organic synthesis, Vol II", Wiley VCH, 1st Edition, Weinheim, Germany.

Duan X., Cheng S., Ai Y., Wu J, (2016), "Enhancing the thermostability of *Serratia plymuthica* sucrose isomerase using B-factor-directed mutagenesis", *PLoS One*, 11.

Dusan Petrovic D., Risso V.A., Kamerlin S.C.L., Sanchez-Ruiz J.M., (2018), "Conformational dynamics and enzyme evolution", *J. R. Soc. Interface*, 15.

El-Zahab B., Donnelly D., Wang P., (2008) "Particle-tethered NADH for production of methanol from CO₂ catalyzed by coimmobilized enzymes", *Biotechnol. Bioeng.*, 99, 508-514.

Graentzdoerffer A., Rauh D., Pich A., Andreesen J.R., (2003), "Molecular and biochemical characterization of two tungsten- and selenium-containing formate dehydrogenases from *Eubacterium acidaminophilum* that are associated with components of an iron-only hydrogenase", *Arch. Microbiol.*, 179, 116-130.

Gul-Karaguler, N., Sessions, R. B., Clarke, A. R., and Holbrook, J. (2001), "A single mutation in the NAD-specific formate dehydrogenase from *Candida methylica* allows the enzyme to use NADP", *Biotechnol. Lett.*, 23, 283-287.

Heinzelman P., Snow C.D., Wu I., Nguyen C., Villalobos A., Govindarajan S., Minshull J., Arnold F.H., (2009), "A family of thermostable fungal cellulases created by structure-guided recombination" *Proc. Natl. Acad. Sci. USA*, 106(14), 5610-5615.

Hirschmann R., Nutt R.F., Veber D.F., Vitali R.A., Varga S.L., (1969), "Studies on the total synthesis of an enzyme. V. The preparation of enzymatically active material", *J. Am. Chem. Soc.*, 91, 507–508.

Horsman G.P., Liu A.M.F., Henke E., Bornscheuer U.T., Kazlauskas R.J., (2003), "Mutations in Distant Residues Moderately Increase the Enantioselectivity of *Pseudomonas fluorescens* Esterase towards Methyl 3-Bromo-2-methylpropanoate and Ethyl 3-Phenylbutyrate", *Chem. Eur. J.*, 9, 1933-939.

Hummel W., (1999), "Large-scale applications of NAD(P)-dependent oxidoreductases: recent developments", *TIBTECH*, 17, 487–493.

Hummel W., Kula M. R., (1989), "Dehydrogenases for the synthesis of chiral compounds", *Eur. J. Biochem.*, 184, 1-13.

Illanes A ed., (2008), "Enzyme Biocatalysis Principles and Applications", Springer, 1st Edition.

Jestin J.L., Kaminski P.A., (2004), "Directed enzyme evolution and selections for catalysis based on product formation", *J. Biotechnol.*, 113, 85-103.

Jiang W., Lin P., Yang R. and Fang B. (2016), "Identification of catalysis, substrate, and coenzyme binding sites and improvement catalytic efficiency of formate dehydrogenase from *Candida boidinii*", *Applied Microbiology and Biotechnology*, 100, 8425-37.

Jiménez-Osés G., Osuna S., Gao X., Sawaya M.R., Gilson L., Collier S.J., Huisman G.W., Yeates T.O., Tang Y., Houk K.N., (2014), "The Role of Distant Mutations and Allosteric Regulation on LovD Active Site Dynamics", *Nat. Chem. Biol.*, 10(6), 431-436.

Jung W.S., Han J., Ha S., (2007), "Analysis of palladium-based anode electrode using electrochemical impedance spectra in direct formic acid fuel cells", *J. Power Sources*, 173, 53-59.

- Kargov I.S., Kleimenov S. Y., Savin S.S., Tishkov V. I. and Alekseeva A.A. (2015). Improvement of The Soy Formate Dehydrogenase Properties by Rational Design. *Protein Engineering, Design and Selection*, 28(6), 171-178.
- Kim J.S., Raines R.T., (1993), "Ribonuclease S-peptide as a carrier in fusion proteins", *Protein Science*, 2, 348-356.
- La Touche C. J., (1948), "A Chaetomium-like thermophile fungus", *Nature*, 161(320).
- Lee H.J., Lee S.H., Park C.B., Won K., (2011), "Coenzyme analogs: Excellent substitutes (not poor imitations) for electrochemical regeneration", *Chem. Commun*, 47, 12538-12540.
- Lee S., Speight J.G.S., Loyalka K., (2007), "Handbook of Alternative Fuel Technology", CRS Press, Taylor & Francis Group, 1st Edition.
- Lehninger A., (1970), "Biochemistry", Worth Publ. Inc., 7th Edition, New York,
- Lehninger A.L., Nelson D.L., Cox M.M., (2008), "Principles of Biochemistry", W.H. Freeman, 5th edition, New York.
- Leisola M, Jokela J., Pastinen O., Turunen O., Schoemaker H., (2001), "Industrial Use of Enzymes", Eolss Publishers, 1st Edition, Oxford.
- Leung D.W., Chen E., Goeddel D.V., (1989), "A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction", *Technique*, 1, 11-15.
- Li A.N., Yu K., Liu H.Q., Zhang J., Li H., Li D.C., (2010), "Two novel thermostable chitinase genes from thermophilic fungi: cloning, expression and characterization", *Bioresour. Technol.*, 101, 5546-5551.
- Li S., Wilkinson M.F., (1997), "Site-directed mutagenesis: a two-step method using PCR and DpnI", *Biotechniques*, 23(4), 588-590.
- Matthews B.W., (1993), "Structural and genetic analysis of protein stability", *Annu. Rev. Biochem.*, 62, 139-160.
- Mendonça L.M.F. and Marana S.R., (2011), "Single mutations outside the active site affect the substrate specificity in a β -glycosidase", *Biochimica et Biophysica Acta*, 1814, 1616-1623.
- Mesentsev A. V., Lamzin V. S., Tishkov V. I., Ustinnikova T. B., Popov V. O. (1997), "Effect of pH on kinetic parameters of NAD⁺-dependent formate dehydrogenase", *Biochem. J.*, 321, 475-480.
- Miguel A.S.M., Martins-Meyer T.S., da Costa Figueiredo E.D., Lobo B.W.P., Dellamora-Ortiz G.M., (2013), "Enzymes in bakery: current and future trends", In: Muzzalupo I., Editor, "Food Industry", InTech., 1st Edition, Rijeka, Croatia.

Mota C.S., Rivas M.G., Brondino C.D., Moura I., Moura J.J.G., Gonzalez P.J., Cerqueira N.M., (2011), “The mechanism of formate oxidation by metal-dependent formate dehydrogenases”, *J. Biol. Inorg. Chem.*, 16, 1255-1268.

Neuhauser W., Steininger M., Haltrich D., Kulbe K. D., Nidetzky B. (1998), “A pH-controlled fed-batch process can overcome inhibition by formate in NADH-dependent enzymatic reductions using formate dehydrogenase-catalyzed coenzyme regeneration”, *Biotechnol. Bioeng.*, 60, 277-282.

Niks D., Hille R., (2018), “Reductive activation of CO₂ by formate dehydrogenases”, *Methods in Enzymology*, 613, 277-295.

Niks D., Hille R., (2019), “Molybdenum- and tungsten-containing formate dehydrogenases and formylmethanofuran dehydrogenases: Structure, mechanism, and cofactor insertion”, *Protein Sci.*, 28(1), 111-122.

Nilov D.K., Shabalin I.G., Popov V.O., Švedas V.K., (2011), “Investigation of formate transport through the substrate channel of formate dehydrogenase by steered molecular dynamics simulations”, *Biochemistry (Moscow)*, 76, 172-174.

Novagen[®], (2009), “pET-45b(+) Vector”.

O'Connor C., Adams J.U., (2014), “Essentials of Cell Biology”, MA: NPG Education, 1st Edition, Cambridge.

Odintseva E. R., Popova A. S., Rojkova A. M., Tishkov V. I., (2002), “Role of cysteine residues in stability of bacterial formate dehydrogenase”, *Chemistry*, 43, 356-359.

Odokonyero D., Sakai A., Patskovsky Y., Malashkevich V.N., Fedorov A.A., Bonanno J.B., Fedorov E.V., Toro R.T., Agarwal R., Wang C., Ozerova N.D.S., Yew W.S., Sauder J.M., Swaminathan S., Burley S.K., Steven C. Almoc S.C., Glasner M.E., (2014), “Loss of quaternary structure is associated with rapid sequence divergence in the OSBS family”, *PNAS*, 111(23), 8535-8540.

Özgün G., Karagüler N.G., Turunen O., Turner N.J., Binay B., (2015), “Characterization of a new acidic NAD⁺-dependent formate dehydrogenase from thermophilic fungus *Chaetomium thermophilum*”, *Journal of Molecular Catalysis B: Enzymatic*, 122, 212-217.

Patel A.K., Singhanian R.R., Pandey A., (2016), “Novel enzymatic processes applied to the food industry”, *Curr. Opin. Food Sci.*, 7, 64-72.

Patel R.N., (2003), “Microbial/enzymatic synthesis of chiral pharmaceutical intermediates”, *Curr. Opin. Drug Discov. Devel.*, 6, 902-920.

Punekar N.S., (2018), “ENZYMES: Catalysis, Kinetics and Mechanisms”, Springer Nature, 1st Edition Singapore.

Rathod J., Dhebar S., Archana G., (2017), “Efficient approach to enhance whole cell azo dye decolorization by heterologous overexpression of *Enterococcus* sp. L2 azoreductase (azoA) and *Mycobacterium vaccae* formate dehydrogenase (fdh) in different bacterial systems”, *International Biodeterioration & Biodegradation*, 1-10.

Reda T., Plugge C.M., Abram N.J., Hirst J., (2008), "Reversible interconversion of carbon dioxide and formate by an electroactive enzyme", *Proc. Natl. Acad. Sci. USA*, 105, 10654-10658.

Reetz M.T., Carballeira J.D., (2007), "Iterative saturation mutagenesis (ISM) for rapid directed evolution of functional enzymes", *Nat. Protoc.*, 2(4), 891-903.

Roberts S., (2000), "Preparative biotransformations", *J. Chem. Soc. Perkin Trans*, 1, 611-633.

Rotberg N.S., Cleland W.W., (1991), "Secondary nitrogen-15 isotope effects on the reactions catalyzed by alcohol and formate dehydrogenases", *Biochemistry*, 30, 4068-4071.

Saito Y., Oikawa M., Nakazawa H., Niide T., Kameda T., Tsuda K., Umetsu M., (2018), "Machine-Learning-Guided Mutagenesis for Directed Evolution of Fluorescent Proteins", *ACS Synth. Biol.*, 7(9), 2014-2022.

Schirwitz K., Schmidt A., Lamzin V.S., (2007), "High-resolution structures of formate dehydrogenase from *Candida boidinii*", *Protein Science*, 16, 1146-1156.

Schwede T., (2013), "Protein modeling: what happened to the 'protein structure gap'?" *Structure*, 21(9), 1531-1540.

Scibioh M.A., Viswanathan B., (2018), "Carbon Dioxide to Chemicals and Fuels", Elsevier, 1st Edition, Chennai.

Seokjoo Hong S., and Spreitzer R.J., (1997), "Complementing Substitutions at the Bottom of the Barrel Influence Catalysis and Stability of Ribulose-bisphosphate Carboxylase/Oxygenase", *The Journal of Biological Chemistry*, 272(17), 11114-11117.

Serov, A.E., Popova, A.S., Fedorchuk, V.V. & Tishkov, V.I., (2002), "Engineering of coenzyme specificity of formate dehydrogenase from *Saccharomyces cerevisiae*", *Biochem. J.*, 367(3), 841-847.

Shabalin I.G., Polyakov K.M., Tishkov V.I., Popov V.O., (2009), "Atomic resolution crystal structure of NAD1-dependent formate dehydrogenase from bacterium *Moraxella* sp, C-1", *Acta. Nat.*, 1, 89-93.

Shaked Z., Whitesides G. M., (1980), "Enzyme-Catalyzed Organic Synthesis: NADH Regeneration Using Formate Dehydrogenase" *J. Am. Chem. Soc.*, 102, 7104-7105.

Sharma, S., Vaid, S., Bhat, B., Singh, S., & Bajaj, B. K., (2019), "Thermostable Enzymes for Industrial Biotechnology. In: Singh R.S., Singhanian R.R., Pandey A., Larroche C., Editors, "Advances in Enzyme Technology".

Simpson B.K., (2012), "Food Biochemistry and Food Processing". In: Kotzia G. A., Platis D., Axarli I.A., Chronopoulou E.G., Karamitros C., Labrou N.E., Editors, "Biocatalysis, Enzyme Engineering and Biotechnology", John Wiley & Sons Inc., 2nd Edition.

Slusarczyk H., Felber S., Kula M. R., Pohl M., (2000), "Stabilization of NAD-dependent formate dehydrogenase from *Candida boidinii* by site-directed mutagenesis of cysteine residues", *Eur. J. Biochem.*, 267, 1280-1289.

Slusarczyk H., Pohl M., Kula M.R., (1998), "Cloning and stabilization of NAD-dependent formate dehydrogenase from *Candida boidinii* by site-directed mutagenesis", *Stability and Stabilization of Biocatalysts*, 15, 331-336.

Souza V.P., Ikegami C.M., Arantes G.M., Marana S.R., (2018), "Mutations close to a hub residue affect the distant active site of a GH1 β glucosidase", *PLoS ONE* 13(6).

Stuwe T., Bley C.J., Thierbach K., Petrovic S., Schilbach S., Mayo D.J., Perriches T., Rundlet E.J., Jeon Y.E., Collins L.N., Huber F.M., Lin D.H., Paduch M., Koide A., Lu V., Fischer J., Hurt E., Koide S., Kossiakoff A.A., Hoelz A., (2015), "Architecture of the fungal nuclear pore inner ring complex", *Science*, 350(6256), 56-64.

Sugai T., (1999), "Applications of enzyme- and microorganism-catalyzed reactions to organic synthesis", *Curr. Org. Chem.*, 3, 373-406.

Taberman H., Parkkinen T., Rouvinen J., (2016), "Structural and functional features of the NAD(P) dependent Gfo/Idh/MocA protein family oxidoreductases", *Protein Sci.*, 25(4), 778-786.

Tamura M., Ito K., Honda M., Nakagawa Y., Sugimoto H., Tomishige K., (2016), "Direct Copolymerization of CO₂ and Diols", *Sci. Rep.*, 6(24038).

Tao H., Cornish V.W., (2002), "Mile Stones indirected enzyme evolution", *Curr. Opin. Chem. Biol.*, 6, 858-864.

Tishkov V. I., (2002), "The Coenzyme Regeneration for Biosynthesis of Chiral Compounds Using Dehydrogenases", *Chemistry*, 43, 380-387.

Tishkov V. I., Galkin A. G., Marchenko G. N., Egorova O. A., Sheluho D. V., Kulakova L. B., Dementieva L. A., Egorov A. M. (1993) "Catalytic properties and stability of a *Pseudomonas* sp.101 formate dehydrogenase mutants containing Cys-255-Ser and Cys-255-Met replacements", *Biochem. Biophys. Res. Commun.*, 192, 976-981.

Tishkov V.I., Popov V.O., (2004), "Catalytic Mechanism and Application of Formate Dehydrogenase", *Biochemistry (Moscow)*, 69(11), 1252-126.

Tong X., El-Zahab B., Zhao X., Liu Y., Wang P., (2011), "Enzymatic synthesis of lactic acid from carbon dioxide and ethanol with an inherent cofactor regeneration cycle", *Biotechnol. Bioeng.*, 108, 465-469.

van der Donk W.A., Zhao H., (2003), "Recent developments in pyridine nucleotide regeneration", *Curr. Opin. Biotechnol.*, 14, 421-426.

van Noort V., Bradatsch B., Arumugam M., Amlacher S., Bange G., Creevey C., Falk S., Mende D.R., Sinning I., Hurt E., Bork P., (2013), "Consistent mutational paths predict eukaryotic thermostability", *BMC Evol. Biol.* 13(7).

- Voet D., Voet J.G., (2010), “Biochemistry”, Wiley Press, 4th Edition, New Jersey.
- Vogel A., May O., (2019), “Industrial Enzyme Applications”, In: Vogel A., “Enzyme Development Technologies”, Wiley, 1st Edition
- Yilmazer B., Isupov M.N., De Rose S.A., Bulut H., Benninghoff J.C., Binay B. Littlechild J.A., (2020), “Structural insights into the NAD⁺-dependent formate dehydrogenase mechanism revealed from the NADH complex and the formate NAD⁺ ternary complex of the Chaetomium thermophilum enzyme”, Journal of Structural Biology, 212(3).
- Wang X., Saba T., Yiu H.H.P., Howe R.F., Anderson J.A., Shi J., (2017), “Cofactor NAD(P)H Regeneration Inspired by Heterogeneous Pathways”, Chem., 2, 621–654.
- Web 1, (2020), <http://biophysics.cs.vt.edu>, (Accessible Date 12.06.2020).
- Web 1, (2020), <https://www.uniprot.org/uniprot/G0SGU4>, (Accessible Date 08.06.2020).
- Wichmann R., Wandrey C., Buckmann A. F., Kula M. R., (1981), “Continuous enzymatic transformation in an enzyme membrane reactor with simultaneous NAD(H) regeneration”, Biotechnol. Bioeng., 23, 2789-2802.
- Wijma H.J., Floor R.J., Janssen D.B., (2013), “Structure-and sequence-analysis inspired engineering of proteins for enhanced thermostability”, Curr. Opin. Struct. Biol., 23, 588–594.
- Williams G.J., Nelson A.S., Berry A., (2004), “Directed evolution of enzymes for biocatalysis and the life sciences”, Cell Mol. Life Sci., 61, 3034– 3046.
- Zheng L., Baumann U., Reymond J.L., (2004), “An efficient one-step site-directed and site-saturation mutagenesis protocol”, Nucleic Acids Res., 32, e115.

BIOGRAPHY

Elif, who completed her high school education in Istanbul Burak Bora Anatolian High School, continued her undergraduate education in the Department of Molecular Biology and Genetics at Istanbul University. She completed her internship Microbiology Laboratory at Onko-Koçsel Pharmaceuticals. After graduation from bachelor's degree Elif started her MSc. program in Institute of Natural and Applied Sciences Department of Molecular Biology and Genetics at Gebze Technical University.

APPENDICES

Appendix A: Publications related with the thesis

Günay E., Binay B., (2020), “Effect of Glu164Ala and Asp188Arg Mutations In *Chaetomium thermophilum* Formate Dehydrogenase Activity”, II. International Enzyme and Bioprocess Days EBDays 2020, 64, Kocaeli, Turkey, 01-03 September.