

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

INVESTIGATION THE EFFECT OF VAL310ASN ON NAD⁺
DEPENDENT CHATEOMIUM THERMOPHILUM FORMATE
DEHYDROGENASE ENZYME BY SITE-DIRECTED MUTAGENESIS

İLKE SELÇUK
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

GEBZE
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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

NAD⁺ BAĞIMLI CHATEOMIUM
THERMOPHILUM FORMAT
DEHİDROJENAZ ENZİMİNİN
ÜZERİNDEKİ SAHAYA YÖNELİK
MUTAJENEZ İLE VAL310ASN ETKİSİNİN
ARAŞTIRILMASI

İLKE SELÇUK
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

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2021

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SUMMARY

Formate dehydrogenases (FDH), which are enzymes of an oxidoreductase group, oxidize formate ion (HCOO^-) to carbon dioxide and at the same time catalyze the reduction of NAD^+ ion to NADH. It is used in coenzyme regeneration systems to produce NADH since NADH production is quite expensive and laborious and the NAD^+ dependent FDH enzyme is more easily available and cheaper. It was also discovered that the NAD^+ dependent FDH enzyme (EC 1.17.1.9) oxidizes formate ions to CO_2 and the enzyme *Chaetomium thermophilum* formate dehydrogenase (CtFDH) has a promising activity for reducing CO_2 to formate.

It was aimed to question the role of valine amino acid in position 310 of CtFDH in the reaction mechanism with site-directed mutagenesis. This non-polar amino acid is located in the active site of CtFDH and has a non-functional R group. The activity of the enzyme was compared to wild type CtFDH by replacing the amino acid valine with the asparagine amino acid, which is both polar and has a functional R group. The Val310Asn mutation did not show a significant change in the CO_2 reduction activity of the enzyme, although a nonfunctional R-grouped amino acid in the active site was replaced with a functional R-grouped amino acid. However, since the CtFDH enzyme is thought to have an important role in reducing CO_2 , enzyme engineering studies that can be developed to oxidize CO_2 to formate will be paved.

Keywords: *Chaetomium thermophilum*, Formate Dehydrogenase, NAD^+ Dependent Formate Dehydrogenase, Site-Directed Mutagenesis, CO_2 Reduction, Enzyme Engineering, Protein Engineering.

ÖZET

Bir oksidoredüktaz grubu enzimleri olan format dehidrogenazlar (FDH) format iyonunu (HCOO^-) karbondioksite yükseltgeyip aynı zamanda NAD^+ iyonunun da NADH 'a indirgenmesini katalize ederler. NADH üretiminin oldukça pahalı ve zahmetli olması ve NAD^+ bağımlı FDH enziminin ise daha kolay elde edilebilir ve ucuz olması nedeni ile NADH üretimi için koenzim rejenerasyon sistemlerinde kullanılmaktadır. Ayrıca NAD^+ bağımlı FDH enzimi (EC 1.17.1.9) format iyonlarını CO_2 'ye oksitler ve *Chaetomium thermophilum* format dehidrogenaz (CtFDH) enziminin de CO_2 'nin formata indirgenmesi için yüksek aktiviteye sahip olduğu keşfedilmiştir.

Bu çalışmada kullanılacak olan CtFDH enziminin seçilmesinin nedeni yakın zamanlarda yapılan çalışmalarda diğer FDH'lere göre daha yüksek aktivite göstermesidir. CtFDH'in 310. pozisyonundaki valin amino asidinin bölgeye yönelik mutajenez ile reaksiyon mekanizmasındaki rolünün sorgulanması amaçlanmıştır. Polar olmayan bu amino asit CtFDH'in aktif bölgesinde bulunur ve fonksiyonel olmayan R grubuna sahiptir. Valin amino asidini hem polar olan hem de fonksiyonel R grubuna sahip olan asparajin amino asidi ile değiştirip enzimin aktivitesi yabanıl tip CtFDH ile karşılaştırılmıştır. Val310Asn mutasyonu, aktif bölgedeki fonksiyonel olmayan R gruplu bir amino asit ile fonksiyonel R gruplu bir amino asidin değiştirilmesine rağmen enzimin CO_2 indirgenme aktivitesine karşı kayda değer bir değişim göstermemiştir. Fakat yine de CtFDH enziminin CO_2 indirgenmesinde önemli bir role sahip olduğu düşünüldüğü için, CO_2 'nin formata oksitlenmesi için geliştirilebilecek olan enzim mühendisliği çalışmalarının önü açılacaktır.

Anahtar Kelimeler: *Chaetomium thermophilum*, Format Dehidrogenaz, NAD^+ Bağımlı Format Dehidrogenaz, Bölgeye Yönelik Mutajenez, CO_2 İndirgenmesi, Enzim Mühendisliği, Protein Mühendisliği.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celcius Degree
Asn	: Asparagine
CH ₃ COOH	: Acetic Acid
CH ₃ COONa	: Sodium Acetate
CO ₂	: Carbon Dioxide
CtFDH	: <i>Chaetomium thermophilum</i> Formate Dehydrogenase
ddH ₂ O	: Double Distilled Water
dH ₂ O	: Distilled Water
dNTP	: deoxyribonucleotide triphosphate
FDH	: Format Dehydrogenase
HCOONa	: Sodium Formate
k _{cat}	: The catalytic rate constants
kDA	: Kilodalton
KH ₂ PO ₄	: Monopotassium Phosphate
K _M	: The Michaelis constant
L	: Liter
LB	: Luria-Bertani
mg	: Milligram
min	: Minute
mL	: Milliliter
mM	: Millimolar
Na ₂ HPO ₄	: di-Sodium Hydrogen Phosphate
NAD ⁺	: Nicotinamide Adenine Dinucleotide
NADH	: Reduced Nicotinamide Adenine Dinucleotide
NaH ₂ PO ₄	: Monosodium Dihydrogen Orthophosphate
NaHCO ₃	: Sodium Bicarbonate
Ni ⁺²	: Nickel
nm	: Nanometer

PCR	:	Polymerase Chain Reaction
PMSF	:	Phenylmethanesulfonyl Fluoride
s	:	Second
SOC	:	Super Optimal broth with Catabolite
Val	:	Valine
WT	:	Wild Type
μL	:	Microliter
μM	:	Micromolar

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1. INTRODUCTION

1.1. Aim and Contribution of Thesis

This study is expected to contribute to future studies on the reduction of CO₂ by *Chaetomium thermophilum* formate dehydrogenase (CtFDH) enzyme and the use of FDH in the industrial field since research on CO₂ conversion is limited and the chosen position has not been studied before.

The non-polar valine amino acid at position 310 selected in this thesis has a non-functional R group. The asparagine amino acid is both polar and has a functional R group. It was aimed to examine the change in binding affinity to the substrate with the V310N mutation planned to be performed by site-directed mutagenesis, and thus the changeover NAD⁺ dependent CtFDH activity.

Within the scope of this thesis, in the first part, the aim, contribution, and content of the thesis are briefly mentioned and in the second part, basic information about enzymes is given, while protein engineering studies for elucidating the active site of formate dehydrogenase (FDH) enzyme and FDHs are explained. In the third part, information about the materials and experiments used in the thesis is given. In the fourth and last part, the results obtained and the comments about these results are explained under the related topics.

2. LITERATURE REVIEW

2.1. Enzymes

Enzymes catalyze chemical reactions. This catalysis is performed with reactants called substrates and products [1]. Enzymes can make chemical reactions a million times faster. The conversion to the product occurs by binding the substrate to the active site of enzymes. The enzyme released after the product is produced can repeat the process by attaching to a new substrate [2].

Enzymes are classified according to the reaction they are catalyzed. This classification is made by the Nomenclature Committee of the International Association of Biochemistry and Molecular Biology by giving four-digit code numbers to enzymes (Table 1). The first number indicates which of the six main enzyme groups the enzyme belongs to, the second number indicates the chemical structure and functional group it acts on, the third number indicates the acceptor, and the fourth number indicates the sequence number of the enzyme in a certain class [3].

Table2.1.: Classification of Enzymes.

Number	Class	Reactions
EC 1	Oxidoreductases	Enzymes that catalyze redox reactions between two substrates.
EC 2	Transferases	Enzymes that catalyze the transfer of groups other than H^+ between two substrates.
EC 3	Hydrolases	Enzymes that catalyze the hydrolysis of ester, ether, peptide, glycoside, acid anhydride, C-C, C-halide, or P-N bonds by the introduction of a water molecule.
EC 4	Lysases	Enzymes that catalyze reactions in which groups are removed from substrates and double bonds are formed by a mechanism different from hydrolysis.
EC 5	Isomerases	Enzymes that catalyze the conversion of geometric, optical, or structural isomers to each other.
EC 6	Ligases	Enzymes that catalyze the binding reactions of two compounds with the help of the energy released as a result of the breakdown of the phosphate bond from high-energy phosphate compounds such as ATP or GTP.

2.1.1. Dehydrogenases

Dehydrogenases, which catalyze the oxidation-reduction reaction with coenzyme such as $NAD^+/NADP^+$ or flavin such as FAD, FMN, are enzymes belonging to the oxidoreductase class, which oxidize organic compounds and generate energy by transferring two hydrogen atoms from organic compounds to electron acceptors. [4,5].

Dehydrogenases can be used together with other oxidoreductases to provide the cofactor regeneration they need, thanks to the property that they need a cofactor as an

electron acceptor for the reaction to start and continue. Dehydrogenases can be used together with other oxidoreductases to provide the cofactor regeneration they need, thanks to the property that they need a cofactor as an electron acceptor for the reaction to start and continue.

2.1.1.1. Formate Dehydrogenases

Aerobic and anaerobic organisms such as eubacteria, archaeobacteria, and eukaryotic yeasts produce or consume formate in a variety of metabolic pathways. All these organisms contain formate dehydrogenase (FDH) enzyme, which catalyzes the reversible two-electron oxidation of formate[6]. FDH activity was first discovered as early as 1921 in plant *Phaseolus vulgaris*[7]. FDHs are a set of enzymes found in anaerobic and aerobic bacteria, yeasts, and plants catalyze the NAD^+ -dependent oxidation of formate to carbon dioxide (CO_2) [8].

NAD(P)^+ dependent dehydrogenases catalyze several key metabolic steps and are one of the most extensively studied protein families. Catalysis of formate anion to CO_2 by NAD^+ dependent FDH is an example of NAD^+ dependent dehydrogenation of carbonyl compounds[9].

Since NADH production is quite expensive and laborious, the NAD^+ dependent FDH enzyme has become lately preferred for NADH production because it is easier and cheaper. NAD^+ dependent FDH enzyme (EC 1.17.1.9) is the last enzyme in the metabolic pathway which reduces the NAD^+ molecule to NADH by oxidizing the formate ions to CO_2 (figure 2.1) [9,10,28]. Also recently discovered that the *Ct*FDH enzyme has high activity for reducing CO_2 to formate. This reduction occurs in a one-step reaction mechanism [11]:



There are also FDHs with W, Mo in the active zones. FDHs can be divided into two groups; metal-dependent and metal-independent. The catalytic mechanism of the enzyme is the direct transfer of the hydride ion in the substrate to the C_4 atom in the nicotinamide portion of NAD^+ . Because the FDHs catalytic mechanism does not have a proton transfer step or acid-base catalysis step, they are model enzymes for studying

the hydride ion transfer mechanism in the active site of dehydrogenases [12]. Besides, FDH has a significant advantage that catalyzes reverse reactions in a 100% yield. In addition, FDHs can adapt to different pH ranges (pH 4 to 10) [13].

If a chemical reaction is catalyzed by FDH, there are no proton release or abstraction steps, and a single carbon-hydrogen bond is cleaved in the substrate, and a single new bond is formed in the product [17].

The NAD⁺ dependent FDH consists of two identical subunits containing the coenzyme binding domain and the substrate-binding domain. The enzyme's active center is at the domain interface [17]. NAD⁺ dependent FDHs which are found in both prokaryotic and eukaryotic organisms are approximately 40 kDa and are highly conserved homodimeric enzymes [14]. It has been shown in previous studies that NAD⁺ dependent *Chaetomium thermophilum* FDH is an enzyme that has high thermostability and can convert CO₂ to formate [15, 16].

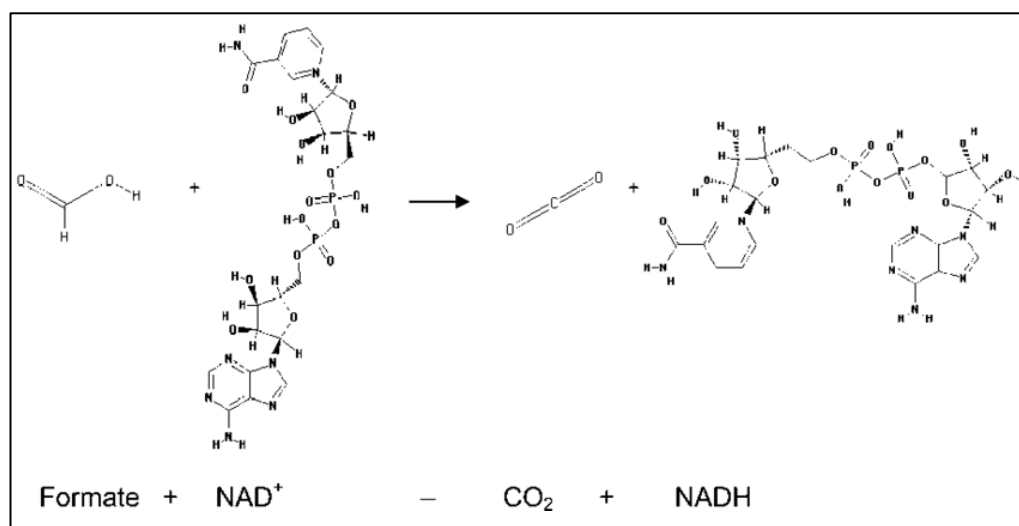


Figure 2.1.: Catalyzing reaction of FDH.

2.1.1.2. CO₂ Reduction

Efforts to reduce CO₂ emission, which is also the main cause of climate change, have increased considerably. Although the energy industry has found ways to obtain energy from carbon-free renewable energy such as geothermal, wind, solar, and biofuels, it is a fact that these forms do not contribute much to the reduction of CO₂ emissions. Therefore, various research groups have developed various enzymatic strategies to transform CO₂ into useful products. One of these enzymatic strategies is

to use FDHs for formate production by CO₂ reduction. However, FDHs have high activity and stability in reducing CO₂ to formate. However, it was revealed that FDH, which has the highest activity among the FDHs examined by previous studies (Aslan et. al., 2017), is *CtFDH* [15].

As mentioned earlier, although FDHs have some drawbacks, they are very important to the industry. These negativities can also be overcome by protein engineering studies. Today, protein engineering studies are continuing rapidly to improve and increase the use of FDHs.

2.2. Protein Engineering

The production of new proteins with the desired structure and function by using molecular biology techniques and by changing their genetic structures is called protein engineering. Protein engineering can also be used to increase the use of enzymes in the industry. For example, if an enzyme's activity is not high enough or its substrate selectivity is low, this problem can be overcome by protein engineering approaches which are shown in figure 2.2.

The substitution of specific nucleotides within a gene sequence can be accomplished by site-directed mutagenesis which is one of the protein engineering approaches [18]. A partially complementary oligonucleotide containing the mutation specially designed for the region to be changed by site-oriented mutagenesis is used as a primer and the mutant DNA is amplified by the PCR method. Thus, genes or proteins with the desired properties are obtained [19].

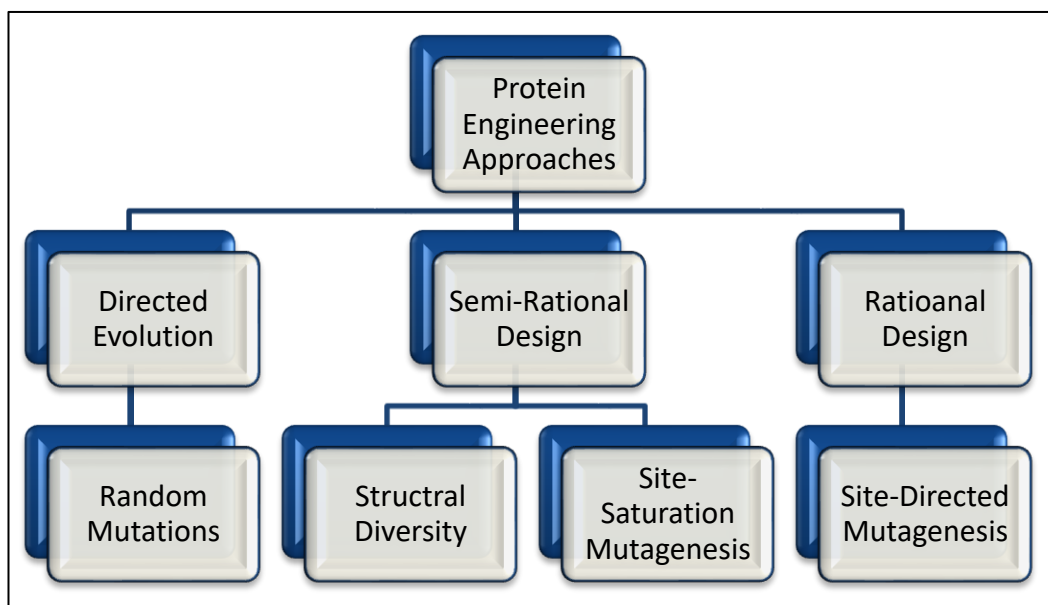


Figure 2.2: Protein engineering approaches.

2.2.1. Directed Evolution

The 2018 Nobel Prize in chemistry has been awarded to three scientists - George P. Smith, Gregory Winter, and Frances Arnold - who use the principle of evolution to produce new compounds. One of the award-winning studies involved the development of enzymes which are proteins that accelerate chemical reactions by directed evolution (Figure 2.3) [29].

Based on organisms that can cope with difficulties due to mutation and natural selection, the method of directed evolution studied in laboratories has been used to optimize or change the activities of genes and gene products[20]. In her study, Arnold used an enzyme called subtilisin and experimented with mutants in different types of bacteria by creating random mutations in the enzyme's codon to accelerate the change in an organic solvent by modifying it [21].

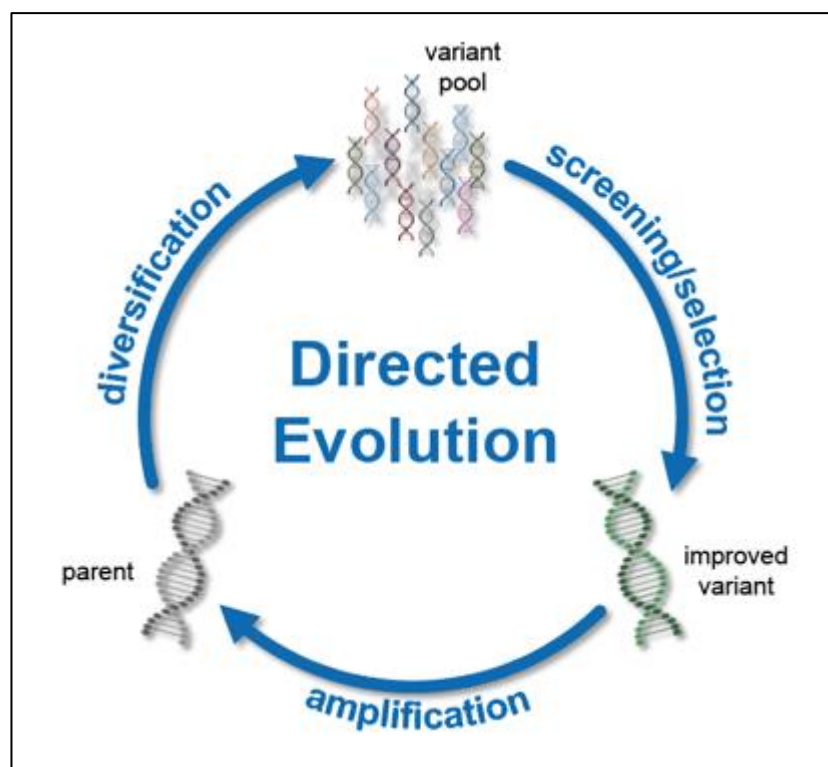


Figure 2.3: Directed evolution bioprocess.

2.2.2. Rational Design

Rational design is the first approach developed to allow the identification of mutant enzymes with desirable traits such as increased activity, modified specificity, selectivity, or cofactor binding [22]. It is necessary to have information about both the function of the protein and its three-dimensional structure to make a rational design. Major processes such as single-point mutation, exchange of elements of secondary structure, exchange of whole domains, creating new hybrid enzymes with enzyme fusion can be managed with the rational design [23].

Rational design by site-directed mutagenesis has also achieved many successes in the past. With the knowledge gained about protein structures and functions, site-directed mutagenesis becomes a very powerful complement to directed evolution. With this approach, a combination of the reshaping of the specificity and reactivity of the substrate or cofactor can be used [24].

2.2.3. Semi Rational Design

One of the difficulties of rational design is that it takes a long time to decide on the selection of the mutation site, as it requires a great deal of knowledge about the protein. Since there is a great need for library scans in directed evolution, the semi-rational design has been developed by combining rational design with directed evolution [25].

Semi-rational protein engineering can significantly increase the efficiency of biocatalyst adaptation. With this design, high-quality libraries can be created, providing an advantage to predict and rationalize experimental findings. Enzyme engineering studies have gained momentum with semi-rational and computer-guided protein designs [26].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Equipments

Micropipettes (Axygen-Isolab), vortex stirrer (N-BioTek), autoclave (N-BioTek), magnetic stirrer (WiseStir), centrifuge (Beckman Coulter), electrophoresis (Biorad), ultrasonic homogenizer (Bandelin), PCR (Techne), deep freezers -80 °C (Arctiko), microplate reader (Biotek), water-bath (Stuart), pH meter (Hanna), ultra-pure water (Merck-Millipore), shaking incubator (BioTek), Ni-NTA HisTrap Column (GE), Concentrators (Sartorius Vivaspinn), sensitive balance (Shimadzu – Radwag), etuve (Nüve).

3.1.2. Enzymes

Phusion High Fidelity DNA Polymerase (BioLabs), *DpnI* (Thermo Sci.), Lysozyme (Serva).

3.1.3. Chemicals and Kits

Yeast extract (LabM), Trypton (LabM), Glucose (LabM), Lactose (Fisher BioReagents), Agar (Fluka), Agarose (Biomax), RedSafe™ Nucleic Acid Staining Solution, dNTP (Thermo Sci.), Imidazole (AppliChem), LB Broth (LabM), Ammonium persulfate (Fisher BioReagents), Sodium chloride (Merck), Sodium Bicarbonate (Hydrogen Carbonate), Sodium Phosphate (Fisher Scientific), Tris-Base (Formedium), Citrate (Riedel-de Haën), Ampicillin (AppliChem), Hydrochloric acid (Sigma-Aldrich), Glycerol (Fisher BioReagents), Sodium Formate (Sigma), NAD⁺, NADH (Sigma), Glycine (Sigma), Plasmid Miniprep Kit (Cat. No. K210010), Invitrogen PureLink (TM), Plasmid isolation kit (Invitrogen).

3.1.4. Plasmids and Cells

The *Ctf_{dh}* gene which contains a hexa-histidine sequence in pET-45b+ was obtained from BERC lab group to use in these experiments. For transformation, *E. coli* BL21 (DE3) and NEB® 5α (NEB) cells were used. The map of the pET45b+ vector is given in Appendix B.

3.1.5. Mediums

- Luria-Bertani (LB) Agar Medium

1.5 g of yeast, 3 g of peptone, 3 g of NaCl, and 4.5 g of agar dissolved in 250 mL of ddH₂O and completed to 300 mL final volume. After autoclaving at 121°C for 15 minutes, the LB agar medium was cooled to 55°C and 1 µL/mL ampicillin was added. The solution was poured into 100 mm Petri dishes under the laminar flow hood. After solidifying to agar plates, they were sealed with parafilm and stored at +4°C.

- Luria-Bertani (LB) Medium

2.5 g of yeast, 3 g of peptone and, 3 g of NaCl were dissolved in 250 mL of ddH₂O and completed to 300 mL final volume and autoclaved at 121°C for 15 minutes.

- Auto-induction Studier Medium

2 g of yeast, 8 g of tryptone, 2 g of NaCl, 2.66 g of Na₂HPO₄, 1.2 g of KH₂PO₄, 0,2 g of Glucose.H₂O, 2.4 mL of glycerol, and 1,26 g of lactose were dissolved in 300 mL of ddH₂O and completed to 400 mL final volume and autoclaved in 121°C for 15 minutes.

3.1.6. Solutions and Buffers

- Ampicillin Solution

1 g ampicillin was dissolved in 10 mL distilled water and passed through a 0.45 μm sterile filter and the solution was aliquoted into 1 mL Eppendorf and stored at -20°C .

- Sodium Phosphate Buffer Solution (100 mM)

13.24 g of Na_2HPO_4 and 0.94 g of NaH_2PO_4 was dissolved in 800 mL dH_2O and pH adjusted to 8 then completed to 1000 mL final volume.

- Sodium Acetate Buffer Solution (100mM)

5.524 g of CH_3COONa and 1.961 g of CH_3COOH were dissolved in 800 mL dH_2O and pH adjusted to 5 then completed to 1000 mL final volume.

- NAD^+ Solution

13.5 mg NAD^+ was dissolved in 30 mL 100 mM pH 5 sodium acetate buffer and kept at $+4^{\circ}\text{C}$.

- NADH Solution

13.8 mg NADH was dissolved in 30 mL 100 mM pH 8 sodium phosphate buffer and kept at $+4^{\circ}\text{C}$.

- Sodium Formate

6.80 g of HCOONa was dissolved in 50 mL 100 mM pH 5 sodium acetate buffer for substrate preparations.

- Sodium Bicarbonate

4.03 g of NaHCO_3 was dissolved in 50 mL 100 mM pH 8 sodium phosphate buffer for kinetic experiments.

- Buffer A

1.38 g NaH₂PO₄·H₂O (20 mM), 14.61 g NaCl (500 mM), and 1.02 g Imidazole (30 mM) were dissolved in 500 mL dH₂O and the pH was adjusted to 7.4 then filtered through a 0.45 µm filter.

- Buffer B

1.38 g NaH₂PO₄·H₂O (20 mM), 14.61 g NaCl (500 mM), and 17.02 g Imidazole (500 mM) were dissolved in 500 mL dH₂O and the pH was adjusted to 7.4 then filtered through a 0.45 µm filter.

3.2. Method

3.2.1. Primer Design

The forward and reverse primers design to convert Valine to Aspartic Acid at position 310 in the *Ctfdh* gene. OligoArchitect™ Primer and Probe Design tool used for oligonucleotide design.

Table 3.1.: Primers designed to generate mutations in this study.

Primer	Sequence (5'-3')
Val310Asn-Fwd	GTGGCAATGCTATGAACCCGCACATGAGCG
Val310Asn-Rev	CGCTCATGTGCGGGTTCATAGCATTGCCAC

3.2.2. Site-Directed Mutagenesis

Site-directed mutagenesis was performed to generate val310asn mutation by PCR reaction using the pET45b+ plasmid, which contains the *Ctfdh* gene as a template. The PCR reaction mix was prepared as in table 2 and the PCR reaction was performed according to the cycling conditions shown in table 3.3.

Table 3.2.: Reactants used for the PCR reaction and volumes.

Reactant	Volume	Final Concentrations
The plasmid containing the <i>ctfdh</i> gene (~70 ng)	1 μ L	~1.4 ng
10 mM Forward Primers	1 μ L	0.5 μ M
10 mM Reverse Primers	1 μ L	0.5 μ M
DMSO	2 μ L	4%
10 mM dNTP mix	1 μ L	200 μ M
Phusion High Fidelity DNA Polymerase	0,5 μ L	1.0 units/ 50 μ l PCR
10X GC Reaction Buffer	10 μ L	1X
ddH ₂ O	33,5 μ L	
Total Volume	50 μ L	

Table 3.3.: PCR cycling conditions for site-directed mutagenesis.

Steps	Cycles	Temperature	Time
Denaturation	1	98°C	30 s
Denaturation	30	98°C	10 s
Annealing		64,8°C	30 s
Extension		72°C	150 s
Final Extension	1	72°C	480 s

3.2.3. Agarose Gel Electrophoresis

After the PCR reaction, the agarose gel electrophoresis method was used to check whether amplification has occurred. To prepare 0.8% agarose gel, 0.4 g agarose was dissolved in 50 mL 1xTBE buffer. After boiling the gel to dissolve, it was cooled slightly and 2.5 μ L of Serva DNA Stain Clear-G was added to the agarose gel. Once the gel was prepared, it was poured into the tank of the electrophoresis system and allowed to freeze after the combs were added. When the gel freeze, the tank was placed in a cuvette filled with 1x TBE, and samples (marker, wild-type *CtFHD*, PCR product)

loaded into wells. Electrophoresis was carried out at 80V. After the electrophoresis, the bands were visualized under UV light.

3.2.4. Enzymatic Digestion of the PCR Products

PCR amplicons were digested by *Dpn* I restriction enzyme by the following procedure; 1 μ L of *Dpn* I enzyme was added onto 40 μ L of PCR amplicon and incubated at 37°C for two hours.

3.2.5. Transformation of CtFDHs

NEB® 5 α competent *E. coli* cells were thawed on ice. 1.5 μ L digested DNA was added onto the competent cells and incubated on ice for 30 minutes then heat shock was applied in a water bath at 42°C for 30 seconds. Transformation tube put on ice for 5 minutes and under the laminar flow hood, 300 μ L preheated SOC medium was added in the tube. After the shaking incubation at 225 rpm at 37°C for one hour, the transformation tube was centrifuged at 4000 rpm for 10 minutes. 200 μ L supernatant was discarded then pellets were resuspended in the remaining 100 μ L medium then spread onto LB agar plate which contains ampicillin for selection and incubated at 37°C overnight.

3.2.6. Plasmid Purification

Single colonies were selected from LB agar plates and inoculated in 5 mL LB medium containing 5 μ L ampicillin at 225 rpm and 37°C overnight. After incubation, plasmid purification was performed by following the manufacturer's instructions for the Invitrogen PureLink™ Quick Plasmid Miniprep Kit.

3.2.7. DNA Sequence Analysis

20 μ L isolated plasmid sample was sent to Eurofins Scientific for DNA sequences analysis. The *T7* promoter and terminator nucleotide sequence were used in the polymerase chain reaction for sequence analysis.

3.2.8. FDH Expression

Mutant *CtFDHs* which are confirmed by DNA sequencing and wild type *CtFDHs* were transformed to BL21 competent cells for protein production. Single colonies were selected from LB agar plates and inoculated in 5 mL LB medium containing 5 μ L ampicillin at 225 rpm and 37°C overnight. Precultures were added into two smaller autoinduction medium erlenmeyer flasks containing 1 μ L / mL ratio of ampicillin for incubation at 225 rpm at 30°C overnight. After incubation, cells were centrifuged at 4000 rpm at 4°C for 15 minutes. Supernatants were discarded and pellets were stored at -20°C.

3.2.9. Purification of Wild Type and Mutant *CtFDHs*

Stored pellets transferred on ice and waited for 30 minutes. Before resuspension of pellets in 3 mL Buffer A solution (20 mM NaPi, 500 mM NaCl, 30 mM imidazole pH 7.4), they were measured in precision scales. Lysozyme (1 mL/mg) and PMSF (4 μ L/gr) were added on pellets according to and incubated on the ice at 500 rpm for 30 minutes. The cell suspension was disrupted by sonication for 10 tours (10 sec waiting and 10 sec disrupting) and centrifuged at 16000 rpm at 4°C for one hour. After centrifugation, lysates were filtered with a 0.45 μ m filter. The HisTrap column was first washed with 15 mL of water and then 15 mL of buffer A was loaded and stabilized the column prepared for purification. After that mutant and wild-type *CtFDH* lysates were eluted with Buffer A containing different imidazole concentrations (100-200-400-500 mM). Collected fractions were analysed by SDS-PAGE to identify fractions containing pure protein. Collected purified proteins were combined and diluted with 100 mM of NaPi pH 7.4 to obtain 50 mL final volume then proteins concentrated with Amicon Millipore centrifugal concentrators which have 30 kDa pore.

3.2.10. SDS-PAGE Analysis

Samples were prepared by mixing 15 μ L sample and 5 μ L 4X sample loading buffer. Samples were heated at 95 °C for 10 minutes then centrifuged at 11000 rpm for 15 minutes.

The SDS gel which is prepared previously placed to tank than the tank was filled with running buffer. 5 μ L protein standard was loaded into the first well and 10 μ L of sample is loaded into the next wells. Then the running was started with 90 V for 10 minutes and it was continued 120 V for 1 hour.

3.2.11. Enzyme Activity Assay

The activity of purified enzymes was measured at 340 nm at room temperature using sodium formate and sodium bicarbonate as substrate and NAD^+ and NADH as cofactors.

Activity calculations were performed as a unit which is the amount of enzyme that converts a μmol formate into a product per minute and under optimum conditions. Specific activity is the number of units per milligram of the enzyme. The specific activity is expressed as Unit/mg protein or $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The following formula was used for activity calculations;

$$\frac{A * B}{C * D * E * F}$$

$$A: \frac{\Delta A_{340\text{nm}}}{\text{min}} \text{Test} - \frac{\Delta A_{340\text{nm}}}{\text{min}} \text{Blank maximum linear rate}$$

$$B: \text{Total volume (in milliliters) of assay} \quad (3.1)$$

$$C: \text{Millimolar extinction coefficient of } \beta\text{-NADH at } 340\text{nm}$$

$$D: \text{Volume (in milliliter) of enzyme used}$$

$$E: \text{Path length of microplate}$$

$$F: \text{Enzyme concentration}$$

3.2.12. Kinetic Analysis

Kinetic measurements were made according to Michaelis-Menten kinetics. Kinetic calculations were performed against substrate and cofactor for both mutant and wild type *CtFDHs*. When the reaction was run at room temperature at 340 nm, the final volume had a range of 0.66 mM NAD^+ and NADH and 0-25 mM sodium formate and sodium bicarbonate for formate consumption and CO_2 reduction. The final

concentration of the enzyme in the reaction medium was used as 0.05 mg/mL. K_M and k_{cat} values of enzymes were calculated using GraphPad Prism8 program.

3.2.13. Molecular Modeling

Molecular modeling was performed to examine the interactions between substrates and mutant *CtFDH* in more detail and to determine the potential effects of structural changes. The published structure of *CtFDH* was obtained from PDB (PDB ID: 6T8Y, Yilmazer et al., 2020). Hydrogen carbonate substrate were docked *CtFDH* active site pockets in silico via the AutoDock Vina (Trott & Olson, 2010). Substrate binding pocket drawings were done by PyMOL (Molecular Graphics System, Version 2.0 Schrodinger, LLC), rotamer of mutated position was predicted with UCSF Chimera (Pettersen et al., 2004; Shapovalov & Dunbrack Jr., 2011)

4. RESULTS

Valine 310 amino acid located in the active site of CtFDH was converted to the Asparagine amino acid which has functional R group by site-directed mutagenesis. The results below show us the kinetic effects of this substitution.

4.1. Mutant and Wild Type CtFHDs Agarose Gel Results

The V310N mutation on the pET45b+ vector containing the *Ctfdh* gene was performed by PCR amplification using primers containing the mutant codon then amplicons were visualized by agarose gel electrophoresis shown in figure 3.1. While a very faint band was observed at 61.1°C annealing temperature, brighter-looking bands were obtained from amplicons that were operated at the other two temperatures.

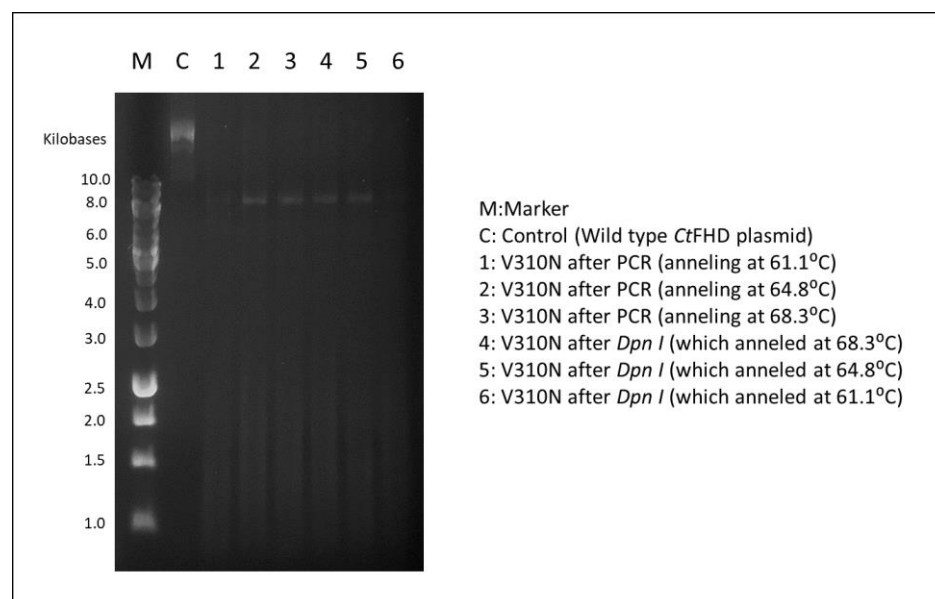


Figure 4.1.: CtFHDs agarose gel image after PCR and after with *DpnI* incubation.

4.2. Expression of CtFDHs

PCR amplicons were transformed into NEB® 5α competent *E. coli* cells. Colony growth that occurred in the amplicon annealed at only 64.8°C can be seen in figure 4.2.

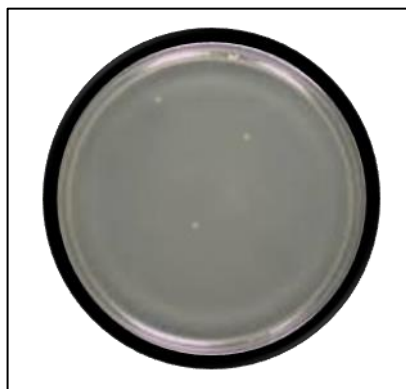


Figure 4.2.: Transformed mutant CtFDH agar plate.

4.3. DNA Sequence Analysis Results

After the PCR amplicons were transformed and purified, they were analyzed by sequencing to confirm that the mutation occurred. As shown in figure 4.3, the analysis results were compared with the wild-type CtFDH in the Snapgene viewer program. The Val310Asn mutation was determined to occur successfully.



Figure 4.3.: Sequence analysis results of mutant CtFDH.

4.4. Expression of mutant and wild-type CtFDHs in BL21

After the confirmation of mutation by sequencing, pET 45b+ vector containing the wild-type and mutant *Ctfdh* gene were successfully transformed into competent *E. coli* BL21 (DE3) cell for overexpression of proteins by inducing lactose at 37 °C (figure 4.4).

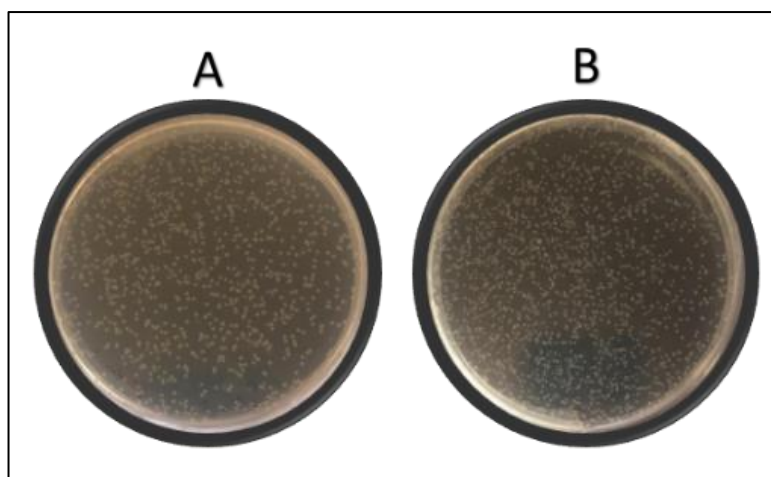


Figure 4.4.: Agar plates of mutant and wild-type CtFDHs transformed into BL21.

4.5. Purification of CtFDHs

After the *pET* 45b+ vectors containing mutant CtFDH genes were transformed into *E. coli* BL21 (DE3) cells, protein expression and purification was performed as described in sections 3.2.8 and 3.2.9. Protein purification was performed by using Ni-NTA HisTrap column and fractions were collected. SDS-PAGE were performed with the collected fractions. SDS-PAGE results showed that CtFDH is ~45 kDa in size which is close to the actual value 45.6 kDa, and pure in fractions were obtained for kinetic measurements.

4.6. Kinetic Parameters Determined for the Wild Type and Mutant CtFDHs

Michaelis-Menten kinetic values of wild-type and mutant CtFDHs whose kinetic measurements were taken at room temperature were determined by hyperbolic non-regression at GraphPad Prism 8 program. Sodium formate and sodium bicarbonate were used as the substrate, and the activity determination was performed by monitoring the reduction of the cofactor NAD⁺ and the oxidation of NADH against time at 340 nm. wild-type and mutant CtFDHs showed kinetic activities against both formate and bicarbonate. While calculating the kinetic activities, the following formula is used, and an example is given from the calculations.

$$\frac{A * B}{C * D * E * F}$$

$$A: \frac{\Delta A_{340nm}}{\min} \text{Test} - \frac{\Delta A_{340nm}}{\min} \text{Blank maximum linear rate}$$

$$B: \text{Total volume (in milliliters) of assay} \quad (4.1)$$

$$C: \text{Millimolar extinction coefficient of } \beta\text{-NADH at 340nm}$$

$$D: \text{Volume (in milliliter) of enzyme used}$$

$$E: \text{Path length of microplate}$$

$$F: \text{Enzyme concentration}$$

Calculation example;

$$\frac{0,1132 * 0,2}{6,22 * 0,55 * 0,05 * 0,793} = 0,16691 \quad (4.2)$$

The corresponding Michaelis-Menten graphs for wild-type and mutants CtFDH toward formate and bicarbonate are shown in figures 4.5, 4.6 and 4.7. K_M , and k_{cat} values for the wild-type and mutant CtFDHs are shown in Table 4.1.

These changes were ignored, since the increase in K_M (1.36 mM to 1.58 mM) and k_{cat} (0.12 to 0.136 s⁻¹) value were very small compared to the wild type in the format direction. When the reaction in the direction of bicarbonate was examined, the changes were ignored since the increase in K_M (0.32 mM to 1.25 mM) and k_{cat} (0.023

s^{-1} to $0.091 s^{-1}$) values was very small, as in the format direction. Thus, the results were interpreted as the V310N mutation had no effect on the substrate binding affinity and catalysis rate in both formate and bicarbonate directions.

As the mutant enzyme is compared, the main features between the two substrates are that sodium bicarbonate (K_M 1.25 mM) binds much better than the formate (K_M 1.58 mM) to the V310N CtFDH enzyme. However, the rate of catalysis is higher than formate ($0.136 s^{-1}$) to sodium bicarbonate($0.091 s^{-1}$).

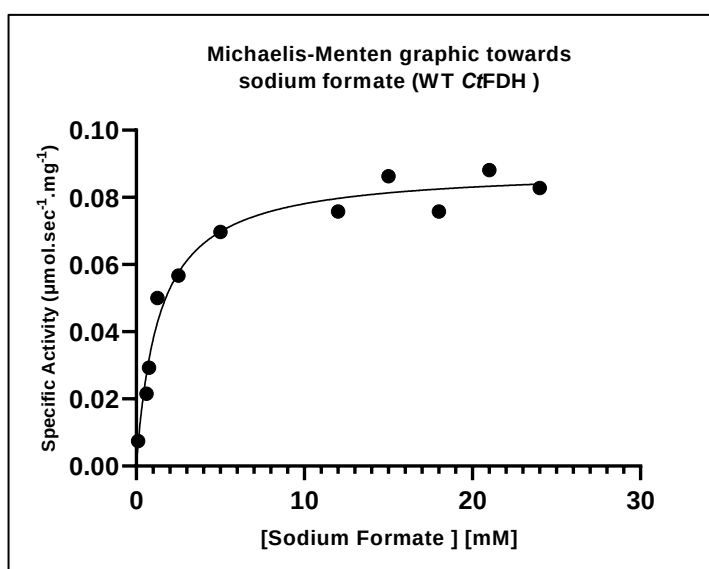


Figure 4.5.: Michaelis-Menten chart versus formate for the wild type CtFDH.

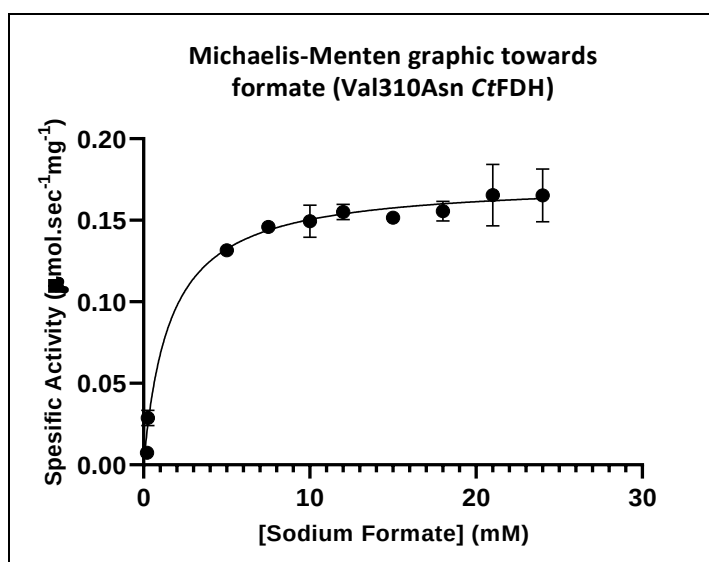


Figure 4.6.: Michaelis-Menten chart versus formate for the Val310Asn CtFDH.

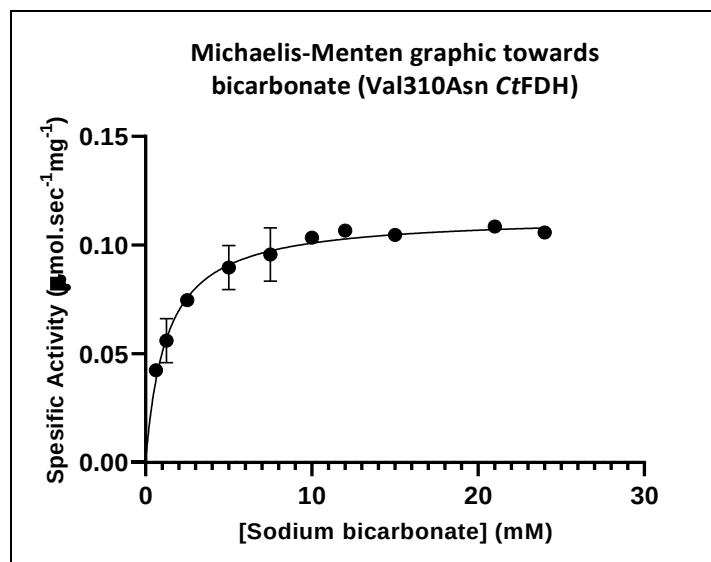


Figure 4.7.: Michaelis-Menten chart versus sodium bicarbonate for the Val310Asn CtFDH.

Table 4.1.: Kinetic properties of mutant and wild-type CtFDH enzymes.

	Sodium Formate			References	Sodium Bicarbonate			References
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)		k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)	
CtFDH								
Wild Type	0.12 ± 0.005	1.36 ± 0.321	0.09 ± 0.015	This Study	0.023 ± 0.000	0.32 ± 0.073	0.069 ± 0.000	[15]
Val310Asn	0.136 ± 0.009	1.58 ± 0.692	0.086 ± 0.013	This Study	0.091 ± 0.005	1.25 ± 0.403	0.072 ± 0.012	This Study

4.7. Molecular Modeling

Molecular modeling results showed that the mutated residue has no effect on CO₂ reduction activity.

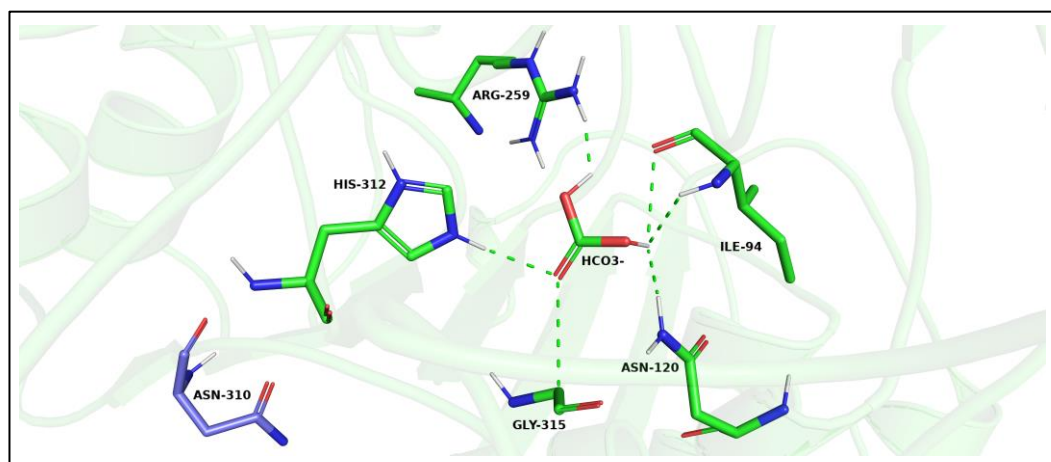


Figure 3.8: Stick representation of CtFDH active site residues involved in the substrate-binding CO₂ reduction reactions (green), mutated residue (blue) and docked substrate (HCO₃⁻).

5. DISCUSSION

In this study, it was aimed to investigate the role of NAD^+ -dependent *Chaetomium thermophilum* formate dehydrogenase enzyme in the reaction mechanism of the V310N position using protein engineering methods. For this purpose, especially to find out how FDH works with hydrogen carbonate as a substrate and to clarify the structure-function relationships in the catalytic reaction, the valine amino acid which is located in the active site of the enzyme and has a nonfunctional R group, is converted the asparagine amino acid which has a functional R group by site-directed mutagenesis designed close to the catalytic center of CtFDH.

According to the results of the literature review, the reason for choosing *Chaetomium thermophilum* formate dehydrogenase was that the specific activity was higher than other FDHs such as *Candida boidinii* (CbFDH), *Thiobacillus* sp. (strain KNK65MA) (TsFDH), *Candida methylica* (CmFDH) and the V310 position in the active site of CtFDH was preserved as threonine in other FDHs, and a mutation in this region was thought to be important [15].

In formate oxidation and CO_2 reduction, the CtFDH Val310Asn mutation enhanced the catalysis rate 1.13 and 3.95-fold respectively, while substrate-binding affinity decreased 1.16-fold and 3.91-fold respectively. Catalytic efficiency on CO_2 reduction increased 1.04-fold with V310N mutation. While the asparagine amino acid is expected to bind much more to the substrate than the valine amino acid due to its functional R group, the results show that the V310N mutation does not have a significant effect on the enzyme activity to reduce CO_2 .

This study shows that a mutation may not differ greatly between wild-type and the mutant CtFDH. Although the valine mutation at position 310 of CtFDH did not yield the expected result in CO_2 reduction compared to wild-type CtFDH, the CtFDH enzyme is still promising in CO_2 reduction and will continue to be investigated. Thus, new and more efficient enzyme engineering approaches can be developed for the conversion of CO_2 into formate.

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BIOGRAPHY

İlke earned her B.Sc degree in Molecular Biology and Genetics from Istanbul Arel University in 2017 after she completed her summer internship at Neurodegeneration lab in Istanbul Technical University. After graduation, she worked as an Erasmus intern for a period of four months at the Neuro Lab of Tallinn University of Technology. She continued her education as a M.Sc student at the Gebze Technical University and worked at the BERC lab. Now she is a molecular biologist at Sentromer DNA Technologies Inc.

APPENDICES

Appendix A: Publications Related with the Thesis

Selçuk İ., (2020) “Increasing the Activity of NAD⁺ Dependent *Chateomium thermophilum* Formate Dehydrogenase Enzyme by Site Directed Mutagenesis”, II. International Enzyme and Bioprocess Dyas (EBDays’2020), (Oral Presentation), Enzyme Testing and Consultancy Center (EDTAM), Gebze Technical University, September 01-03,2020.

Appendix B: Vector Map of Recombinant *Ctfdh* Gene

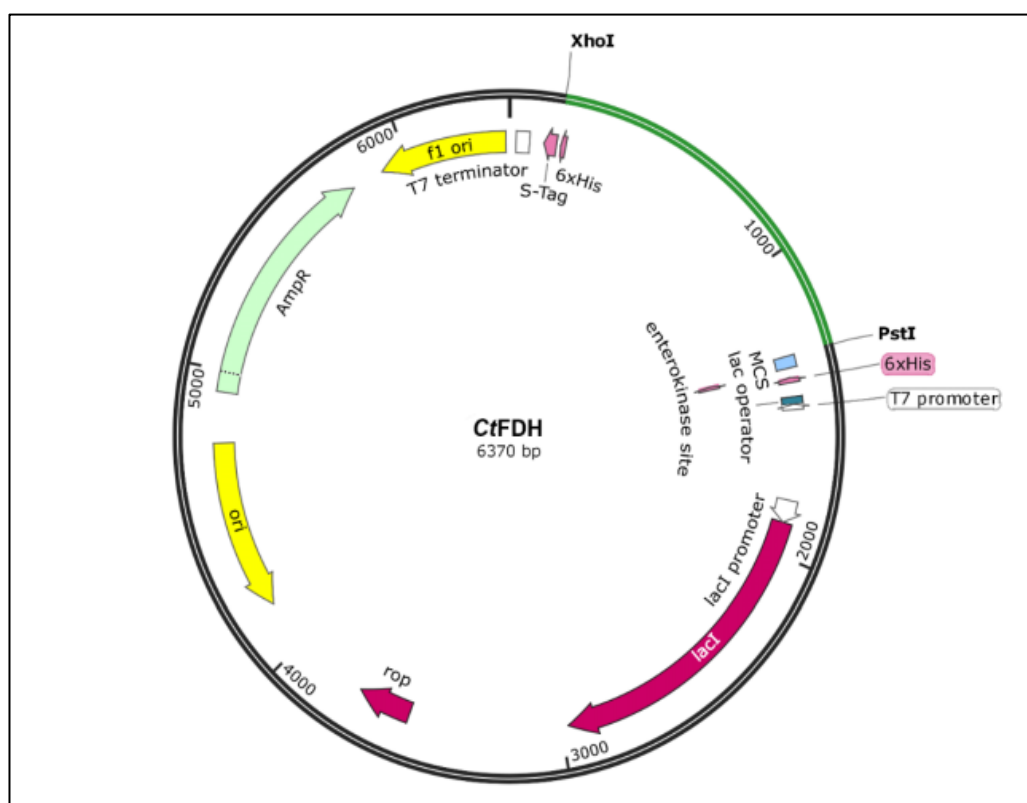


Figure B1.1: Cloned *Ctfdh* gene into pET45b+ vector.