

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

**THE EFFECT OF SITE DIRECTED MUTATIONS ON THE
ACTIVITY OF NAD⁺ - DEPENDENT FORMATE
DEHYDROGENASES**

YASİN ADEM WEDAJO
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF CHEMISTRY

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THESIS SUPERVISOR
ASSOC. PROF. DR. BARIŞ BİNAY

GEBZE
2021

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

YÖNLENDİRİLMİŞ BÖLGE
MUTASYONLARININ NAD^+ - BAĞIMLI
FORMAT DEHİDROGENAZLARIN
AKTİVİTESİNE ETKİSİ

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2021

GEBZE TEKNİK ÜNİVERSİTESİ	DOKTORA JÜRİ ONAY FORMU
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SUMMARY

Formate dehydrogenases (FDH) catalyze the reversible oxidation of formate to carbon dioxide; in the forward reaction it oxidizes formate ion to CO₂ while reducing NAD⁺ to NAD(P)H. Also, the reverse reaction of FDHs reduces CO₂ to formate simultaneously oxidizing NAD(P)H to NAD⁺.

However, NAD⁺-dependent FDHs show low catalytic efficiency for the reduction of CO₂. Besides, regeneration of cofactors requires that FDHs have stability in extreme process conditions and high cofactor turnover rates. These enzymes would need to be engineered before they could be used at an industrial scale for the conversion of CO₂ to hydrocarbons and to have more efficient cofactor regeneration.

The effects of amino acid changes on catalytic properties and thermal stability of *Chaetomium thermophilum* (CtFDH) and *Ceriporiopsis subvermispota* (CsFDH) formate dehydrogenases revealed some interesting results compared the literature. Therefore, this study was designed to investigate the effects of specific mutations on the kinetic properties of selected FDH enzymes. The selected enzymes were aligned with well-known FDHs and four non-aligned residues were selected for mutation based on their relative proximity to the active site. The kinetic values were analysed and structural analyses of the substrate-binding site were done by homology modelling.

The strongest effect was observed in CsFDH Val313Pro against HCO₃⁻. The K_M value of the CsFDH Val313 enzyme for substrate and coenzyme slightly decreased, and its activity is almost three-fold higher than the wildtype. Analyses of results of mutant FDHs give some promising results for CO₂ reduction compared to the literature.

Key Words: NAD⁺-Dependent Formate Dehydrogenase, CO₂ Reduction, Site-Directed Mutagenesis, Molecular Modelling.

ÖZET

Format dehidrogenazlar (FDH), formatın karbon dioksit tersinir oksidasyonunu katalize ederler; ileri reaksiyonda format iyonunu CO₂'e oksitlerken, NAD⁺'ı, NAD(P)H'ye indirgemektedir. Ayrıca tersinir reaksiyon aynı zamanda CO₂'i indirgeyerek NAD(P)H'nin NAD⁺ 'ya oksitlenmesini sağlamaktadır.

Ancak NAD⁺ bağımlı FDH'ler, CO₂'nin indirgenmesi için düşük katalitik verimlilik göstermektedirler. Ek olarak, kofaktörlerin rejenerasyonu, FDH'lerin aşırı işlem koşullarında kararlı ve yüksek katalitik aktiviteye sahip olmasını gerektirmektedir. Bu enzimlerin, CO₂'nin hidrokarbonlara dönüştürülmesi ve daha verimli kofaktör rejenerasyon eldesi açısından endüstriyel ölçekte kullanılabilmesi için bu enzimlerin yeniden tasarlanmaları gerekmektedir.

Amino asitlerin *Chaetomium thermophilum* (CtFDH) ve *Ceriporiopsis subvermispora* (CsFDH) format dehidrogenazların katalitik özellikleri ve termal kararlılığı üzerindeki etkileri, literatür ile karşılaştırıldığında ilginç sonuçlar ortaya çıkarmıştır. Bu nedenle, bu çalışma, spesifik mutasyonların seçilen FDH enzimlerinin, kinetik özellikleri üzerindeki etkilerini araştırmak için tasarlanmıştır. Seçilen FDH'ler iyi bilinen FDH'ler ile birlikte hizalanmıştır ve aktif bölgeye göreceli yakınlıklarına göre mutasyon için dört hizalı olmayan amino asit seçilmiştir. Kinetik değerler analiz edilmiştir ve substrat bağlama bilgisinin yapısal analizleri homoloji modellemesi ile yapılmıştır.

En güçlü etki HCO₃'e karşı CsFDH Val313Pro'da gözlemlenmiştir. Substrat ve koenzim için CsFDH Val313 enziminin K_M değeri az oranda azalmıştır ayrıca aktivitesi, doğal tipten neredeyse üç kat daha yüksektir.

Mutant CtFDH ve CsFDH'lerin sonuçlarının analizleri, literatür ile karşılaştırıldığında CO₂ indirgenmesinde bazı umut verici sonuçlar göstermektedir.

Anahtar Kelimeler: NAD⁺-bağımlı format dehidrogenaz, CO₂ indirgenmesi, Yönlendirilmiş Bölge Mutasyonu, Moleküler Modelleme.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
μ	: Micro
°C	: Celsius Degree
BCA	: Bicinchoninic Acid
CO ₂	: Carbon Dioxide
Cys	: Cysteine
FDH	: Formate Dehydrogenase
HCl	: Hydrochloric Acid
His	: Histidine
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
L	: Liter
LB	: Luria-Bertani
Mo	: Molybdate
NAD ⁺	: Nicotinamide Adenine Dinucleotide
NADP	: Nicotinamide Adenine Dinucleotide Phosphate
NaOH	: Sodium Hydroxide
NaPi	: Sodium Phosphate
NaAce	: Sodium Acetate
OD	: Optical Density
PCR	: Polymerase Chain Reaction
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC	: Super Optimal Broth with Catabolite Repression
Ser	: Serine
kDa	: Kilodalton
mM	: Millimolar
mg	: Milligram
min	: Minute
sec	: Second

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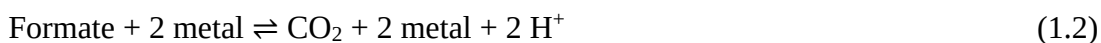
1. INTRODUCTION

Formate dehydrogenases (FDHs) are a set of enzymes that catalyse the oxidation of formate to carbon dioxide, donating the electrons to a second substrate, such as NAD^+ in NAD^+ (equation 1) - or to a metal in metal-dependent (equation 2) oxidoreductase [1,2]. In both types, a single carbon–hydrogen bond is broken in the formate and a new carbon–hydrogen bond is formed in the NADH, but the proton does not associate with the metal in the metal dependent oxidoreductase [3,4].

- NAD^+ -dependent reaction:



- Metal-dependent reaction:



NAD^+ dependent FDHs are mostly seen in industrial regeneration systems, where they are usually used for the synthesis of valuable optically active chiral compounds such as drugs and amino acids [5]. Among the enzymes used for cofactor regeneration, formate dehydrogenase has been reported as the most suitable due to having many advantages in the recycling process of NADH: an appropriate thermodynamic equilibrium, the immobility of the substrate, and an inert by-product (CO_2). Since the formed CO_2 can be easily removed from the reaction environment, NADH is obtained purely. Until now, FDHs have been evaluated only as excellent NADH regenerators. In addition, recent studies have shown that some FDHs have the ability to reduce carbon dioxide to formate as a reverse reaction [6-9].

Biotransformation of CO_2 has the benefit of global warming reduction and also is a powerful alternative to produce renewable energy [10]. Because of the ability of biocatalysts to efficiently catalyse processes under mild conditions with limited by-product formation, they play a critical role in the transformation of CO_2 by lowering activation barriers [7,11]. However, only a few biocatalysts, such as formate dehydrogenase, can capture CO_2 . The reverse reaction of FDHs has promising potential for methods of carbon fixation from the atmosphere and the oceans. Formate,

which can be produced from CO₂ as a feedstock, is also an economically and environmentally valuable chemical [7,12,13].

It has been reported that NAD⁺/NADH dependent FDHs show low catalytic efficiency for the reduction of CO₂ [8]. In addition, regeneration of cofactors requires that FDHs have stability in extreme process conditions and high cofactor turnover rates. However, the reported FDHs have limited properties such as thermostability and catalytic efficiency in order to fulfil the industrial requirements [4,7,8,11,14-18]. These enzymes would need to be engineered before they could be used at an industrial scale for the conversion of CO₂ to hydrocarbons and to have more efficient cofactor regeneration. For protein engineering improvements, study of the structure–function relationships at the FDH active site, and especially the catalytic mechanism, is essential.

2. LITERATURE REVIEW

2.1. Enzymes

Proteins that function as biological catalysts (biocatalysts) are called enzymes. The main purpose of a catalyst is to accelerate chemical reactions [19]. Substrates are the molecules that enzymes react with. As the result of a reaction, enzymes convert substrates into different molecules known as products. Enzymes, like all catalysts, increase the rate of the reaction by decreasing its activation energy [20]. The rate can be really increased, such that the conversion of substrate to product fastens millions of times [21]. Chemically, enzymes are like any catalyst and are not consumed in chemical reactions. Enzymes do not alter the equilibrium of a reaction.

Amino acids are the building blocks of proteins. Amino acids bond with each other to form short chains of polymers called peptides or longer chains of polymers which are called polypeptides or proteins. In order to the enzyme become functionally active, the long amino acid chain must be folded properly into its proper three-dimensional shape and must be successfully post-translationally modified [22].

All amino acids have the same fundamental structure, which consists of a central carbon atom, which is also known as alpha (α) carbon, bonded to a carboxyl group (COOH), an amino group (NH_2) and to a hydrogen (H) atom. Besides these, amino acids also contain a side chain, represented by R and a side chain is specific to each amino acid.

Domains are regions of the proteins that are functional and can self-stabilize. Usually, a domain is responsible for a specific function or interaction and many proteins contain several domains for different or same functions. Similar domains can have different functions in same or different proteins [23]. Generally, length of domains ranges from around 50 amino acids to 250 amino acids in length [24].

One of the most beneficial sides of enzymes is the substrate specificity. Before catalyzing any chemical reaction, enzymes have to bind their substrates. Specificity is achieved by binding pockets that allows the substrates to fit with a complementary shape, charge and hydrophilic/hydrophobic features.

In 1894, Emil Fischer suggested that both enzyme and substrate has complementary three-dimensional shape that allows them to fit into each other [25].

This became a model, which is called as "the lock and key" model. Even though the model explains enzyme's substrate specificity, it fails when the transition state of enzymes is considered. Daniel Koshland proposed an update to the lock and key model in 1958: because enzymes are very versatile structures, interactions with the substrate consistently reshape the active site as the substrate interacts with the enzyme [26]. Until the substrate is fully bound, the active site continues to shift, at which point the final form and charge distribution is determined. Induced fit will enhance the fidelity of molecular recognition through the conformational proofreading mechanism in the presence of competition and noise [27].

To show optimum activity, some enzymes do not need additional components. Others require non-protein molecules to be bound to the protein in order to function. These non-protein molecules are called cofactors. Cofactors may be either inorganic (e.g., iron-sulfur clusters and metal ions) or organic compounds (e.g., flavin and heme). Such cofactors serve many purposes; metal ions, for example, may assist to stabilize nucleophilic organisms within the active site. Organic cofactors may either be coenzymes that are released during the reaction from the active site of the enzyme or prosthetic groups that are closely bound to an enzyme [28,29]

2.2. Enzyme Classification

Enzymes are categorized into six functional classes in accordance with the rules of Nomenclature Committee established by International Union of Biochemistry and Molecular Biology (IUBMB) and are classified depending on the type of reaction in which they catalyze. The six functional classes are:

- **Oxidoreductases:** Oxidoreductase is an enzyme that catalyzes oxidation reduction (redox) reactions by utilizing electron acceptor such as NAD^+ in dehydrogenases, H_2O_2 in peroxidases and oxygen in oxidases. Oxidoreductases have the highest subclass number of other functional groups. There are 22 subclasses of oxidoreductases, some of which are technologically important, such as dehydrogenases that oxidize a substrate with a cofactor (NAD^+ , NAD(P)^+ , FAD^+ , FMN) [30] that serves as an acceptor, by transporting hydrogen atoms. In the other

hands, oxidoreductases produced and secreted inside the cell take place in various reactions in the living cell.

- **Transferases:** Transferase is an enzyme that achieves the transfer of specific functional groups (for example, a methyl, carboxyl, phosphoryl, glycosyl) from a donor to a suitable acceptor. These enzymes, which are the heads of transferase enzymes such as acyltransferases, methyltransferases, transaminases, glycosyltransferases, have been understood to play an important role in different cell metabolisms.

- **Hydrolases:** Hydrolase is an enzyme that uses water to break a chemical bond in which usually results in separation of larger molecule to smaller molecules. Hydrolases are related to some of the enzymes that used in biotechnology: glycosidases, esterases and proteases are noticeable.

- **Lyases:** Lyase is an enzyme that catalyzes the breaking (called as “elimination” reaction) of different chemical bonds through mechanisms other than hydrolysis and oxidation, occasionally forming a new double bond or a new ring structure. Lyases are distinct from other enzymes because they only require one substrate in one direction for reaction, yet two substrates for the reverse reaction.

- **Isomerases:** Isomerase is an enzyme that catalyzes changes within one molecule. It modifies one isomer to another, implying that the same molecular formula but a different structure in the end product. It promotes intramolecular rearrangement in which bonds are broken and formed to produce other isomers of the molecule. Too few isomerases are used in biotechnological areas, however the case with glucose isomerase [31] (indeed xylose isomerase) is an exception.

- **Ligases:** Ligase is an enzyme that catalyzes the joining (ligation) of two large molecules by forming a new chemical bond which is usually covalent bond. Despite its metabolic compatibility, ligases have no application in the industry due to their complex and unstable structure.

A nomenclature for enzymes has been developed by the IUBMB, with EC numbers (for “Enzyme Commission”). Each enzyme is defined by “EC” followed by

a sequence of four numbers representing the enzymatic activity hierarchy (from very general to very specific). The first digit vastly classifies the enzyme due to its mechanism while the other digits append more and more specificity. For example, formate dehydrogenases (EC 1.17.1.9) is a oxidoreductase (EC 1) that acts on CH or CH₂ groups (EC 1.17) with NAD or NADP as acceptor (EC 1.17.1).

For the continuation of life in living cells, oxidoreductases, which catalyze the reactions of reduction and oxidation, are necessary. These enzymes in natural or non-natural substrates may also catalyze reactions. There are distinct oxidoreductases that can be obtained from animals, such as oxidases, which use molecular oxygen as an oxidizing agent, but typically show a very small specificity of the substrate, and oxygenases, monooxygenases, and dehydrogenases. They are important enzymes in the conversion of substrates into products, even though they use complex coenzymes. Through the acquisition of optically active compounds and the efficient development of chiral building blocks, these enzymes are especially involved in synthetic pathways in drug production.

2.3. Dehydrogenases

Oxidoreductases are the biological catalysts (enzymes) that catalyze the transfer of an electron from the reductant to the oxidant. These enzymes are consisting of six subclasses: oxygenases, reductases, peroxidases, oxidases, hydroxylases, and dehydrogenases.

Dehydrogenases (DH or DHase) are the most common types of biological catalysts that catalyze oxidation-reduction reactions of their specific substrate through the transfer of proton ([H]) to an acceptor or co-enzyme.

DHase are take parts in both catabolic and anabolic pathways by using nicotinamide adenine dinucleotide (NAD⁺ or NADH), nicotinamide adenine dinucleotide phosphate (NADP⁺ or NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as a co-enzyme to catalyze reduction reactions [32].

2.3.1. NAD⁺-Dependent FDHs

Properties of FDHs isolated from yeasts and bacteria were compared in the mid-1990s, and bacterial FDHs were found to be more active and stable. FDH from *Pseudomonas* sp. 101 occurred to be the most stable, and up to now it is the most known thermostable natural FDH and best studied [33]. The active centre of FDH-*Ps* is presented in Figure 2.1 with formate anion occupying the azide binding site revealed by crystallographic studies [34]. Oxygen atoms of formate form H-bonds to Arg-284 and Asn-146, while the direction of the presumed hydride ion transfer makes an angle of about 90 with the pyridine plane (similar to other dehydrogenases). The H atom of the substrate is within 1.4 Å of the C₄N position of the coenzyme.

2.3.1.1. Active Site of FDHs

The active site of FDH comprises negatively and positively charged clusters of residues as well as hydrophobic patches. The 'negatively' charged region (Figure. 2.1) is made up of residues Thr-282, Asp-308 and Ser-334 holding in place the carboxamide group of NAD⁺. They belong to the coenzyme binding domain, are already properly oriented in the apo enzyme and do not undergo considerable shifts on holo complex formation.

Another cluster of residues can be designated as 'positively' charged and comprises the Arg-284 and Asn-146 side chains and the imide nitrogen of Ile-122. All three residues undergo substantial displacement on the transition from apo to holo. Arginine and asparagine move about 0.7-0.8 Å towards the substrate binding site in holo FDH, while the wall comprising the Ile-122-Gly-123 pair as well as several neighbouring residues advances about 1.8 Å towards the substrate. The role of the positively charged cluster is mostly to bind formate anion and to fix it in the proper position relative to NAD⁺.

There are several hydrophobic clusters in the active site. The first is composed of residues Val-150 and Ile-202 and provides a hydrophobic environment for one face of the NAD⁺ pyridine ring. The position of these residues in holo FDH differs by less than 0.4 Å from that in the apo form. The second cluster comprises Pro-97 and Phe-98. Their side chains in holo FDH move more than 1 Å and point to the formate binding

site. The third hydrophobic region is the Ile-122 side chain hanging over the edge of the pyridine ring. The fourth hydrophobic area is the Val-309 side chain that supports the proper conformation of the NAD⁺ carboxamide.

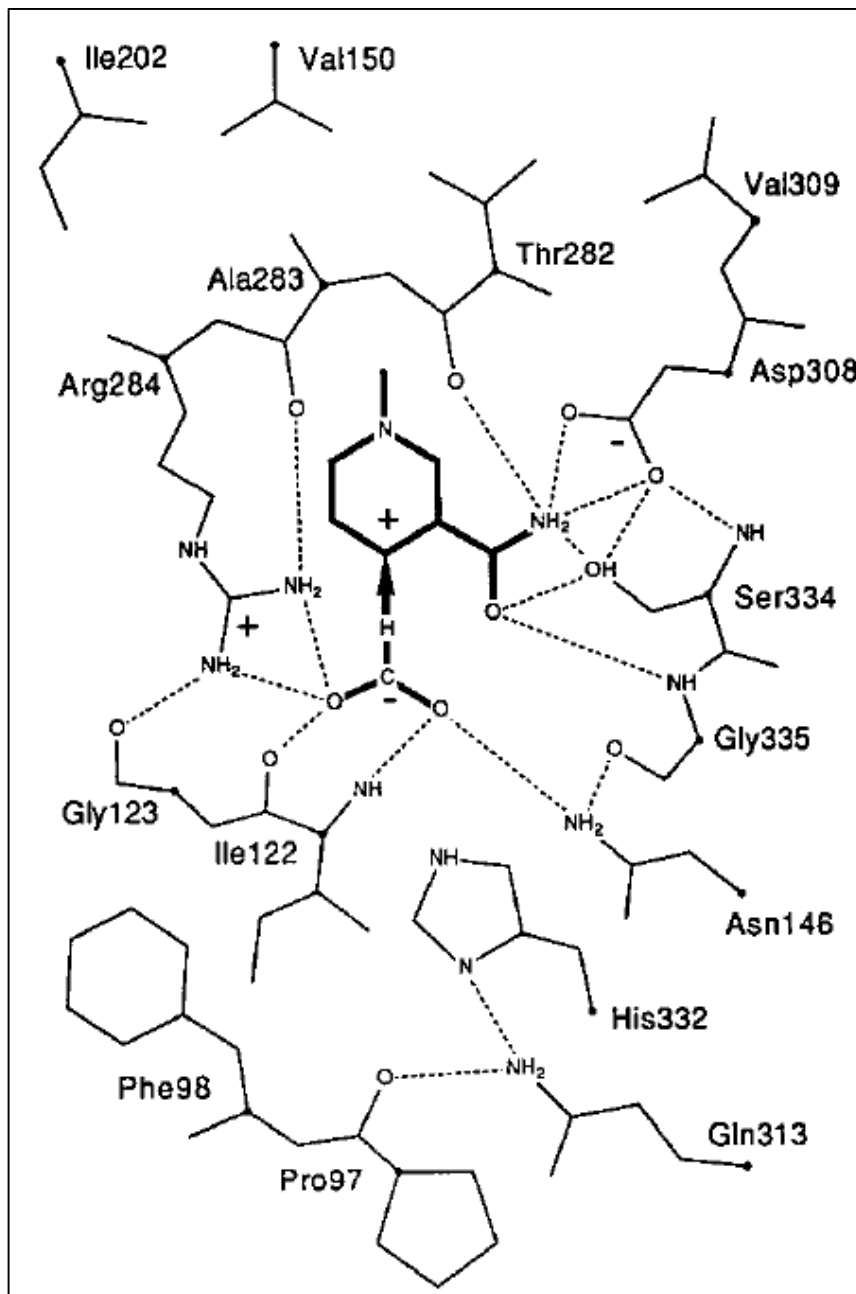


Figure 2.1: Scheme of the FDH active centre near the point of catalysis H-bonds are indicated by dashed lines. The pointer shows the direction of hydride anion transfer.

2.3.1.2. Reaction Mechanism

The mechanism of hydrogen transfer in the FDH-catalysed reaction is mainly governed by electrostatic effects. The positively charged nicotinamide unit of NAD^+ is properly oriented in the active centre by multiple interactions with a 'negatively' charged cluster and hydrophobic side chains, whereas formate is bound and oriented by multiple H-bonds with a 'positively' charged cluster. Several stabilizing and destabilizing interactions take place in the active centre in the course of catalysis (Figure 2.1 and Table 2.1).

An important factor for catalysis by all NAD^+ -dependent dehydrogenases, including FDH, is enhancement of the electrophilic properties of C_4N of the nicotinamide moiety of the coenzyme (Figure 2.3). This might be achieved through sufficient polarization of the NAD^+ carboxamide group via interactions with negatively charged ligands (Figure 2.2) and perturbation of its ground state due to the twist of the carboxamide with respect to the pyridine plane. Such out-of-plane positioning of the carboxamide group of the coenzyme is quite common among NAD^+ -dependent dehydrogenases.

Three hydrophobic walls comprising Pro-97-Phe-98, Ile-122 and Val-150/Ile-202 are pressed over the active site as a result of the conformational change in the course of the reaction. The Pro-Phe pair may perturb the formate ground state and provide a more suitable environment for neutral CO_2 , thus accounting for better binding of the latter compared with the charged formate anion. The Val-150 and Ile-202 side chains face the other side of the pyridine ring and thus favour better binding of neutral NADH compared with charged NAD^+ . This wall also facilitates possible bending of the nicotinamide moiety towards the substrate, forcing it to acquire the 'boat' conformation assumed to result in lowering of the activation barrier. However, the hypothesis of changing the pucker of the nicotinamide in the transition state is in conflict with recent observations of a lack of change in the N-1 hybridization state in the FDH and alcohol dehydrogenase (ADH) reactions.

Besides being an anchor group for the substrate, Arg-284 can play two additional roles. First, the positive charge on Arg-284 may stabilize a partial negative charge on the migrating hydride ion and, secondly, it may perturb positively charged nicotinamide.

An important role in the mechanism is ascribed to Ile-122, which together with the flanking parts of the polypeptide chain moves by nearly 2 Å on transition from apo to holo. This movement is essential for several reasons: (1) it prevents unproductive substrate binding due to pairing of the guanidinium group of Arg-284 and the formate carboxylic acid group; (2) the main chain carbonyl of Ile-122 stabilizes the partial positive charge on the carbon atom of the formate; and (3) the hydrophobic side chain of Ile-122 might perturb positively charged NAD⁺.

By combining all these features, FDH provides a very effective machinery for abstraction of the hydride ion from formate. The C₄N position of NAD⁺ in the active centre is an at least 105-fold better acceptor of hydride than stable thriaryl methylcarbonium cations in model reactions with formate anion proceeding via a similar mechanism of hydride ion transfer [35].

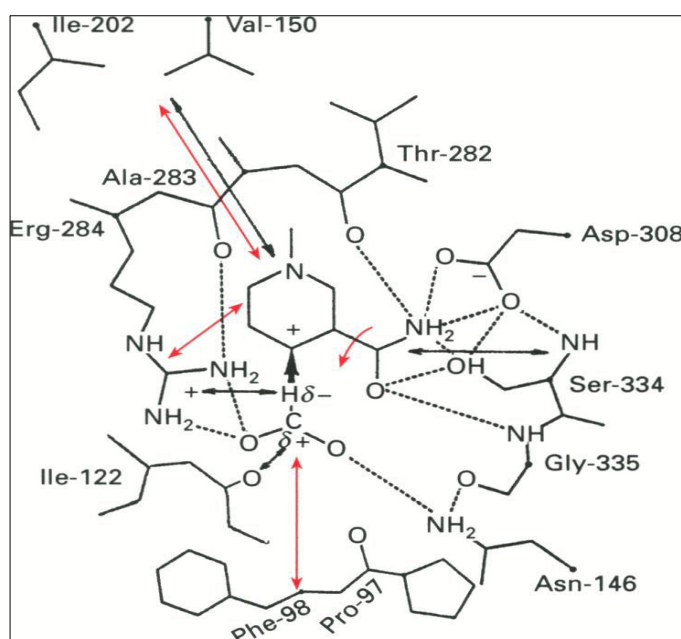


Figure 2.2: Scheme of the FDH active centre near the point of catalysis. H-bonds are indicated by dashed lines. The pointer shows the direction of hydride anion transfer. Black arrows show stabilizing interactions, whereas red arrows show destabilizing interactions within the active centre.

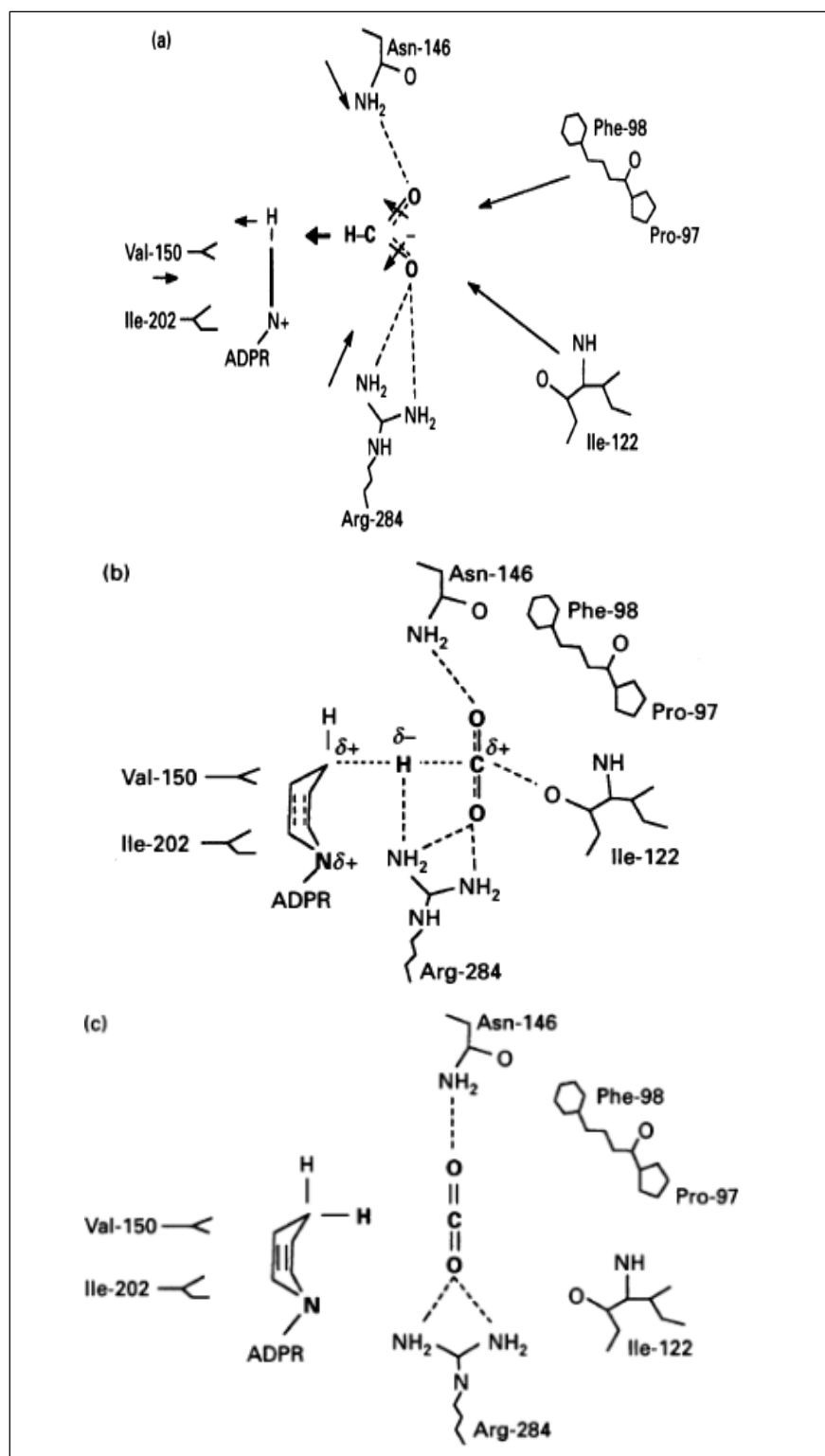


Figure 2.3: The main steps of the FDH molecular mechanism. Pre-transition (a), transition (b) and post-transition (c) states of the reaction. Arrows indicate the movements of parts of the enzyme active centre and the reactants during the course of the catalytic transformation. The size of the arrows represents the relative displacement of the respective amino acid residues on the transition of FDH from the apo to the holo state.

Table 2.1: Roles of active site residues

Destabilizing	Stabilizing
• Twist of the carboxamide group enhances polarization of C₄N • Hydrophobic wall (Val-150, Ile-202) perturbs the nicotinamide moiety of NAD⁺ • Ile-122 perturbs the nicotinamide moiety of NAD⁺ • Positive charge of Arg-284 perturbs the nicotinamide moiety of NAD⁺ • Formate anion is destabilized by the hydrophobic wall (Pro-97, Phe-98)	• 'Negative cluster' (Thr-282, Asp-308, Ser-334) enhances polarization of C₄N • Hydrophobic wall (Val-150, Ile-202) stabilizes neutral NADH and forces bending of the nicotinamide moiety towards the substrate ('boat' conformation) • Ile-122 prevents unproductive substrate binding • Arg-284 stabilizes the negative charge on migrating hydride ion • Ile-122 oxygen stabilizes the positive charge on the formate carbon

2.3.2. Metal-Dependent FDHs

The other class comprises prokaryotic enzymes whose active sites harbour one tungsten (W) or molybdenum (Mo) ion [36] that mediates the reaction catalyzed by the enzyme. The active site of all presently known metal-dependent FDHs is well conserved [4]. Mo-FDH from *E. coli* is the best characterized metal dependent FDH, and easy to produce in a stable, reduced form yielding significant advantages for the EFC field [37]. The hydride transfer mechanism proposed for formate dehydrogenases is shown below in Figure 2.4. Active site of all known metal-dependent FDHs are well

conserved. The active site of oxidized FDH harbours consist of one molybdenum atom or one tungsten atom coordinated by selenium atom from a cysteine or selenocysteine residue. The only variation in the active site of metal-dependent FDH is the presence of one selenocysteine or one cysteine residue. A proposed hydride ion transfer mechanism can be seen in Figure 2.4 [4].

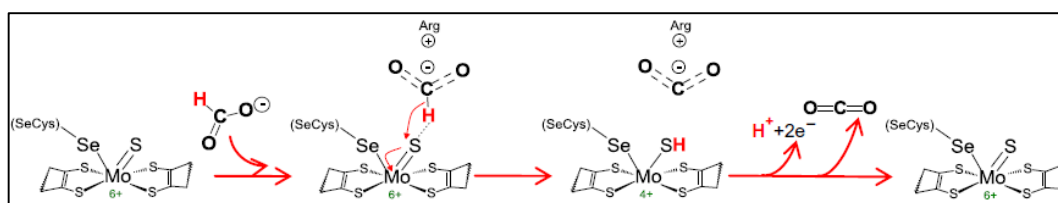


Figure 2.4: Reaction mechanism proposed for the FDH-catalyzed formate oxidation.

2.4. Practical Applications of FDH

2.4.1. Industrial applications of FDH

Formate dehydrogenase (FDH, EC 1.2.1.2) is well-established as a redox enzyme for the regeneration of NADH in cofactor dependent biocatalysis [38]. A major advantage of the FDH-based cofactor regeneration is the irreversible step of carbon dioxide formation and removal, thus shifting the equilibrium toward (complete) product formation [39,40]. In fact, it is irreversibility which renders this reaction indispensable in industrial biocatalysis. Typically, enzymatic reactions are characterized by being reversible, thus in most instances only incomplete conversion can be achieved. As a consequence, space time yields are unfavorable and the processes are economically less attractive. In addition, the circumstance of the oxidation product CO_2 being chemically inert [38] and does not inactivate enzymes in reaction systems nor harm products [40]. A further advantage is the simple work-up, since (ideally) no organic byproduct remains in the reaction mixture [39].

Optically active compounds are the most selective and efficient regulators of vital activity of the cell because of chirality of the living world. Among the 500 drug best sellers all over the world more than 58% are chiral compounds, and among new

preparations, their fraction achieves 70% [33]. According to prescriptions of the Food and Drug Administration of the United States of America, from 1999 the optical purity of all chiral compounds used as drugs has to be no less than 99%. Therefore, the number of studies concerning the application of the enzyme in pharmaceutical industry sharply increased within the last decade. Virtually all classes of enzymes can be used for synthesis of optically active compounds.

Dehydrogenases can be used to produce optically active compounds from nonchiral ones because they are extremely stereospecific in the transfer of hydride ion between the substrate and coenzyme (optical purity in the range 99.9–99.99%) [41]. Because of the high price of NADH and especially of NADPH (>US\$ 12,000/kg), processes involving only dehydrogenases is economically unprofitable. To decrease the contribution of the cost of reduced coenzymes to the cost of the final product, another reaction has been proposed be used which converts NAD(P)^+ back to NAD(P)H . This reaction can be catalyzed by both the major enzyme (e.g., addition into the system of excess isopropanol in processes with involvement of alcohol dehydrogenases) and other additional enzyme. Figure 2.5 presents the general scheme of such a process, with FDH as the second enzyme.

The reaction catalyzed by FDH fits the best requirements for the system of NADH regeneration.

- The reaction of formate oxidation to carbon dioxide is irreversible, and this allows us to use thermodynamic pressure on the major process and obtain 99–100% yield of the desired product.
- The FDH activity does not change in the pH range from 5.5 to 11.0 and values of Michaelis constants for NAD^+ and formate are unchanged in the pH range from 6.0 to 9.5, thus FDH can be used in combination with any dehydrogenase that has activity optimum in this range.
- Formate salts are very low-cost and available substrates. Formate ion does not influence activities of enzymes used in the proposed process.
- Carbon dioxide, which is the product of the FDH catalyzed reaction, also does not inhibit the majority of dehydrogenases, does not interfere with purification of the desired compound, and can be easily removed from the reaction mixture at decreased pressure.

- FDHs from bacteria and yeasts are highly stable enzymes and can work in systems for weeks and months.

FDHs are inexpensive and available, and the price for the production of the enzyme is low. The content of FDH in the starting strains of methylotrophic bacteria and yeasts can be up to 10-18% of the total cell protein, and technology of recombinant DNA provides for obtaining of still higher expression of the desired product.

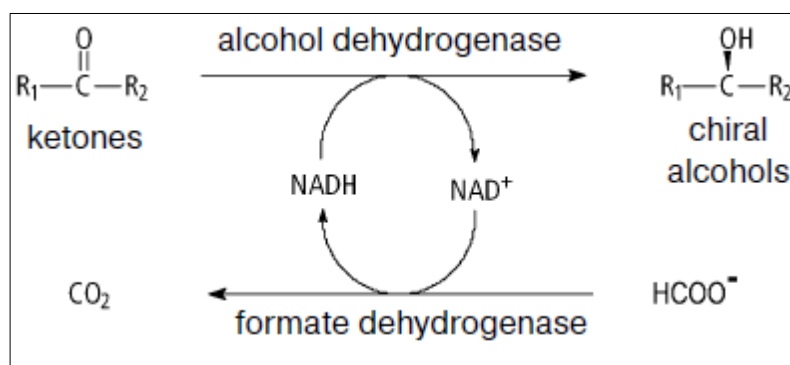


Figure 2.5: A scheme for synthesis of optically active compounds using dehydrogenases and formate dehydrogenase for regeneration of NADH.

Owing to irreversibility of the formate dehydrogenase reaction and the wide pH optimum of the activity, this enzyme is a versatile biocatalyst efficient at pH optima for many processes. The company Degussa (Germany) has developed an industrial scale process for production of *tert*-L-leucine with FDH as a catalyst of NADH regeneration, and this is one of the largest enzymatic processes in pharmaceutical chemistry [33].

2.4.2. Biological transformations of carbon dioxide

Rapid growth in the world's economy depends on a significant increase in energy consumption. As is known, most of the present energy supply comes from coal, oil, and natural gas. The overreliance on fossil fuels has led to the discharge of more and more carbon dioxide into the atmosphere accompanied with the rapid development of modern industry. This has resulted in serious environmental problems in addition to the scarcity of energy. Additionally, the excessive consumption of fossil fuels and

deforestation have interrupted the carbon cycle on the earth and accelerated the environmental deterioration and resource depletion.

In order to mitigate the issue of global warming, most countries have signed the Paris Agreement, which aims to abate the net atmospheric CO₂ levels by 2050. Therefore, efficient measures have to be made to reduce atmospheric CO₂ levels, such as reducing the direct emission of CO₂ by developing clean and sustainable energy resources to replace traditional fossil fuels, capturing and storing CO₂, and converting the anthropogenic CO₂ into useful chemicals or fuels by reducing it [42]. Among them, biological transformations of carbon dioxide are key processes in the global carbon cycle and have lately attracted great attention, because they may be used to combat the greenhouse effect [36] the fact that it is the most abundant C1 compound [35]. The conversion of CO₂ to value-added fuels or chemicals is important in recycling carbon species and simultaneously solving environmental problems and energy crises.

The reduction of CO₂ via biocatalytic processes received particular attention because of their special substrate and product selectivity as well as high conversion efficiency [42]. In 1993 and 1994, Yoneyam et al. [43,44] demonstrated that CO₂ can be biocatalyzed into CH₃OH in a CO₂-saturated phosphate buffer solution, in which pyrroloquinoline quinone (or methyl viologen) was used as an electron mediator, and formate dehydrogenase, formaldehyde dehydrogenase (FaldDH), and alcohol dehydrogenase were used as biocatalysts. Subsequently, Obert [45] presented the reduction of CO₂ to methanol using three different dehydrogenases in three consequent reductions, in which reduced nicotinamide adenine dinucleotide (NADH) molecules were required at each step. Such a multi-enzyme system was composed of three different dehydrogenases (Figure 2.6) that catalyze the conversion of CO₂ to CH₃OH in the presence of NADH.

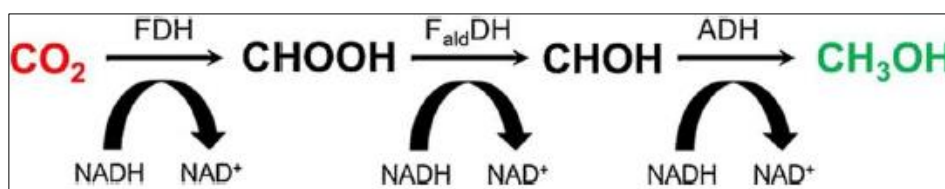


Figure 2.6: Biocatalytic transformation pathway of CO₂ to CH₃OH via stepwise reverse enzymatic catalysis by FDH, FaldDH, and ADH.

2.5. Protein Engineering

Enzymes, which have been used for many years, are still included in the processes of many products today. It used at many fermentation processes including baking, production of dairy products (cheese, yoghurt etc.) and wine making. Today several biocatalytic activities are exist like synthesis of amides or alcohols, addition of fermentation process [46]. However, it is not always easy to identify and purify new enzymes resistant to industrial harsh conditions. The full use of enzymes as catalysts in chemical processes requires effective expression and production methods, without limiting availability. Protein engineering is a scientific method that improves enzymatic activity, increase enzyme stability, and broaden product spectra in natural product biosynthesis. Protein engineering is an important approach for the development of sustainable industrial processes [46,47].

The general definition of protein engineering is an approach that understanding the functions and structure of the protein in molecular level and changing the genetics of the protein, so obtaining new protein that has desired function and structure. The designed change is occurred thanks to changes in genes encoding proteins and improved new protein is obtained [48].

2.5.1. Directed Evolution

Directed evolution involves the introduction of molecular diversity at the DNA level, on cloned genes coding for the protein of interest, through a variety of mutagenic techniques. Libraries of variant genes, whose size can be adjusted from a few to many millions of clones, are produced and then passed into the crucial selection or screening system [49].

Directed evolution is based on the generation of protein variants by recombining gene fragments in vitro using PCR followed by selection or screening of those that possess desired properties like activity, selectivity, stability [50].

2.5.2. Semi-Rational Design

The search for reduction in modeling studies required for rational design has shown that a new approach is needed. Thus, the semi-rational design technique has been used in order to develop and investigate protein functional properties. Semi-rational design is an effective and a novel approach. It facilitated the directed evolution method.

It requires full or partial amino acid sequence information and three-dimensional structure knowledge. The use of selection and scanning techniques is necessary to be able to choose from the acquired smart library. Semi-rational design has some advantages compared to other directed evolution techniques. Large libraries are created in methods such as DNA shuffling. Finding the descriptors of the phenotype that are looked for from these libraries takes a lot of time and effort. In semi-rational design, the amino acid sequence can be changed with 22 different amino acids at predetermined target positions on the gene. In this way, the library is made smaller and the library size becomes more rational. Thanks to the three-dimensional structure of the protein, the position responsible for the targeted change can be found. This method has been used successfully in developing many protein properties [51]. The use of this method is formed by combining the determination of mutation sites with a rational approach and an effective screening method [52].

2.5.3. Rational Design

Rational design is a method that required the three-dimensional structure data as well as functions and biophysical information of protein. The research topics about single-point mutation, exchange of elements of secondary structure and creating hybrid enzymes is studied by rational design method [51,53].

In order to apply the rational design method, it is necessary to have information about the amino acid sequence information, three-dimensional structure and function of the protein to be studied. Information about protein sequences and its three-dimensional structure is not adequate to design improved protein [53,54]. Controllable amino acid changes like insertion, deletion or displacement can be provided by rational design method. A single amino acid change can be made or a sequence of a certain

length can be changed. A single amino acid change or replacement of a particular length of sequence can also be provided [55].

Estimation of sequences that can accept all the confirmation to require for change between predefined states is necessary for rational design of the protein. Multiple designs can be applied to large structural ensembles, and the imminent development of this approach enables the evaluation of protein sequence energies in the context of many structural states. Nevertheless, the fact that the structural properties of the relevant conformational states for dynamic change are known in advance and the incorporation of the functionally relevant conformational change into a stable protein layer is a difficult design problem to solve [56,57].

Active site substitution by site-directed mutagenesis is a rational design method and a common approach in protein engineering. Site-directed mutagenesis is based on structural information. Site-directed mutagenesis, which has been successfully used in reshaping the substrate or cofactor specificity and reactivity, can also become a powerful complementary approach to directed evolution, thanks to the ever-increasing knowledge of protein structure and function [58,59]. Site-directed mutagenesis that based on polymerase chain reaction methods has several protocols that developed to obtain efficient mutagenesis of a target DNA sequence. Among of the PCR based protocols of the site-directed mutagenesis are overlap extension and all plasmid single round. All plasmid DNA is oriented differently but overlapping at their 5' ends, by the mutagenic primers. This approach is based on the PCR amplification of these plasmids. The aim of the approach is to solve the problem of length limitation due to plasmid size and to minimize the rate of unwanted mutations. It also provides the increase in the mutagenesis efficiency [59].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Equipment and Apparatus

Micropipettes (Axygen-Isolab), SDS-PAGE electrophoresis system (Bio-Rad), autoclave (N-BioTek), vortex (N-Biotek), magnetic stirrer / heater (Wisd), electrophoresis power supply (Biorad), mini centrifuge (Sigma), centrifuge (Beckman Coulter), PCR-thermal cycler (Techne-5PRIMEG / 02), -80 °C deep freezer (Arctiko), refrigerator (Vestel), pure water device (Merck Millipore), shaking incubator (N-Biotek) pH meter (Hanna), oven (Nüve), water bath (Stuart), sonicator (Bandelin) microplate reader (Tecan), Syringe Filters (Sartorius) Concentrators (Amicon Millipore), microplate shaker incubator (Bio-Rad), HisTrap Column (GE), sensitive balance (Shimadzu – Radwag).

3.1.2. Enzymes

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

3.1.3. Chemicals, Reagents and Kits

Ultrapure, distilled water, Yeast extract (LabM), Acrylamide (Fisher BioReagents), Bis acrylamide (Fisher BioReagents), Ammonium persulfate (Fisher BioReagents), Sodium dodecyl sulphate (Fisher BioReagents), Trypton (LabM), Glucose (LabM), Lactose (Fisher BioReagents), 2-mercaptoethanol (Alfa Aesar), Agar (Fluka), Agarose (Biomax), Imidazole (AppliChem), LB Broth (LabM), LB Agar, Ethidium bromide, Sodium chloride (Merck), Sodium Chloride, Sodium Bicarbonate, Sodium Phosphate (Fisher Scientific), Tris-Base (Formedium), Ampicillin (AppliChem), Hydrochloric acid (Sigma-Aldrich), Glycerol (Fisher BioReagents), Sodium Formate (Sigma), NAD⁺, NADH (Sigma), Citrate (Riedel-de

Haën), Glycine (Sigma), SDS-PAGE Gel Stain (Generon), SDS-PAGE Gels, SDS-PAGE Running Buffer (CBS Scientific) dNTP (Thermo Sci.), Quick Plasmid Miniprep Kit (Cat. No. K210010), BCA Assay Kit (Serva), Plasmid isolation kit (Invitrogen), Invitrogen PureLink (TM).

3.1.4. Plasmids and Cells

csfdh and *ctfdh* gene containing hexa-histidine sequence in pET-45b+ plasmid that was previously constructed by GERC lab group was used in the experiments [8]. Commercial *E. coli* BL21 (DE3) and NEB® 5 α (NEB) cells were used for transformation.

3.1.5. Mediums

The following three types of mediums were used to conduct the experiment.

- Luria-Bertani (LB) Medium: 12 g of LB was weighed with sensitive balance and dissolved in 500 mL ddH₂O. Right after, the solution was sterilized at 121 °C for 15 minutes in autoclave.
- Luria-Bertani (LB) Agar Feeder: 20 g of LB Agar was weighed and dissolved in 500 mL of ddH₂O and then autoclaved at 121 °C for 15 min. 1 μ L/1 mL ampicillin was added to it after it was cooled to 55 °C. Then it was poured to petri dishes in a sterile cabinet. Finally, the dishes were covered with their lids and parafilm and stored at +4 °C for later use.
- Autoinduction Studier Medium: A mixture of 5 g of yeast, 20 g of tryptone, 5 g of NaCl, 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g of glucose, 6.9 mL of 87 % glycerol and 2.1 g of lactose were dissolved in distilled water. After its pH was adjusted to 7, the solution was filled up to 1 L. The autoinduction Studier medium was sterilized in an autoclave at 121 °C for 15 minutes.

3.1.6. Buffers and Solutions

- Ampicillin Solution: 1 g of ampicillin was dissolved in 10 mL ddH₂O and was filtered using a 0.45 mm sterile filter. Finally, the solution was aliquoted into 1 mL eppendorf tubes and kept at -20 °C for later use.

- Protein Purification Buffers

Buffer 1: 1.38 g NaH₂PO₄·H₂O (200 mM), 14.61 g NaCl (500 mM) and 1.02 g Imidazole (30 mM) were dissolved in 500 mL dH₂O and the pH was fixed to 7.4 using NaOH.

Buffer 2: 1.38 g NaH₂PO₄·H₂O (200 mM), 14.61 g NaCl (500 mM) and 17.02 g Imidazole (500 mM) were dissolved in 500 mL dH₂O and the pH was fixed to 7.4 using NaOH.

Imidazole: a linear gradient of 50–250 mM imidazole in a buffer consisting of the above buffers.

3.1.7. Enzyme Activity Assay Buffers

- Sodium Phosphate Buffer Solution (100 mM): A mixture of 14.19 g Na₂HPO₄ and 13.79 g NaH₂PO₄·H₂O (100 mM) were dissolved in dH₂O and it was filled up to 1 L. The pH of the solution was adjusted to 8 using Na₂HPO₄ as a base and NaH₂PO₄·H₂O as an acid.

- Sodium Acetate Buffer Solution (100mM): A 6.81 g of CH₃COONa was dissolved in dH₂O and its pH was adjusted using CH₃COOH (100 mM). The solution was filled up to 1 L using dH₂O.

3.1.8. Enzyme Activity Solution

- 0.5 and 1mM NAD⁺ Solution: 1.7 and 3.4 mg NAD⁺ were dissolved in 5 ml of 100 mM pH 5 sodium acetate buffer in different falcons in order to prepare 0.5 mM and 1 mM solution respectively.

- 0.5 mM NADH Solution: 1.77 mg NADH was weighed and dissolved in 5 mL of 100 mM NaPi, pH 8.

3.2. Methods

3.2.1. Bioinformatic Analysis

Four positions, Val331Thr, Asn312Phe and Val313Pro for CsFDH and Val310Thr for CtFDH were identified rationally for site-directed mutagenesis. Both the CsFDH and CtFDH was aligned with well-known FDHs and a sum of four non-aligned residues were selected for mutation based on their relative proximity to the active site (Figure 3.1). The numbering of identified candidates of CsFDH is in 2NAD numbering system, where for CtFDH is in its own sequence.

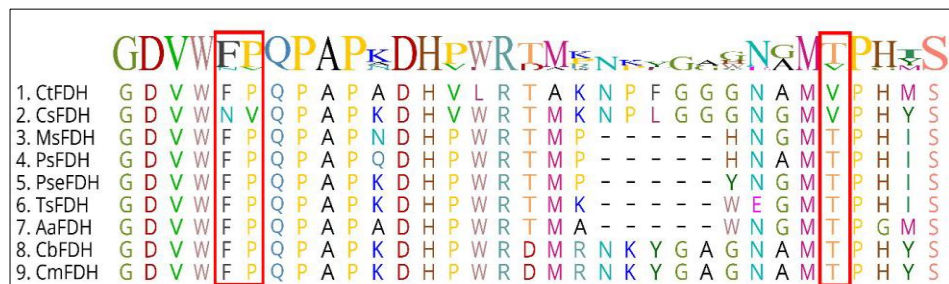


Figure 3.1: Alignment of formate dehydrogenases. Symbols: *Pse*, *Pseudomonas* sp. (strain 101) (*Pse*FDH); *Cb*, *Candida boidinii* (*Cb*FDH); *Ts*, *Thiobacillus* sp. (strain KNK65MA) (*Ts*FDH); *Cm*, *Candida methylica* (*Cm*FDH); *Ct*, *Chaetomium thermophilum* (*Ct*FDH), *Cs*, *Ceriporiopsis subvermisporea* (*Cs*FDH, BAF98206.1), *Ps*, *Paracoccus* sp. 12-A (*Ps*FDH, BAB64941.1), *Ms*, *Moraxella* sp. C-1 (*Ms*FDH, CAA73696.1), *Aa*, *Ancylobacter aquaticus* KNK607M (*Aa*FDH, BAC65346.1).

3.2.2. Rational Design

3.2.2.1. Primer Design

The primers were designed using OligoArchitect™ Primer and Probe Design Tool and are listed below.

Table 3.1: Primers used for site-directed mutagenesis.

Cs FDH Val331Thr Forward Primer	5'GTGGCAATGGTATG ACCCCG CACTATAGCG'3
Cs FDH Val331Thr Reverse Primer	5'CGCTATAGTGCGG GGT CATACCATTGCCAC'3
Cs FDH Asn312Phe Forward Primer	5'GCAGGCGATGTGTGG TTT GTTCAGCCGGCG'3
Cs FDH Asn312Phe Reverse Primer	5'CGCCGGCTGAAC AA ACCACACATCGCCTGC'3
Cs FDH Val313Pro Forward Primer	5' GCGGATGTGTGGAAT CCG CAGCCGGCGC'3
Cs FDH Val313Pro Reverse Primer	5' GCGCCGGCTG CGG ATTCCACACATCGCC'3
Ct FDH Val310Thr Forward Primer	5' GGTGGCAATGCTATG ACCCCG CACATGAGCG'3
Ct FDH Val310Thr Reverse Primer	5' CGCTCATGTGCGG GGT CATAGCATTGCCACC'3

3.2.2.2. Site-Directed Mutagenesis

The wild-type CtFDH and CsFDH genes were subjected to site-directed mutagenesis according to the standard PCR method (98 °C for 30 seconds, 98 °C for 10 seconds, 65 °C for 20 seconds, 72 °C for 2.5 minutes, 72 °C for 7 minutes for the final extension for one cycle) in the presence of 1 µL dNTPs mix, 2.5 µL primer mix, 0.5 µL Phusion High Fidelity DNA polymerase in a total 50 µL reaction.

3.2.2.3 *DpnI* Digestion of the PCR Products

After PCR was run, 10 μ L of PCR products were taken for agarose gel control. 1 μ L of *DpnI* restriction enzyme was added into the remaining 40 μ L PCR product and incubated at 37 °C for 2 hours.

3.2.2.4. Agarose Gel Electrophoresis

Agarose gel electrophoresis was applied to separate the targeted DNA fragments. A 1 % agarose gel was prepared, 0.5 g agarose was weighed on calibrated model ATX224 electronic balance and transferred to 250 ml Erlenmeyer flask containing 50 ml TBE buffer. Then the mixture was heated on hot plate until the agarose was completely dissolved (but not overboiled). 2 μ L of SERVA staining was added to the agarose solution after allowing it to be cooled down to 50 °C (about when hands can comfortably be kept on the flask). Then the agarose was poured into a gel tray with the well comb and waited for 20-30 mins at room temperature until it was completely solidified. The solidified agarose gel was placed into the gel box and subsequently, the box was filled with 10x TAE until the buffer surpassed gel. A molecular weight ladder (2-Log DNA Ladder) was added into the first lane of the gel. The samples mixed with loading dye in 5:1 ratio were loaded into the additional wells of the gel.

Finally, the gel was electrophoresed at 90 V until the dye line was approximately 75-80% of the way down the gel (was about 1.5 hours). Thereafter the gel was carefully removed from the gel box and visualized using LED transilluminator for its DNA fragments (bands).

3.2.2.5. Transformation of *CtFDH* and *CsFDH* to *E. coli*, Purifying Their Plasmids and Expression

Following observations of band on the agarose gel, both the PCR products and wild types were transformed into NEB® 5-alpha Competent *E. coli* cells using the New England Biolabs High Efficiency Transformation Protocol.

The plasmids were isolated from the colonies using MN Nucleospin Plasmids Kit after inoculating a colony into ampicillin contained LB medium and overnight growth at 37 °C and 180 rpm. Finally, they were sent for sequencing.

The confirmed plasmids of were transformed into BL21 using the aforementioned transformation protocol. Discrete colonies were picked from LB-agar plates supplemented with 100 µg/mL ampicillin, inoculated into 5 mL of LB medium containing ampicillin (100 µg/mL) and incubated at 37 °C at 225 rpm for overnight.

4 mL of the overnight cultures were cultivated in a larger autoinduction studier medium (400 mL) which were incubated at 30 °C at 225 rpm for 16 hours. After that, the cells were centrifuged at 4000 rpm for 15 minutes for harvesting.

3.2.2.6. Purification of CtFDH and CsFDH Wild type and Mutants with Affinity Chromatography

The obtained pellets were incubated on ice for 30 min after their suspension in Buffer 1 (20 mM NaPi, 500 mM NaCl, 30 mM imidazole pH 7.4, 1.0 mg/mL lysozyme). Then they were subjected to sonication for their cell wall break. The solution was clarified by centrifugation (16000 rpm, 20 min). The lysate was filtered through a 0.45 µm filter then applied to a HisTrap column which was pre-equilibrated with the Buffer 1. The column was washed with 5 mL of Buffer 1 and the CtFDH and CsFDH were eluted with Buffer 1 containing different imidazole concentrations (50-250 mM).

SDS-PAGE was run for all the fractions to identify the fractions that contain our interest protein. Then those fractions were combined and diluted to 50 mL by addition of 100 mM of NaPi pH 7.4. Proteins were concentrated with Amicon Millipore centrifugal concentrators. The concentration of the purified CtFDH and CsFDHs were determined by BCA Assay Kit. The expression efficiency was monitored by SDS-PAGE.

3.2.2.7. SDS-PAGE Analysis

A mixture of 15 µL sample and 5 µL 4X sample loading buffer were heated at 95 °C for 10 minutes then centrifuged at 13500 rpm for 10 minutes.

The SDS gel was placed in to SDS-PAGE tanker that was filled with its running buffer. 5 μ L protein standard was loaded into the first well and 5 μ L of mixtures were loaded into the next wells. Finally, SDS-PAGE was run at 80 V for 10 minutes firstly and then it was at 100 V for 1 hour.

3.2.2.8. Enzyme Activity Assay

The FDH activity was determined by monitoring the absorbance changes at 340 nm during the redox reactions catalyzed by the FDHs at 25 °C. The activity of wild type and mutants of *CsFDH* and *CtFDH* were measured against 40 mM sodium formate and 20 mM sodium bicarbonate in 100 mM NaAce buffer at pH 5 and 100 mM NaPi pH 8 containing 2 mM NAD^+ , 0.5 NADH respectively. For both, oxidation and reduction, one unit was equal to the amount of pure enzyme required to consume 1 μ M NADH/ NAD^+ per minute by monitoring the change in absorbance at 340 nm. All experiments were performed in triplicates.

3.2.2.9. Effect of pH

The relative activity of wild type and all the mutant FDHs were measured in different 100 mM buffers for different pH ranges at 25 °C in a solution containing 10 mM formic acid buffer and 1.0 mM NAD^+ . The selected 100 mM buffers were citrate for pH 3.0–6.0, NaPi for pH 6.0–7.5, Tris–HCl for pH 7.5–9.0, Glycine for pH 9.0–10.0, sodium carbonate pH 9.5–11.0 and KCl–NaOH pH 11.0–13.0.

3.2.3.10. Effect of Temperature

The thermostability of enzymes was measured by incubating the purified FDHs for 15 min at temperatures from 25 °C to 65 °C. The residual activity of the incubated samples was measured in specific activity assays at 24 °C. The specific activity of FDHs was measured in optimum 100 mM buffers containing 20 mM sodium formate and 1.0 mM NAD^+ for formate oxidation or 20 mM sodium bicarbonate and 0.5 mM NADH for CO_2 reduction.

3.2.2.11. Enzyme Kinetic Assays

Kinetic parameters of wild type and all the mutant FDHs were determined through monitoring the change in absorbance at 340 nm due to NADH formation or consumption in 100 mM of optimum buffers for each FDH. The reaction mixtures contained sodium formate and 1.0 mM NAD^+ for formate oxidation and sodium bicarbonate and 0.5 mM NADH for CO_2 reduction. In the case of the CO_2 reduction reaction, NaHCO_3 was used as a substrate to supply CO_2 because the concentration of gaseous CO_2 cannot be accurately determined. Therefore, the substrate concentration for CO_2 reduction was represented as that of NaHCO_3 . The substrate concentration ranges for the different assays were 0.5–50 mM for formate, 0.5–40 mM for sodium bicarbonate. All reactions were performed at 25 °C. The Michaelis–Menten kinetic constants were determined from triplicate measurements of the initial velocities using GraphPad Prism software.

4. RESULTS AND DISCUSSION

4.1. Post-PCR Control of Site-Directed Mutagenesis

Mutations were applied on the pET45b+ vector containing the *ctfdh* and pET-23b(+) vector containing *csfdh* genes using the designed degenerate primers. Asn312, Val313 and Val331 positions are converted to Phe, Pro and Thr on CsFDH gene, meanwhile Val310 position is converted to Thr position on CtFDH gene. Obtained PCR products of both CsFDH and CtFDH genes are visualized on agarose gel electrophoresis. According to the gel images obtained, the expected PCR product bands are shown in Figure 4.1 and 4.2, with the pET-45b(+) vector containing the mutant *ctfdh* gene of approximately 4500 kb and the vector containing the wildtype and mutant *csfdh* gene of approximately 4100 kb size.

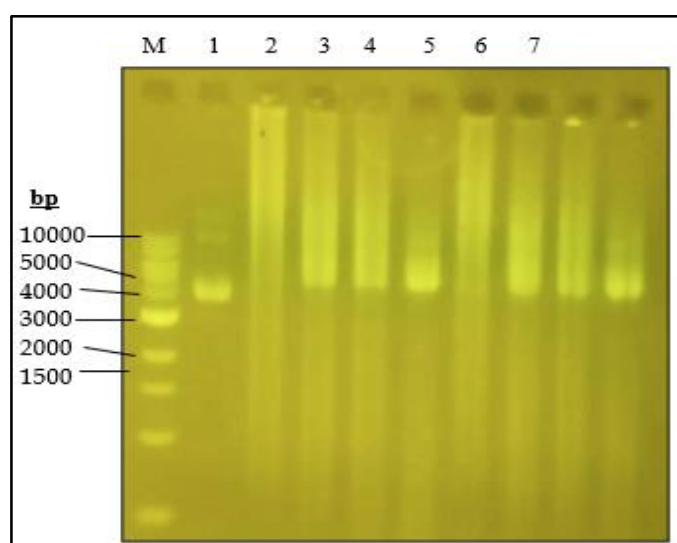


Figure 4.1: PCR results analysis on agarose gel electrophoresis. M: Marker, 1: wild type CsFDH plasmid, 2: CsFDH Asn312Phe after PCR, 3: CsFDH Val313Pro after PCR, 4: CsFDH Val331Thr after PCR, 5: CsFDH Asn312Phe after *DpnI*, 6: CsFDH Val313Pro after *DpnI*, 7: CsFDH Val331Thr after *DpnI*.

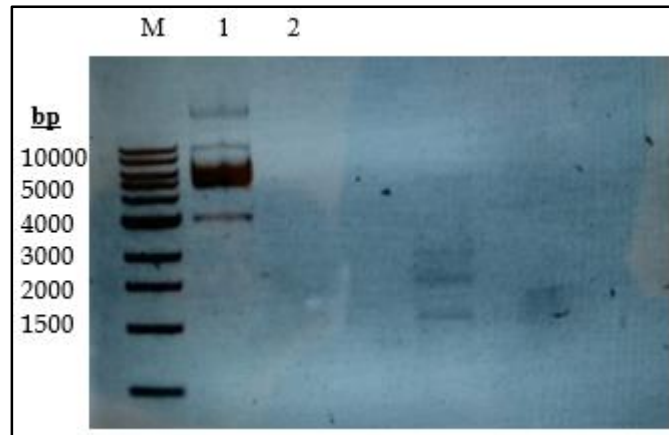


Figure 4.2: PCR results analysis on agarose gel electrophoresis of CtFDH Val310Thr
M: Marker, 1: wild type CtFDH plasmid, 2: CtFDH Val310Thr after *DpnI*.

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

Transformation of PCR products into *E.coli* DH5 α strain is successfully performed. Bacterial colonies on LB-Agar containing ampicillin (100 ug/mL) can be seen in Figure 4.3.

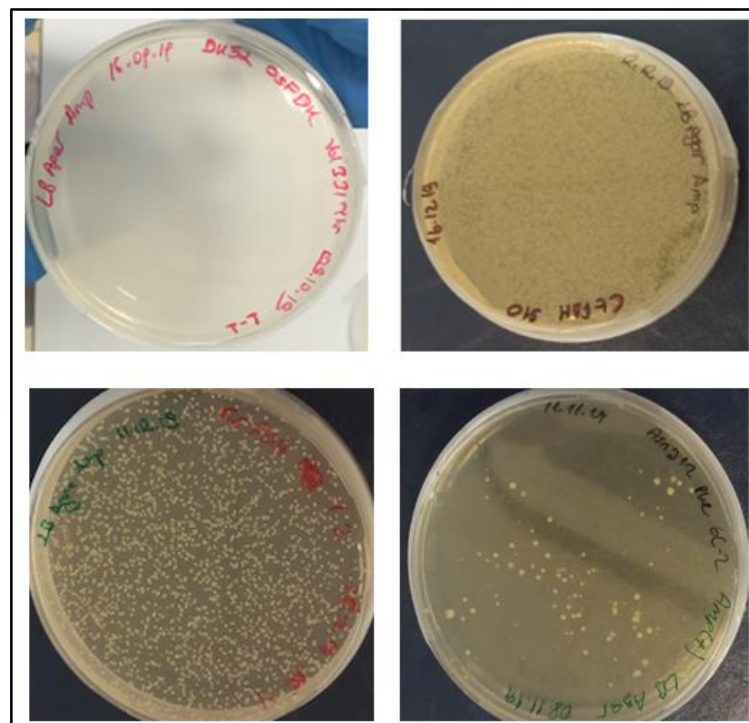


Figure 4.3: Transformation plates of Cs and CtFDHs. Top left is stand for Cs FDH Val331Thr, top right for Ct FDH Val310Thr, bottom left is for Cs FDH Val313Pro and bottom right for Cs FDH Asn312Phe.

4.3. DNA Sequence Analysis Results of Mutations

The plasmids containing *ctfdh* and *csfdh* genes are purified as described on Section 3.2.2.5. and they were sent for DNA sequence analysis. The analysis results aligned with their wild-types via BioEdit Sequence Alignment Editor program. CsFDH Val331Thr, CsFDH Asn312Phe, CsFDH Val313Pro, CtFDH Val310Thr mutations have been identified. Aligned analysis results can be seen in Figure 4.4.

CsFDH wildtype	G	C	A	G	G	C	G	A	T	G	T	G	T	G	G	A	A	T	G	T	T	C	A	G	C	C	G	G	C	G	
Asn312Phe	G	C	A	G	G	C	G	A	T	G	T	G	T	G	G	T	T	T	G	T	T	C	A	G	C	C	G	G	C	G	
CsFDH wildtype	G	G	C	G	A	T	G	T	G	T	G	G	A	A	T	G	G	T	C	A	G	C	C	G	G	C	G	C	G	C	
Val313Pro	G	G	C	G	A	T	G	T	G	T	G	G	A	A	T	C	C	G	C	A	G	C	C	G	G	C	G	C	G	C	
CsFDH wildtype	G	T	G	G	C	A	A	T	G	G	T	A	T	G	G	T	T	C	C	G	C	A	C	T	A	T	A	G	C	G	
Val331Thr	G	T	G	G	C	A	A	T	G	G	T	A	T	G	A	C	C	C	C	G	C	A	C	T	A	T	A	G	C	G	
CtFDH wildtype	G	G	T	G	G	C	A	A	T	G	C	T	A	T	G	G	T	G	C	C	G	C	A	C	A	T	G	A	G	C	G
Val310Thr	G	G	T	G	G	C	A	A	T	G	C	T	A	T	G	A	C	C	C	C	G	C	A	C	A	T	G	A	G	C	G

Figure 4.4: Sequence analysis results of wild-type and mutant CsFDHs.

4.4. Results of Mutant Ct and CsFDH Protein Expression

pET-45b(+) vectors containing mutant *csfdh* and *ctfdh* genes were transformed into *E. coli* BL21 (DE3) cells. Protein expression and isolations are performed within given procedure in Section 3.2.2.5 and 3.2.2.6. The SDS-PAGE gel image obtained by loading the collected fractions at the end of purification is shown in Figure 4.5. Figure 4.5 shows that CsFDH is ~41 kDa and CtFDH is ~45 kDa in size and pure in fractions were obtained for kinetic measurements.

Site-directed mutagenesis was used to construct mutations at selected positions in the CtFDH and CsFDH genes. The desired mutations were confirmed by DNA sequencing. FDH protein variants containing the N-terminal 6xHis-tag were isolated by Ni-NTA HisTrap column chromatography. The fractions were analysed with SDS-PAGE (Figure 4.5) and pure fractions were collected. Wild-type and the mutant enzymes were obtained with high yield.

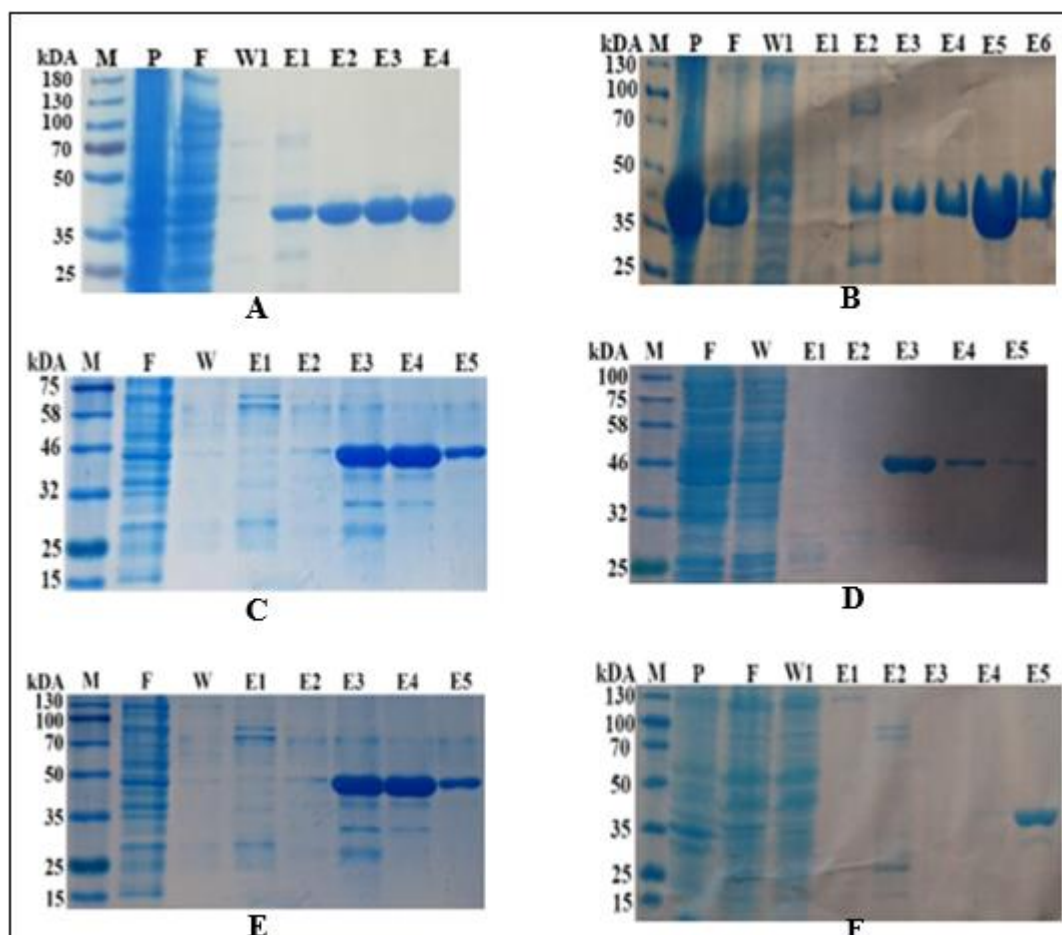


Figure 4.5: SDS-PAGE analysis of purified FDHs. (A) CsFDH wild-type; (B) CtFDH wild-type; (C) CsFDH-Val313Pro; (D) CtFDH-Val310Thr; (E) CsFDH-Asn312Phe; (F) CsFDH Val331Thr. Lanes: M, marker; P, cell pellet; F, flow through; W, column washing solution; E, eluted fractions.

4.5. Effect of pH on *Ct* and *CsFDH* Enzyme Activity

The optimum pH rates of CsFDH, CtFDH, and their mutants for formate oxidation and CO₂ reduction are shown in Table 4.1. The measurements show that the optimum pH for formate oxidation is 6.5 for the wildtype, Asn312Phe and Val313Pro mutants, whereas 6.0 is the pH optimum for the Val331Thr mutant of CsFDH. The optimum pH for formate oxidation is 7.0 for the wild-type CtFDH, whereas the optimum pH is 5.0 for Val310Thr mutant.

The optimum pH of activity towards HCO₃⁻ measured as 6.0 for the wild-type CsFDH, 7.0 for Asn312Phe, and 6.5 for Val313Pro mutants. Even though the wild-type and mutants show similar patterns, the Asn312Phe mutant has lower CO₂

reduction activity than the wild-type. The optimum pH towards HCO_3^- is 8.0 for the wild-type *CtFDH*.

The optimum pH rates of *CsFDH*, *CtFDH*, and their mutants for formate oxidation and CO_2 reduction are shown in Table 4.1. The measurements show that the optimum pH for formate oxidation is 6.5 for the wild-type, Asn312Phe and Val313Pro mutants, whereas 6.0 is the pH optimum for the Val331Thr mutant of *CsFDH*. The Val331Thr mutant showed higher affinity towards formate than the wild-type *CsFDH* between pH 6.0–10.0. Although they show similar patterns between pH 6.0 and 10.0, the formate oxidation activity of the mutant is higher than the wild type in acidic pH (4.5, 5.0). On the other hand, no significant differences in pH optimum were found for the *CsFDH*-Val313Pro and *CsFDH*-Asn312Phe mutants. whereas for its mutant, Val310Thr, is 5.0.

The measurements show that the optimum pH of activity towards HCO_3^- is 6.0 for the wild-type *CsFDH*, 7.0 for Asn312Phe, and 6.5 for Val313Pro mutants. Even though the wild-type and mutants show similar patterns, the Asn312Phe mutant has lower CO_2 reduction activity than the wild-type. The optimum pH towards HCO_3^- is 8.0 for the wild-type *CtFDH*, whereas its mutant did not show activity at all.

4.6. Effect of Temperature on *Ct* and *CsFDH* Enzyme Activity

The relative thermostability of the enzymes was calculated by the ratio of initial rate constants of wild-type and the mutants (Table 4.1). Rate constants of wild-type enzymes were measured at 55 °C in forward reaction (formate oxidation) and 40 °C against HCO_3^- for the *CtFDH* wild-type, while it was measured at 45 °C in forward reaction and 30 °C against HCO_3^- for the *CsFDH* wild-type. In the formate-direction reaction, the *CtFDH*-Val310Thr mutant is more stabilized than the wild-type at 55 °C. At 35 °C, *CsFDH*-Asn312Phe mutants were more stable in formate oxidation than the wild-type, but drastically lost stability in CO_2 reduction. The Val313Pro mutant was also more stabilized than its wild-type at 35 °C against HCO_3^- .

Table 4.1. Relative thermostability of CsFDH and CtFDH mutants against formate and hydrogen carbonate.

Temperature (°C)	Substrates					
	Formate				Hydrogen carbonate	
	Cs N312F	Cs V313P	Cs V331T	CtVal310Thr	Cs N312F	Cs V313P
25	1.8	6.2	1.5	4.6	10.3	2.2
30	1.0	6.1	1.5	5.8	2.4	1.0
35	0.9	5.8	1.3	4.6	4.6	0.8
40	2.7	5.3	1.1	4.0	5.5	1.7
45	28.5	4.6	1.2	4.6	11.9	1.9
50	211	4.1	3.8	2.7	10.7	2.1
55	0	24.8	19.2	0.3	30.8	5.1
60	0	46.9	97.4	2.9	0	0
65	0	0	0	2.9	0	0
- for CsFDH, the initial rate constants that used for thermal stability calculation are wild type enzyme measured at 45 °C for formate oxidation and at 30 °C for CO₂ reduction. - for CtFDH, The initial rate constants that used for thermal stability calculation are wild type enzyme measured at 55 °C against formate and at 40 °C for CO₂ reduction.						

4.7. Results of Kinetic Analysis for Wild Type and Mutant *Ct* and *Cs*FDHs

Site-directed mutagenesis at non-conserved amino acid positions in the active site was used to explore the functional properties of these three positions in *Cs*FDH and *Ct*FDH. The kinetic analysis took place at room temperature as described in Section 3.2.3.11 and Michaelis-Menten kinetic values were determined. *Cs*FDH Val331Thr and *Ct*FDH Val310Thr lost their interest against CO₂, meanwhile *Cs*FDH Val313Pro shows significantly high interest against CO₂.

Michaelis-Menten graphs for formate and bicarbonate wild-type and mutants *Cs* and *Ct*FDH are shown in Figures 4.6. and 4.7. K_M and k_{cat} values for the wild-type and mutant *Cs* and *Ct*FDHs are shown in Table 4.1.

The *Cs*FDH Val331Thr mutation had higher K_M and k_{cat} values, but low substrate affinity prevented an increase in catalytic efficiency (k_{cat}/K_M decreased from 0.107 to 0.079 s/mM). The same mutation on *Ct*FDH, which is Val310Thr, had higher K_M and lower k_{cat} values, this mutation caused slightly decreasing in catalytic efficiency. Though these two mutations did not show activity at all in the CO₂ reduction reaction and *Cs*FDH-Val331Thr had the lowest catalytic efficiency in oxidizing formate, but the maximum speed of its catalysed reaction can be reached with smaller amounts of formate than the others. A different mutation on the same position which is Val310Asn *Ct*FDH mutation resulted in decreased k_{cat} and K_M values but was followed by a low catalytic efficiency due to a drastic decrease in the k_{cat} value.

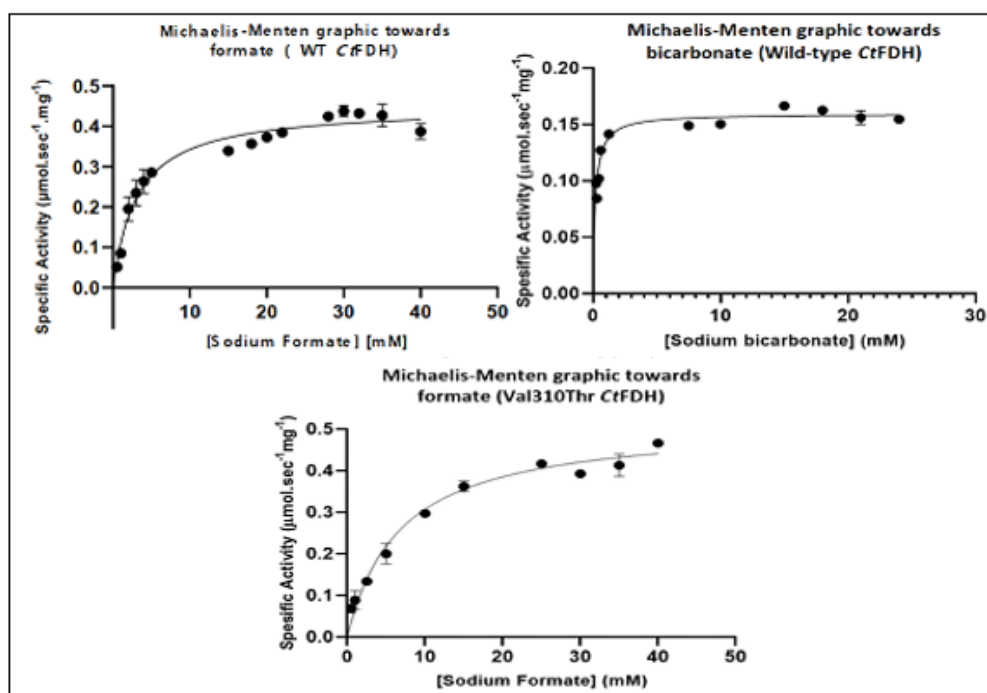


Figure 4.6: Michaelis-Menten plots for determination of kinetic parameters of CtFDH.

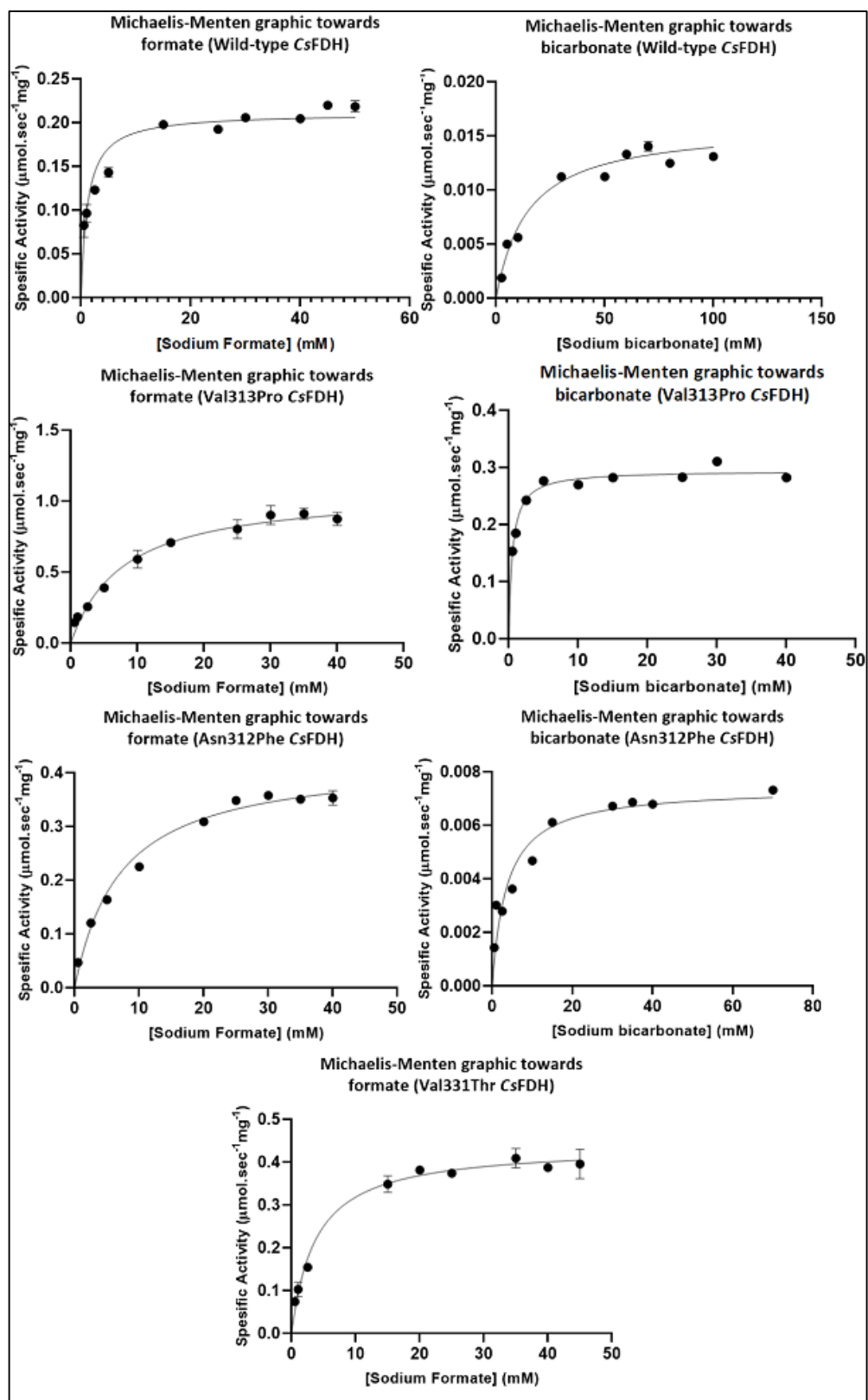


Figure 4.7: Michaelis-Menten plots for determination of kinetic parameters of CsFDH.

Table 4.2: The kinetic constants of Cs and CtFDH for hydrogen carbonate and formate.

		Substrate							
		Formate				Hydrogen carbonate			
		Optimum pH	k_{cat} (sec ⁻¹)	K_{M} (mM)	$\frac{k_{\text{cat}}}{K_{\text{M}}}$ (sec ⁻¹ mM ⁻¹)	Optimum pH	k_{cat} (sec ⁻¹)	K_{M} (mM)	$\frac{k_{\text{cat}}}{K_{\text{M}}}$ (sec ⁻¹ mM ⁻¹)
CtFDH	Wild-Type	7.0	1.5 ± 0.001	3.22 ± 0.460	0.465 ± 0.002	8.0	0.023 ± 0.000	0.32 ± 0.070	0.0690
	Val310Thr	5.0	0.387 ± 0.028	6.80 ± 1.90	0.057 ± 0.014	No activity			
CsFDH	Wild-Type	6.5	0.554 ± 0.008	5.20 ± 0.630	0.107 ± 0.007	6.0	0.011 ± 0.001	14.46 ± 6.97	0.001 ± 0.000
	Asn312Phe	6.5	0.254 ± 0.007	4.126 ± 0.453	0.062 ± 0.015	7.0	0.005 ± 0.001	3.64 ± 2.87	0.001 ± 0.000
	Val313Pro	6.5	0.736 ± 0.059	7.794 ± 2.18	0.094 ± 0.027	6.5	0.201 ± 0.008	0.51 ± 0.16	0.394 ± 0.05
	Val331Thr	6.0	0.292 ± 0.015	3.776 ± 0.881	0.079 ± 0.017	No activity			

4.8. Molecular Modelling

CsFDH and CtFDH enzymes are modelled for further understanding on the positions on substrate binding. Figure 4.8 and 4.9 highlight the substrate-binding pockets of CsFDH and CtFDH which are built by residues Pro97, Phe98, Thr120, Gly124, Asn146, Val150, Arg284, Val309, His332, and Gly335.

Formate and hydrogen carbonate substrates were bound in the active site of CsFDH with H-bonds with Val93, Asn117, Arg256, His309 and Gly312 (Figure 4.8). According to the crystal structure (formate) and docking information (hydrogen carbonate) of CtFDH, the substrates were bound in the active site of CtFDH with H-bonds with Ile94, Asn120, Arg259, His312, and Gly315 (Figure 4.9) [9]. Figure 4.8 and 4.9 show that CsFDH Asn312, Val313, Val331 and CtFDH Val310 positions don't have direct interaction with substrates. CsFDH Val313, Val331 and CtFDH Val310 positions affect the orientation of His309 (His312 for CtFDH) by van der Waals interactions.

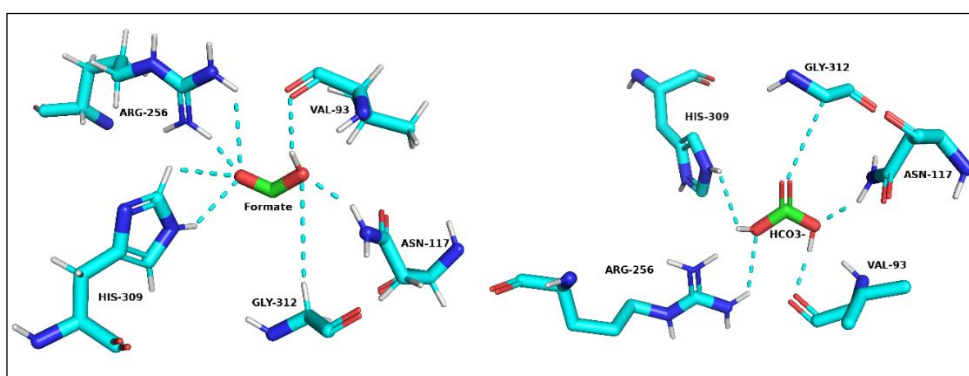


Figure 4.8: CsFDH active site residues involved in the substrate-binding for formate oxidation and CO₂ reduction reactions.

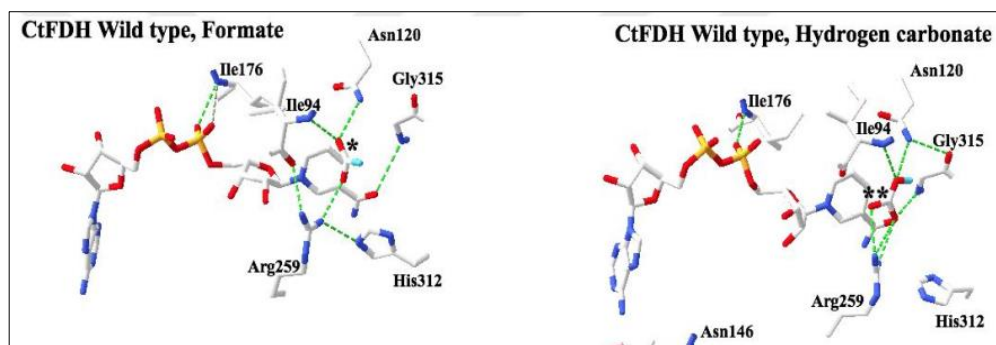


Figure 4.9: CtFDH active site residues involved in the substrate-binding for formate oxidation and CO₂ reduction reactions. *Formate. **Hydrogen carbonate.

Although the CsFDH-Asn312Phe mutation slightly increased the substrate affinity for formate, the catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of this mutant decreased to some degree, based on lowered k_{cat} values (Table 4.2).

The CsFDH-Val313Pro mutant showed decreased substrate affinity to formate (K_{M} : 5.2 to 7.8 mM), whereas k_{cat} was slightly increased. It had comparable efficiency to the wild type in formate oxidation, while CO₂ reduction was four times as efficient as both the wild type and CsFDH-Asn312Phe. It also had more affinity towards hydrogen carbonate than the two other enzymes.

The mutations Phe311Asn in *Pse*FDH and Phe290Asn in *Soy*FDH similarly showed a decrease in catalytic efficiency due to their loss of affinity against formate [60,61]. The mutations studied on *Pse*FDH-Phe311 by Tishkov *et al.* revealed the formation of additional H-bonds. *Pse*FDH wild-type forms only one H-bond in between Phe311 residue and nitrogen of Arg290 residue. However, this bond is also present in the replacement of Phe311 residue by Ser or Thr but disappears in mutants *Pse*FDH-Phe311Asn. The same mutation on *Soy*FDH studied by Alekseeva *et al.* and Tishkov *et al.* [60,61] resulted in increased K_{M} (formate). The reason for this is that Phe is a hydrophobic residue and contains a benzene ring, while Asn is an uncharged amino acid. It is thought to be that the Phe residue on the CsFDH-Asn312Phe mutant may cause a slight change in the folding of the protein, causing new H-bonds to form, positively affecting the substrate affinity.

5. CONCLUSION

In conclusion, we have examined the functional effects of site mutations on the activity of *Ct*FDH and *Cs*FDH. These effects were clarified by pH and thermostability assay, measuring kinetics parameters (K_M , k_{cat}), and molecular modelling. Obtained results were compared with structurally equivalent positions which have been studied in the literature. The present study indicates that the effect of mutations can cause small structural changes which create significant differences. Previous studies have shown that alanine mutation in the active site of *Ct*FDH has a similar effect. Changing to a less-bulky amino acid disturbs the H bonding of the substrate and weaker binding provides flexibility for substrate release, which causes an increase in the catalytic rate. The Proline mutation at the 313 position of *Cs*FDH shows very promising results on CO_2 reduction, even though this mutant is not directly involved in substrate binding. By this, it was understood that the orientation of the key residues with the amino acids having hydrophobic side chains is significant for the FDH catalysis. Results of this study enlighten the role of catalytic residues of *Cs*FDH and provide a further understanding of the catalytic reaction mechanism of FDHs. Besides, this mutant enzyme would increase the possibility of FDH using for CO_2 reduction. FDHs are considered as unfavourable for electrochemical reduction of CO_2 due to their low catalytic rate constants (k_{cat}) for CO_2 reduction. However, the obtained mutant in this study and further engineering of FDHs in the light of this study can be integrated into electrochemical CO_2 reduction systems. FDHs are dimeric enzymes and can be easily inactivated depending on the process conditions by separating their subunits. Since the enzyme stability can be improved by immobilization to enable enzymes to be applicable in industrial processes, immobilization of these enzymes can be beneficial for reducing CO_2 on an industrial scale [62-67]. Therefore, immobilized mutant enzyme might provide development of feasible and reusable CO_2 fixation processes.

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BIOGRAPHY

After completing Bachelor degree at Wolaita Sodo University in Chemistry, Yasin Adem joined Bahir Dar University to peruse his Msc. degree in Inorganic Chemistry, Bahir Dar, Ethiopia. He perused his PhD program at Gebze Technical University in Institute of Natural and Applied Sciences department of Chemistry, Gebze, Turkey.

APPENDICES

Appendix A: Publications related with the thesis

Koçdemir K., Şen F., Wadejo Y.A., Bilgici M.Ç., Bayram M., Selçuk İ., Yılmaz B., Çakar M.M., Aslan E.S., Binay B., (2020), “Investigation new positions for catalytic activity of *Chaetomium thermophilum* and *Ceriporiopsis subvermispora* Formate Dehydrogenases” Biocatalysis and Biotransformation, doi: 10.1080/10242422.2020.1863951

Şen F., Koçdemir K., Yılmaz B.A., Wedajo Y.A., Binay B., 2020, “Protein Engineering Based Catalytic Site Analysis of NAD⁺ Dependent Mutant *Ceriporiopsis subvermispora* Formate Dehydrogenase Enzyme” II. International Enzyme & Bioprocess Days, 01-03 September 2020 (Poster Presentation).

Koçdemir K., Şen F., Aktar B.Y., Wedajo Y.A., Binay B., 2020, “Investigation of the roles of V331 position of NAD⁺ dependent *Ceriporiopsis subvermispora* Formate dehydrogenase and V310 position of NAD⁺ dependent *Chaetomium thermophilum* Formate dehydrogenase enzyme on the reaction mechanism” II. International Enzyme & Bioprocess Days, 01-03 September 2020 (Poster Presentation).

Yasin Adem, Berin Yelmazer, Barış Binay, 2018, “Enlightening Catalytic Mechanism of *Ceriporiopsis Subvermispora* (CsFDH) With Site Directed Mutagenesis”, 1st Eurasia Biochemical Approaches & Technologies (EBAT) Congress, 27-30 October 2018 (Oral and Poster Presentation).