

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

**MOLECULAR CHARACTERIZATION OF NAD⁺-
DEPENDENT FORMATE DEHYDROGENASE FROM**
Clostridium ljungdahlii

MEHMET MERVAN ÇAKAR
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF CHEMISTRY

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THESIS SUPERVISOR
ASSIST. PROF. DR. BARIŞ BİNAY

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

NAD⁺-BAĞIMLI *Clostridium ljungdahlii*
FORMAT DEHİDROGENAZIN
MOLEKÜLER KARAKTERİZASYONU

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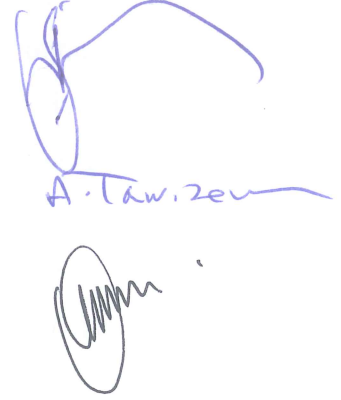
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ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

...../...../..... tarih ve/..... sayılı kararı.

SUMMARY

NAD⁺-dependent formate dehydrogenases (FDHs) are oxidoreductases catalysing the conversion of formate into CO₂. Therefore they have been extensively used for cofactor recycling in different chemoenzymatic processes. With the growing concern of rising atmospheric CO₂ levels, the importance of investigating all methods for its reduction is at an all-time high. One such possibility that has recently emerged is in the enzymatic conversion of CO₂ into a biologically useful carbon source. It has been recently discovered that the FDHs enzymes also have high activity in the reverse direction, the reduction of aqueous CO₂ to formate.

Therefore, the *fdh* gene from anaerobic *Clostridium ljungdahlii* (Cl) was cloned for the expression in *E. coli*. Experimental studies were done to determine the optimum conditions for overexpression. Pure enzyme was obtained by using the TAGzyme purification system and effect of metals on the activity of ClFDH was investigated according to the metal centers presence in the active site of the enzyme. Effect of pH on the activity of ClFDH was studied to determine the optimum pH range. The effect of temperature on the activity was detected to determine the thermostability of the enzyme. Purified enzyme was stored at different temperatures in order to determine the best storage condition. Kinetic analysis of ClFDH activity were done to detect enzyme's affinity. Structural analysis of the enzyme via homology modeling is also presented.

Biochemical and kinetic characterization of FDH from *Clostridium ljungdahlii* (ClFDH) has been shown to possess activity in the reverse reaction: the conversion of CO₂ into formate. The obtained results show that ClFDH is a good template to engineer the enzyme to increase the activity for CO₂ reduction.

Key Words: NAD(P)H-Dependent Formate Dehydrogenase, Metalloenzyme, Catalytic Mechanism, CO₂ Reduction, Cofactor Regeneration, Homology Modeling

ÖZET

NAD⁺-bağımlı format dehidrogenazlar, formatın CO₂'ye dönüşümünü katalizleyen NAD(P)H'ye bağımlı oksidoredüktazlardır. Bu nedenle, kemoenzimatik işlemlerde kofaktör geri dönüşümü için yaygın şekilde kullanılırlar. Atmosferik karbondioksit seviyelerinin gittikçe artmasının yarattığı kaygı nedeniyle bu seviyenin azaltılması için yöntemlerin geliştirilmesine yönelik araştırmaların önemi yüksektir. Son zamanlarda ortaya çıkmış olan olasılıklardan biri, CO₂'nin biyolojik açıdan faydalı karbon kaynaklarına enzimatik olarak dönüştürülmesidir. Bununla birlikte, yakın zamanda FDH enziminin ters yönde CO₂'nin formata indirgenmesi için yüksek aktiviteye sahip olduğu keşfedilmiştir.

Bu nedenle, bu çalışmada, aneorobik *Clostridium ljungdahlii* organizmasından *fdh* geni, *E. coli*'de ifade edilmek için klonlanmıştır. Proteinin yüksek miktarlarda ifadesini sağlayabilmek adına optimizasyon çalışmaları yapılmıştır ve optimum koşullar belirlenmiştir. Saf enzim, TAGzyme saflaştırma sistemi kullanılarak elde edilmiştir. Metal bağımlı FDH'ler aktif bölgelerinde metal kümelerine sahip olabildikleri için, metallerin, C/FDH aktivitesi üzerindeki etkisi araştırılmıştır. Optimum pH aralığının belirlenmesinin ardından, C/FDH'nin termostabilitesini saptamak amacıyla sıcaklığın C/DH aktivitesi üzerine etkisi araştırılmıştır. Saflaştırılmış enzimin farklı sıcaklıklarda depolanmasının ardından aktivite ölçümleri yapılarak en iyi saklama koşulu ve kinetik analizlerle enzimin substratlarına karşı ilgisi belirlenmiştir.. Ayrıca, homoloji modellemesi çalışması ile enzimin yapısal analizi de sunulmuştur.

C/FDH'nin biyokimyasal ve kinetik karakterizasyonu, enzimin ters yöndeki reaksiyonda, CO₂'nin formata çevriminde de etkinliğe sahip olduğu göstermiştir. Biyoinformatik çalışmalar, C/FDH'nin CO₂ indirgenmesinde iyi bir şablon olarak kullanılabileceğini göstermiştir.

Anahtar Kelimeler: NAD(P)H-Bağımlı Format Dehidrogenaz, Metalloenzim, Katalitik Mekanizma, CO₂ İndirgemesi, Kofaktör Yenilenmesi, Homoloji 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
OD	: Optical Density
PCR	: Polymerase Chain Reaction
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC	: Super Optimal Broth with Catabolite Repression
SeCys	: Selenocysteine
Ser	: Serine
W	: Tungsten
kDa	: Kilodalton
mM	: Millimolar
mg	: Milligram
min	: Minute
sec	: Second

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1. INTRODUCTION

1.1. Enzymes

Enzymes are macromolecules of amino acids with an ability to catalyse particular chemical reactions and responsible in the metabolic pathways in species. Enzymes increase the biochemical reactions rate by decreasing the activation energy. Catalytic activities of enzymes depend on their essential protein conformation. There are many factors that affect the activity of enzymes, and thus the specificity and velocity of the reactions they catalyze such as temperature, pH, substrate concentration, enzyme inhibitors etc.

All enzymes apart from a few catalytic RNA molecules are proteins. The molecules that the enzymes act on are called substrates and enzymes transform substrates into different molecules called products. Important metabolic activities such as construction and destruction of the organic substances, digestion, muscle contraction, cell respiration in cells are the result of various reactions and these reactions are possible by the catalytic effect of enzymes.

Some enzymes can work alone without requiring any other molecule, while others require molecules called cofactors or coenzymes. A holoenzyme is the enzyme which is catalytically active when it is bounded to a cofactor or coenzyme.

1.2. Enzyme Classification

The Enzyme Commission number is a numerical classification scheme based on chemical reactions catalyzed by enzymes. Enzyme code numbers consist of four digits separated by dots and the first number indicates which of the six main enzyme groups the enzyme has entered, the second number indicates the chemical structure or functional group the enzyme affects, the third digit indicates the acceptor, and the fourth digit indicates the order number the enzyme has received in a certain class.

- Oxidoreductases (EC 1.X.X.X)

An oxidoreductase is an enzyme that catalyzes the transfer of electrons from one molecule, the oxidant; to another, the reductant.

- Transferases (EC 2.X.X.X)

A transferase is an enzyme that transfer specific functional groups from one molecule to another.

- Hydrolases (EC 3.X.X.X)

A hydrolase is an enzyme that catalyzes the hydrolysis of a chemical bond or the transfer of a functional group to the water.

- Lyases (EC 4.X.X.X)

A lyase is an enzyme that catalyzes the separation of a group from a substrate by a route other than hydrolysis or oxidation.

- Isomerases (EC 5.X.X.X)

An isomerase is an enzyme that convert a substrate from one isomer to another.

- Ligases (EC 6.X.X.X)

A ligase is an enzyme that catalyze the incorporation of two large molecules by formation of the bonds between C, O, S and N.

2. FORMATE DEHYDROGENASE

Formate dehydrogenase is a member of oxidoreductase class of enzymes. NAD^+ -dependent formate dehydrogenase is a member of the superfamily of D-specific 2-hydroxy acid dehydrogenases and catalyse the oxidation of formate anion to CO_2 while reducing the NAD^+ to NADH . Formate dehydrogenase was firstly found in a plant *Phaseolus vulgaris* at 1921. The first study of FDH was done by Davidson for the formation of NADH by ethanol, succinate and glutamate [1].

The popularity of the enzyme has increased in the 1970s to provide cofactor recycling during the reaction to synthesize valuable chiral compounds. FDH is widely used for NAD(P)H regeneration in the synthesis of optically active compounds by enzymatic methods. The reaction catalyzed by formate dehydrogenase can be seen in Figure 2.1 [2].

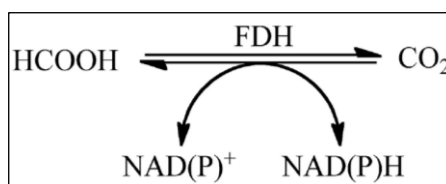


Figure 2.1: The reaction catalyzed by FDH.

2.1. General Properties of FDH

FDHs belong to D-specific 2-hydroxy acid dehydrogenases superfamily, contains differences in structure and substrate affinity from other enzymes involved in this family. Because the reaction catalyzed by the FDH enzyme is simple and single-step relative to other dehydrogenases. The FDH enzyme is common in industrial and scientific use. Since the format is a cheap substrate and it is possible to easily remove CO_2 from the reaction in gaseous form as a product, the use of FDH for the easy and cheap regeneration of NADH become attractive [3].

FDHs divided into two major classes. The first group is NAD^+ -dependent FDHs which are metal-independent FDHs and use NAD^+ molecule as a cofactor. The second group is Metal-dependent FDHs which are using a metal ion and NAD^+ as cofactor together [4].

2.2. Structural Properties of FDH

NAD⁺-dependent FDHs are highly specific for both formate and NAD⁺ contains two identical subunits which range from 70 kDa to 100 kDa [5].

Comparisons between the FDHs from different organisms suggest that FDHs have strong similarities between the amino acid sequences. Especially the amino acid residues where the substrate and coenzyme bound and the catalytic activity occur are highly conserved in evolutionary period. Popov [6] has shown that the residues Phenylalanine 98, Isoleucine 122, Asparagine 146, Alanine/Glycine 198, Glycine 200, Glycine 203, Arginine 284, Glutamine 313, and Histidine 332 are catalytically important and can be seen in Figure 2.2 which is created by BIOVIA Discovery Studio.

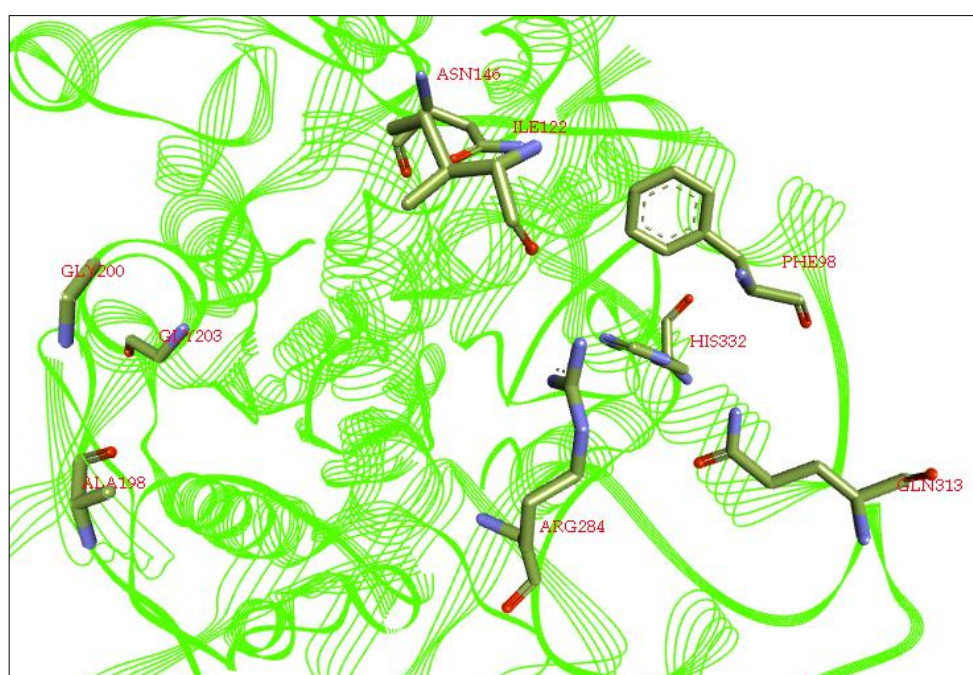


Figure 2.2: Catalytically important residues in the active site of *PseFDH*.

2.3. Catalytic Properties of FDH

NAD⁺-dependent formate dehydrogenase enzyme shows a Bi-Bi reaction which formate and NAD⁺ are used as substrate. Affinity to one substrate can be increased 3.5-fold by binding of the other substrate. Catalytic properties of FDHs from different organisms are shown in Table 1.1 [6–8].

Table 2.1: Kinetic features of FDHs from different species.

Source	Specific Activity-Formate (U/mg)	Optimum pH (Formate)	Specific Activity-Carbon Dioxide (U/mg)	Optimum pH (Carbon Dioxide)
Bacteria				
<i>Paracoccus</i> sp. 12-A	12.2	5.5	6.5	5.5
<i>Thiobacillus</i> sp. KNK65MA	10.9	6.5	12.2	5.5
<i>Moraxella</i> sp. C-1	14.3	5.5	2.8	5.5
<i>Methylomonas methylica</i> (x32)	3.1	7.7-8.	-	-
<i>Methylomonas extorquens</i> AM1 (x3)	0.42	8.4	-	-
<i>Pseudomonas</i> sp. 101	16.0	6-9	-	-
<i>Morexella</i> sp. C-1	6.0	6.0-9.0	-	-
<i>Paracoccus</i> sp. 12-A	11.6	6.5-7.5	-	-
<i>Ancylobacter aquaticus</i>	21.6	6	4.5	6.0
<i>Mycobacterium vaccae</i> 10	6.0	6-9	-	-
Yeast				
<i>Candida boidinii</i>	6.1	7	1.6	5.5
<i>Chaetomium thermophilum</i>	3.1	5	34.6	5
<i>Ceriporiopsis subvermispota</i>	1.3	6.5	0.8	6
<i>Candida methylica</i>	13.2	8	4.7	8
<i>Candida methanolica</i>	7.5	6.5-9.5	-	-
<i>Kloeckera</i> sp. 2201	0.14	7-8	-	-
<i>Pichia pastoris</i> NRRL-Y-7556	8.2	6.5-7.5	-	-
<i>Pichia pastoris</i> IFP 206	2.8	7.5	-	-
<i>Hansenula polymorpha</i> (x16)	2.8	7	-	-

FDHs optimum pH range is between 6.0-9.0 and show 50% lower activity at temperatures higher than 50 °C. FDH is able to catalyse at wide pH ranges but has sensitivity to high temperatures. Most of the NAD⁺-dependent FDHs are highly specific to NAD⁺ and do not use NAD(P)⁺ as a coenzyme. Besides, *Pseudomonas* has a potential to give reaction with both NAD⁺ and NADP⁺ by possessing two times more coenzyme specificity [9].

2.4. Metal-Dependent FDHs

NADH-dependent FDHs contain metal ion cofactors such as molybdenum (Mo) and oxidative-prone catalytic components such as iron-sulfur groups and selenocysteine [10–13]. The Mo-dependent FDH class comprises only prokaryotic enzymes that hold these different redox-active centers and whose active site accommodates a Mo atom that mediates the formate oxidation. The FDHs which are metal dependent are limited and only a few Mo-FDH have been reported to date [14–17].

All known metal-dependent FDHs active site is well conserved. The active of oxidized FDH harbors 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 2.3 [4].

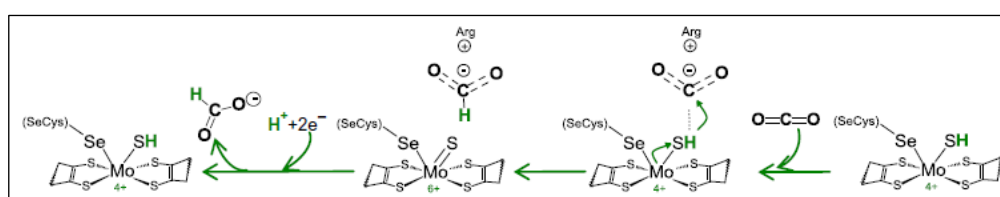


Figure 2.3: The hydride transfer mechanism proposed for FDHs for carbon dioxide reduction.

2.5. Practical Applications of FDH

2.5.1. Cofactor Regeneration with FDHs

Oxidoreductases are highly stereospecific in hydride ion transfer between substrate and coenzyme. The use of dehydrogenases in cofactor regeneration reduces the cost of cofactors such as NAD(P)^+ and NAD(P)H . A coupled reaction between FDH and ADH in order to maintain NAD(H) can be seen in Figure 2.4.

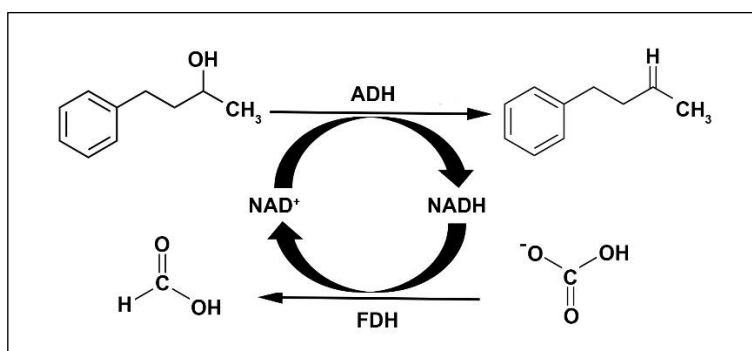


Figure 2.4: NAD(P)H regeneration system.

FDH has many advantages in the application of NAD(P)H regeneration process, which can be listed as follows:

- The product of the FDH reaction, CO_2 , can be easily eliminated since formate is a cheap substrate.
- Working in a wide range of pH is possible.
- The reaction is irreversible and shifting the equilibrium of the main reaction by regeneration results in 99% yield.
- FDHs from bacteria and yeast are able to be used in flow-through reactors for a while without being destabilized [3].

2.5.2. CO₂ Reduction with FDHs

Formate production from CO₂ provides a method to eliminate the greenhouse effect of CO₂ and production of valuable chemical feedstocks. FDHs have a potential to catalyze CO₂ reduction, however, they usually prefer the reverse reaction, oxidation of formate. On the other hand, it has been found that FDHs from acetogens catalyze preferably the reduction of CO₂. Wood-Ljungdahl pathway uses the CO₂ as a carbon source and as a result of this, FDH is naturally reducing the CO₂. The condition determining the catalysis direction is that the enzyme has low affinity against formate. Aslan et. al. demonstrated that therefore, FDH is an attractive candidate for using for biotechnological applications intend to produce formic acid and chemical feedstocks from CO₂ [18, 19]. The oxidized form of the active site of a metal dependent FDH can be seen in Figure 2.5 [20].

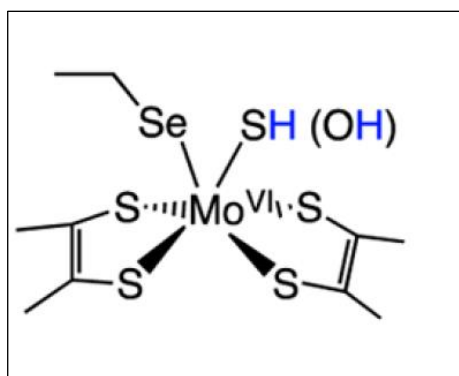


Figure 2.5: The oxidized form of the active site of a metal-dependent FDH.

2.6. Recombinant DNA Technology

Recombinant DNA technology is a technology that involves the cutting of different DNA molecules from different biological species and combining them together by genetic engineering technology. DNA molecules formed by combining molecules from different species are called recombinant DNA. Genetic analysis and manipulations such as endonuclease assays, DNA sequencing and directed evolution studies can be performed on recombinant DNA. The scientific basis of this technology is called recombination which is one of the important reasons for the diversity seen in nature.

Recombinant DNA technology make it possible to isolate a gene or sequence DNA, define the nucleotide sequence, study its transcripts, mutate it in highly specific ways and reinsert the modified DNA into a living organism.

2.7. Gene Cloning

Molecular cloning is a molecular biology method which is used for bringing together of recombinant DNA molecules and direction of that molecules' replication in host organisms. Cloning is a replication of a DNA molecule to produce a cell population with the same genetic material. Molecular cloning usually uses DNA molecules from two different species: the species which is the origin of the DNA molecule to be cloned, and the species which will used as the living host for replication of the cloned DNA. Steps of molecular cloning are as follows:

- Choice of host organism and the proper vector
- Treatment with restriction endonucleases to the DNA to be cloned and the vector.
- Ligating the DNA molecule and the vector together by ligases.
- Transformation of recombinant DNA into host organism
- Selection of organisms which contain the recombinant DNA by their antibiotic resistance etc.
- Screening of transformed cloned with desired DNA inserts and attributes.

Steps of molecular gene cloning are as follows and can be seen in Figure 2.6.

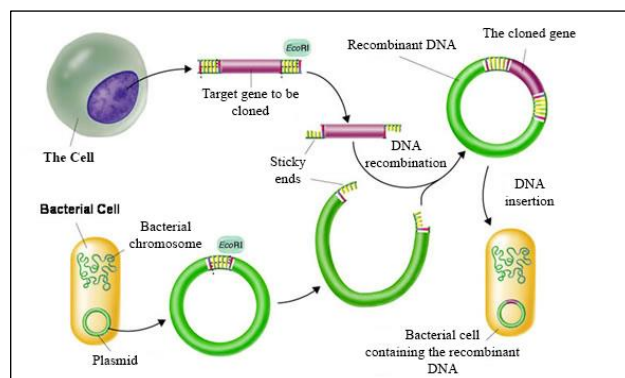


Figure 2.6: Flow chart of recombinant DNA technology.

2.8. Protein Purification

Protein purification is a process that aims to isolate one or more proteins from a mixture of other protein and non-protein portions. usually. Protein purification is crucial for the characterization of a protein's function, to understand the structure and interactions with other molecules. Protein separation frequently based on the differences of size, binding affinity and physico-chemical properties of the protein. Strategies for protein purification are as follows:

- Chromatography techniques: size exclusion, immunoaffinity, hydrophobic interaction, affinity and ion exchange; HPLC
- Metal binding-Polyhistidine-tag

Use of affinity tags have a benefit on the purification of recombinant proteins by simplifying the purification process. This method relies on affinity chromatography. Nickel-nitrilotriacetic acid (Ni-NTA) purification system has an extraordinary selectivity and affinity to the proteins tagged with 6 sequential histidine. Imidazole is a part of histidine's side chain. For protein purification, imidazole is responsible for binding to Ni^{2+} atoms. Using free imidazole at high concentrations outcompetes the binding of the histidine imidazole ring. The interaction between 6xHis tag and Ni^{2+} can be seen in Figure 2.7.

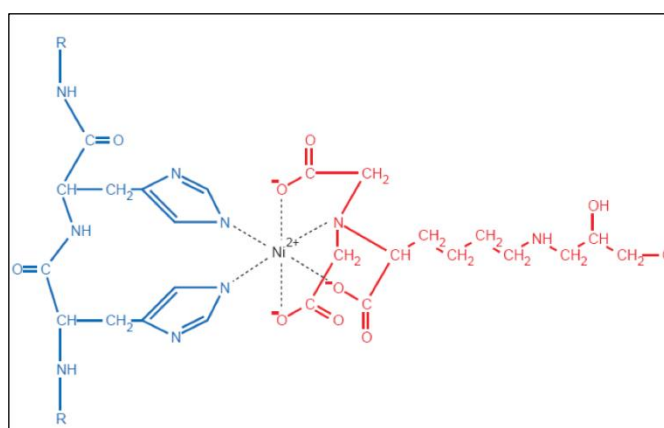


Figure 2.7: Interaction between Ni-NTA and a 6xHis-tagged protein.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Equipments

Laboratory equipments used in this study are given in Table 3.1.

Table 3.1: Laboratory equipments

Autoclave	BioTek
Centrifuge	Beckman Coulter
Deep freezers (-80 °C)	Eppendorf
Electrophoresis Power Supply	Bio-Rad
Magnetic Stirrer	WiseStir
Micropipettes	Axygen
Microplate Reader	Tecan
Microplate Shaker Incubator	BioTek
pH Meter	Hanna
SDS-PAGE Electrophoresis System	Bio-Rad
Shaking Incubator	BioTek
Sonicator	Bandelin
Thermal Cycler	Techne
Vortex	BioTek
Water-Bath	Stuart

3.1.2. Strains, Chemicals and Kits

The chemicals and kits used in this study are given in Table 3.2.

Table 3.2: Chemicals, competent cells, equipments and kits.

	Material	Trademark
Chemicals	Auto Induction Medium	Formedium
	BCA Assay Kit	Serva
	Citrate	Riedel-de Haën
	Glycine	Sigma
	Imidazole	Alfa Aesar
	IPTG	Sigma
	LB-Agar	Formedium
	LB-Broth	Formedium
	NAD ⁺	Prozomix
	NADH	Prozomix
	SDS-PAGE Gel Stain	Generon
	SDS-PAGE Gels	CBS Scientific
	SDS-PAGE Running Buffer	CBS Scientific
	Sodium Bicarbonate	Fisher Scientific
	Sodium Chloride	Fisher Scientific
	Sodium Formate	Sigma
	Sodium Molybdate	Sigma
	Sodium Phosphate	Fisher Scientific
	Sodium Tungstate	Sigma
	Tris-Base	Formedium
Competent Cells	BL21 (DE3)	NEB
	NEB [®] 5α	NEB
Equipment	Concentrators	Sartorius
	HisTrap Column	GE
	Syringe Filters	Sartorius

Table 3.2: (continued)

Kits	Plasmid Miniprep	Qiagen
	PCR Clean-up	Macherey-Nagel
	Western Blotting Kit	Life Technologies

3.1.3. Buffers and Solutions

3.1.3.1. Protein Purification Buffers

Purification of proteins from bacterial lysate was done by using HisTrap protein purification columns. Buffers and preparations of purification buffers are given below:

- Buffer 1: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM) and 1.02 g Imidazole (30 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 2: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM) and 17.02 g Imidazole (500 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 1 with 100 mM Imidazole (50 mL): 42.50 mL Buffer 1 and 7.50 mL Buffer 2 were mixed.
- Buffer 1 with 200 mM Imidazole (50 mL): 32 mL Buffer 1 and 18 mL Buffer 2 were mixed.
- Buffer 1 with 400 mM Imidazole (50 mL): 10.50 mL Buffer 1 and 39.50 mL Buffer 2 were mixed.

3.1.3.2. Enzyme Activity Assay Buffers

- Sodium Phosphate Buffer Solution (100 mM)

14.19 g Na_2HPO_4 and 13.79 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (100 mM) were dissolved in dH_2O and the final volumes were completed to 1 L. pH adjustments for NaPi buffer were done by using Na_2HPO_4 as a base and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ as an acid.

- Tris-HCl Buffer Solution (100 mM)

12.11 g $\text{C}_4\text{H}_{11}\text{NO}_3$ were dissolved in dH_2O and the final volume was completed to 1 L. pH adjustments were done by using HCl (100 mM).

- Glycine-HCl Buffer Solution (100 mM)

7.50 g $\text{NH}_2\text{CH}_2\text{COOH}$ were dissolved in dH_2O and the final volume was completed to 1 L. pH adjustments were done by using HCl (100 mM).

- Sodium Molybdate Solution (1.6 mM)

0.66 mg Na_2MoO_4 were dissolved in 2 mL of dH_2O .

- Sodium Tungstate Solution (1.6 mM)

1.05 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ were dissolved in 2 mL of dH_2O .

3.2. Methods

3.2.1. Cloning of *Clostridium ljungdahlii fdh* Gene into pET45b(+) Vector

The *fdh* gene (approximately 2.1-kb) from *Clostridium Ljungdahlii* (strain ATCC 55383/DSM 13528) was optimized by codon usage preference for *E. coli* expression. The codon optimized gene was synthesized (Genscript) based on the amino acid sequence (UniProt accession number: D8GIZ1) except SeCys139 residue was modified to Cys139. *Nco*I and *Xho*I sites for cloning were added to the synthesized *fdh* gene by the following primers:

Table 3.3: Primers used for *fdh* gene cloning.

Forward Primer	5'CCATGGATAAAAAAGTGCTGACCGTTTGCCCGT'3
Reverse Primer	5'CTCGAGTTAGTGATGGTGATGGTGATGCACATTCAT'3

Gradient thermocycler conditions were as follows: 95 °C for 10 minutes; 30 cycles of 95 °C for 30 seconds; 60-72 °C for 30 seconds; 72 °C for 2 minutes; with a final extension at 72 °C for 10 minutes. PCR products were purified by using the PCR purification kit. The purified PCR products and pET-45b+ vector were digested with *Nco*I and *Xho*I endonucleases, then ligated for the transformation process.

3.2.2. Transformation of *ClFDH* to *E. coli*

The plasmid containing *Clfdh* was transformed into chemically competent NEB[®] 5 α competent *E. coli* cells. Competent cell tubes were thawed on ice for 10 min. 1-5 μ L containing 1-100 ng of DNA was added to cell mixture and flicked 4-5 times to mix DNA and cells. Mixture was placed on ice for 30 min then heat shocked at 42 °C for 30 sec. Mixture was placed on ice for 5 min. 300 μ L of room temperature SOC medium was added to the mixture and incubated at 37 °C at 250 rpm for 1 hour. Cells were mixed by flicking the tube then 100 μ L of the mixture was spreaded to the selection plates and incubated overnight at at 37 °C. Individual colonies were picked and inoculated into overnight cultures for optimization of the expression conditions.

3.2.3. Optimization of *ClFDH* Expression in *E. coli*

E. coli BL21 (DE3) cells were used as the host to express *ClFDH* from pET-45b+ plasmid. Individual colonies were picked from LB-agar plates supplemented with 100 μ g/mL ampicillin, inoculated into 5 mL of LB medium containing ampicillin (100 μ g/mL) and incubated at 37 °C at 225 rpm for overnight. The overnight cultures were added to the large cultures (500 mL) which were incubated at 37 °C at 225 rpm. When the cell density had reached to OD₆₀₀=0.6, 1.0 mM IPTG was added for induction and the cultures were incubated at 15 °C for 16 hours or at

37 °C for 4 hours. After cultivation, the cells were harvested by centrifugation at 4000 rpm for 15 minutes and purification process was started.

3.2.4. Enzyme Activity Assay

The obtained pellets were resuspended in the lysis buffer (20 mM NaPi, 1.0 mg/mL lysozyme) and incubated on ice for 30 mins. The resuspended cells were disrupted by sonication. The sonicated cells were clarified by centrifugation (4000 rpm, 20 min) and the supernatant was used as a source of enzyme. The activity of *C/FDH* was measured against 20 mM sodium formate and 20 mM sodium bicarbonate in 100 mM NaPi pH 7.4 containing 0.5 mM NAD⁺, NADH respectively. A unit was equal to the amount of pure enzyme required to consume 1 μM NADH/NAD⁺ per minute by monitoring the change in absorbance at 340 nm. All experiments were performed in triplicates.

3.2.5. Purification of *C/FDH*

Cell pellets were resuspended in Buffer 1 (20 mM NaPi, 500 mM NaCl, 30 mM imidazole pH 7.4, 1.0 mg/mL lysozyme) and incubated on ice for 30 min. The resuspended cells were disrupted by sonication, and 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 *C/FDH* was eluted with Buffer 1 containing different imidazole concentrations (100-200 and 400 mM). Collected fractions were analysed by SDS-PAGE to identify fractions containing pure protein, which were then combined and diluted to 50 mL by addition of 100 mM of NaPi pH 7.4. Proteins were concentrated with Vivaspin® 20 centrifugal concentrators. The concentration of the purified *C/FDH* was determined by BCA Assay Kit. The expression efficiency was monitored by SDS-PAGE. Proteins on the SDS-PAGE gel were transferred to nitrocellulose membrane for Western blot analysis, where the expressed His-tagged *C/FDH* protein was identified by anti-His antibody using a commercially available Western blotting kit.

3.2.6. Effects of Metals on *C/*FDH

The effects of both molybdate and tungsten on the enzyme activity was studied. Sodium molybdate or sodium tungstate (10 μ M final concentration) was added to medium for overnight cultures and the reaction mixture of the to see effect of the selected metals on the enzyme activity in different levels. Activity of the obtained supernatant and purified enzyme were calculated as mentioned before.

Effect of different metal concentrations (10, 20, 30 and 40 μ M final) of most beneficial metal on enzyme activity were also studied to determine its optimum concentration. Enzyme activity was calculated from initial slopes as mentioned before.

3.2.7. Effect of Different Conditions on Activity and Stability Studies of *C/*FDH

3.2.7.1. Effect of pH

The activity of *C/*FDH was measured in different pHs to see the effect of them on *C/*FDH activity. The specific activity of *C/*FDH was measured in selected 100 mM buffers containing 50 mM sodium formate or sodium bicarbonate with 0.5 mM NAD⁺ or NADH, respectively, and 5 μ M sodium molybdate. The selected 100 mM buffers were NaPi for pH 6.2–8, Tris–HCl for pH 8-9 and glycine for pH 8.5–10.

3.2.7.2. Effect of Temperature

Effect of temperature on activity and stability of the *C/*FDH was measured. Purified *C/*FDH was incubated for 15 min at temperatures between 25–45 °C. The remaining activity of the incubated enzyme was measured in a solution containing 3 mM sodium bicarbonate, 0.25 mM NADH, 5 μ M sodium molybdate and the purified protein at incubation temperatures.

In addition, effect of temperature on activity and stability of the enzyme was measured commercially over the temperature range of 20-95 °C using a Chirascan-

plus qCD Spectrometer with data collection between 200 to 250 nm. The assay was performed on a 0.33 mg/mL *C*/FDH sample in 20 mM NaPi buffer (pH 7.4).

3.2.7.3. Effect of Storage Conditions

Effect of different storage temperatures on enzyme activity and stability was determined by incubation at four different temperatures: -80, -20, 4, 22 °C in glycine buffer (pH: 9). Aliquots of the purified *C*/FDH in 100 mM glycine pH 9 were incubated at -80, -20, 4, 22 °C. The remaining specific activity for formate oxidation was measured as described above after seven and 15 days of the incubation period.

3.2.8. Kinetic Analysis

The kinetic parameters of purified *C*/FDH were determined through monitoring the change in absorbance at 340 nm due to NADH formation or consumption in optimum buffer. The reaction mixtures contained optimized metal concentration, 0.5 mM NAD⁺ or NADH, or 5 mM sodium formate when the kinetic parameters were measured for sodium formate, sodium bicarbonate and NAD⁺, respectively. The concentration ranges for measuring kinetic parameters was 0-5.6 mM for formate, 1-19.2 mM for sodium bicarbonate and 0.3-7.5 mM for NAD⁺. All reactions were performed at 25 °C with their control reactions in the absence of related substrates. Michaelis-Menten kinetic constants were determined from triplicate measurements of the initial velocities. The k_{cat} and K_M values were determined by using GraphPad Prism.

3.2.9. Bioinformatic analysis and molecular modeling of *C*/FDH

Amino acid sequences of Mo and NAD⁺-dependent characterized FDHs from Uniprot were aligned with Clustal Omega [21] (Uniprot accession number for each FDH amino acid sequence; *Cn*: SCU75131, *Des_v*: Q727P3, *Rc*: KQB13064, *E. coli*: P24183, *Ac_w*: H6LB59, *Cl*: D8GIZ1, *Des_d*, *Mf*: WP_048072979 [22]). The homology model of *C*/FDH was created based on the published structure of *E. coli* FDH (PDB ID: 1FDO) [23] using SWISS-MODEL [24].

4. RESULTS AND DISCUSSION

4.1. Cloning, Expression and Purification of *C*/FDH

Transformation of *C*/FDH to BL21 (DE3) and NEB[®] 5 α competent *E. coli* cells were successfully done. Bacterial colonies on the selection plates containing ampicillin (100 μ g/mL) can be seen in Figure 4.1.

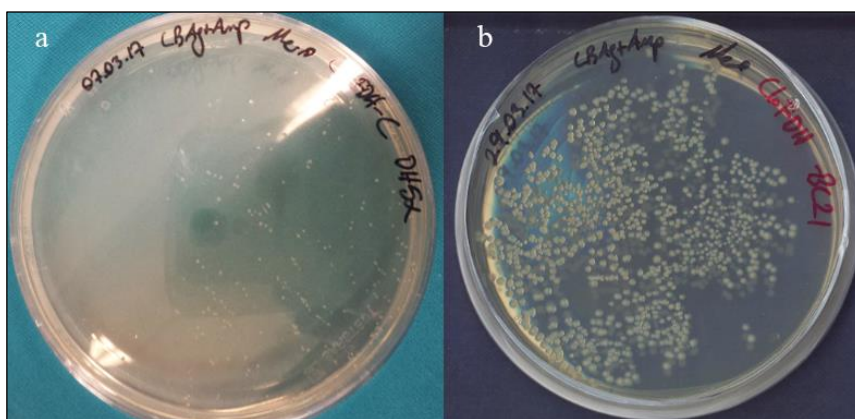


Figure 4.1: Transformation plates of *C*/FDH. a) Transformed colonies of NEB[®] 5 α competent cells b) Transformed colonies of BL21 (DE3) competent cells.

Overexpression of *C*/FDH was accomplished in *E. coli* BL21 (DE3), leading to high level of recombinant enzyme production. Expression at 15 °C lead to a high level of soluble protein, whereas incubation at 37 °C resulted in predominantly insoluble expression of *C*/FDH. This is very clearly not the case since expression at 37 °C leads to completely insoluble protein (Figure 4.2.A lanes 5 and 6). The SDS-PAGE and Western blot analysis show that *C*/FDH expressed in *E. coli* (Figure 4.2.A-B), with soluble protein accounting for approximately 40% of the total protein concentration (Figure 4.2.A, sample lane 1). Soluble *C*/FDH was expressed to approximately 50 mg/L (Figure 4.2.A, sample lane 1) and purification process was started to obtain the pure enzyme.

The C-terminal His-tag allowed purification of the protein using immobilized metal affinity chromatography (IMAC) (Figure 4.2.C) This construct has a calculated molecular weight of 79701 Da, including the C-terminal hexa histidine-tag. As

determined by SDS-PAGE analysis, the purified protein had a molecular mass of about 80 kDa, which is close to the predicted molecular mass. The purity and quantity of *Ci*FDH from this expression were sufficient to allow further characterization (Figure 4.2.C, lanes E4-E6).

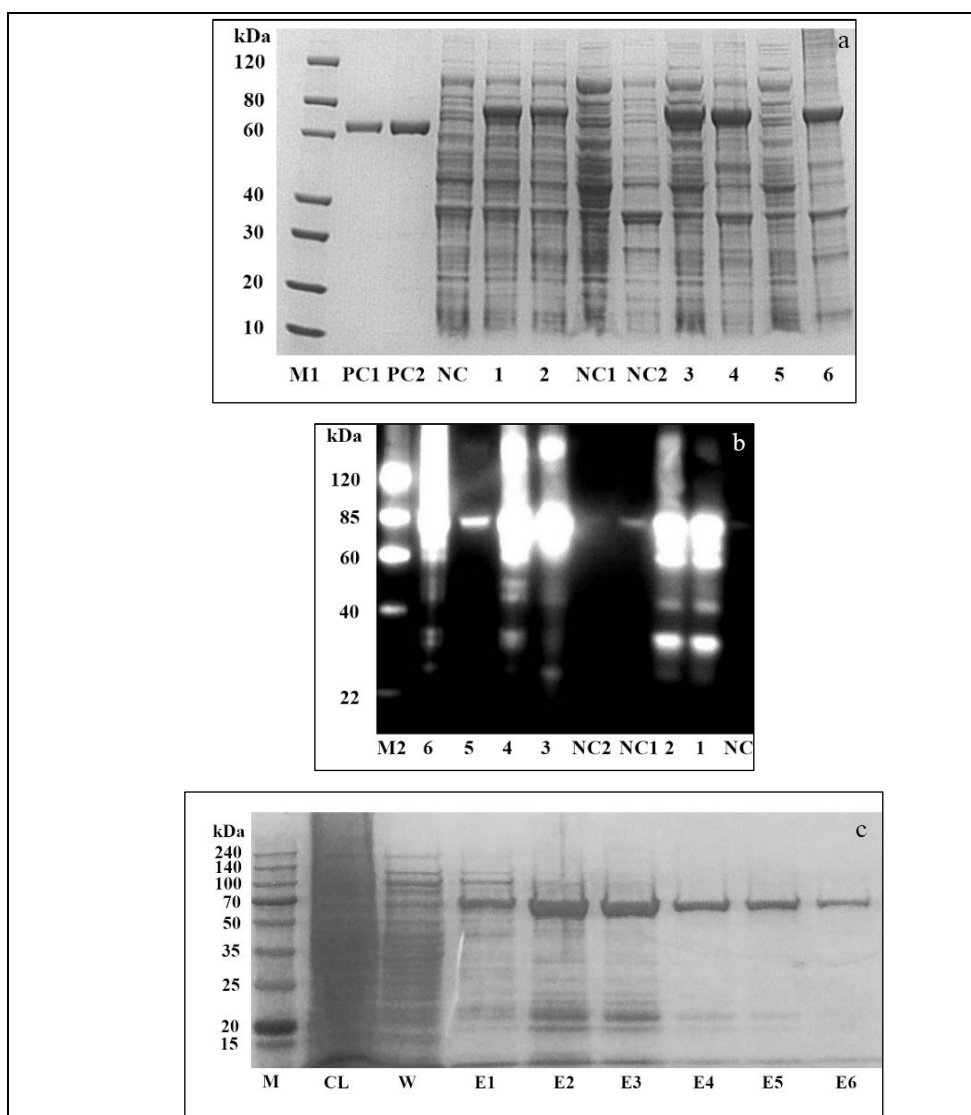


Figure 4.2: The expression and purification results of *Ci*FDH a) SDS-PAGE analysis of *Ci*FDH expression and purification. Lanes: M: Molecular weight marker; PC1: 1 μ g BSA; PC2: 2 μ g BSA; NC: Cell lysate without induction; 1: Cell lysate with induction for 16 h at 15 $^{\circ}$ C; 2: Cell lysate with induction for 4 h at 37 $^{\circ}$ C; NC1: Supernatant of cell lysate non-induced; NC2: Pellet of cell lysate non-induced; 3: Supernatant of cell lysate with induction for 16 h at 15 $^{\circ}$ C; 4: Pellet of cell lysate with induction for 16 h at 15 $^{\circ}$ C; 5: Supernatant of cell lysate with induction for 4 h at 37 $^{\circ}$ C; 6: Pellet of cell lysate with induction for 4 h at 37 $^{\circ}$ C. b) Western blotting analysis of *Ci*FDH protein expression. Lanes: M, Molecular weight marker; lanes 1-6 are the same than in A. c) SDS-PAGE of fractions from the purification of *Ci*FDH. Lanes: M, Molecular weight marker; CL, cell lysate; W, washing fraction; E1–E6, eluted fractions.

4.2. Effect of pH on *C/FDH* Activity

The effect of different pH on *C/FDH* activity for formate oxidation and CO₂ reduction was measured (Figure 4.3.A-B). The enzyme assays were performed at 25 °C using the following buffers: pH 5-6.2; 100 mM NaPi pH 6.8-7.4; 100 mM Tris-HCl, pH 8-8.5; 100 mM glycine, pH 8.5-10. *C/FDH* has its optimal activity between pH 8 and 10. Therefore, *C/FDH* can be called as alkaline enzyme. *C/FDH* loses 40% activity at slightly acidic environment (pH 6.8) (Figure 4.3.A). The enzyme showed activity toward CO₂ in a wide pH range, however reduction of CO₂ by *C/FDH* gave the greatest activity at pH 8.5-9 (Figure 4.3.B).

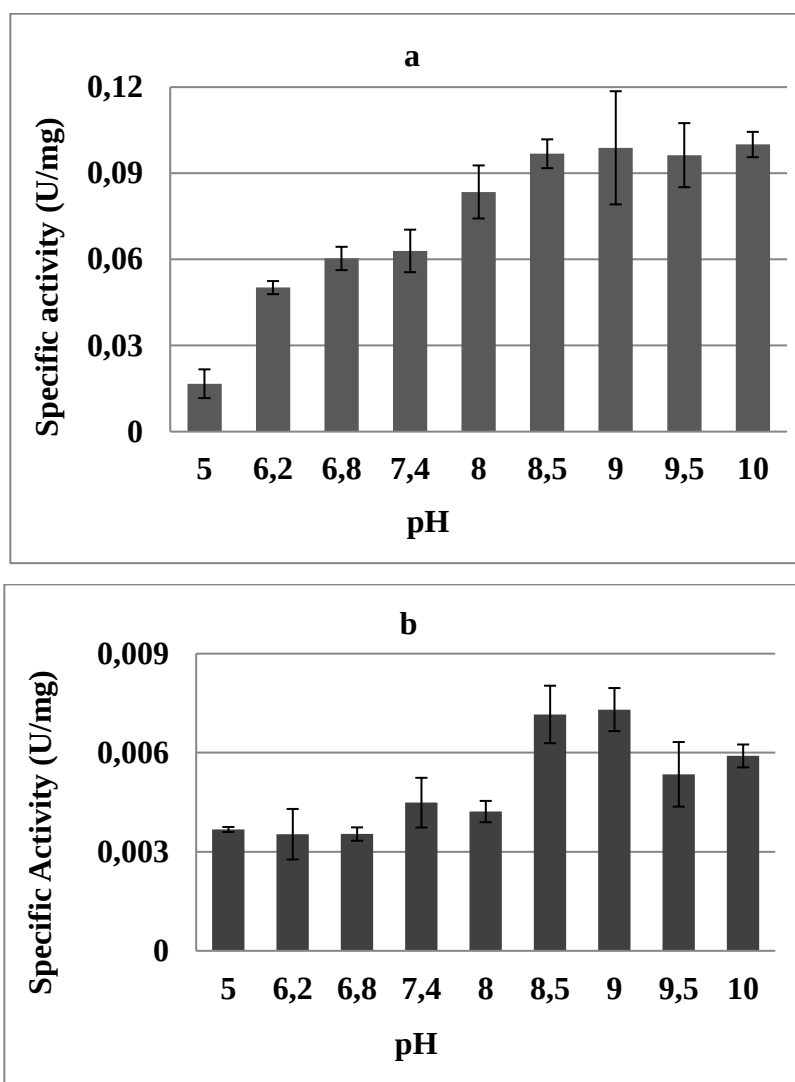


Figure 4.3: Effect of pH and buffer on the activity of *C/FDH*. a) Formate oxidation b) CO₂ reduction (as bicarbonate).

4.3. Thermal Analysis of *C*/FDH

The influence of temperature on the purified enzyme activity was investigated by determining the thermotolerance of the enzyme as well as the melting point. *C*/FDH showed a slight decrease in formate oxidation activity after incubation for 15 min at up to 35 °C, but the enzyme retains high activity (>80%) between 25-35 °C. At 40-45 °C the activity decreased rapidly to approximately half of the maximal activity (Figure 4.4A). This corresponded well with the transition temperature of 45.7 ± 0.4 °C for *C*/FDH calculated through qCD measurements. Figure 4.4B shows the proportion of low temperature form present (grey curve) and high temperature form (red curve) present at different temperatures.

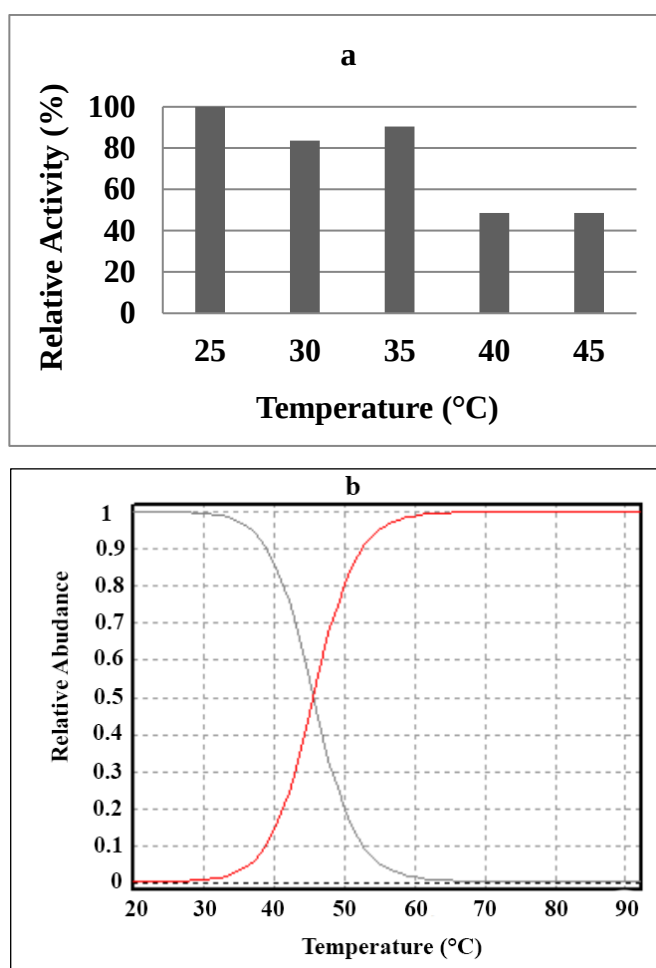


Figure 4.4: Effect of temperature on the activity of *C*/FDH. a) Residual activity after incubation at different temperatures. b) Measurement of the *C*/FDH transition temperature through qCD.

4.4. Effects of Suspected Metals on the Activity of C/FDH

The previously identified acetogenic bacteria requires Mo for better FDH activity [2]. Since the organism source of *Clostridium ljungdahlii* FDH is an acetogenic bacteria, the effect of W and Mo were measured on the activity of C/FDH for formate oxidation [25, 26]. The purified enzyme shows much more activity than the control in presence of both Mo and W, however it reached its maximum activity in presence of only molybdate (Figure 4.5).

The effect of different Mo concentrations on C/FDH activity was also tested. Maximum activity of the enzyme was found in the presence of 10 μM molybdate (Figure 4.6). Therefore, the conducted reactions were set up in the presence of 10 μM Mo.

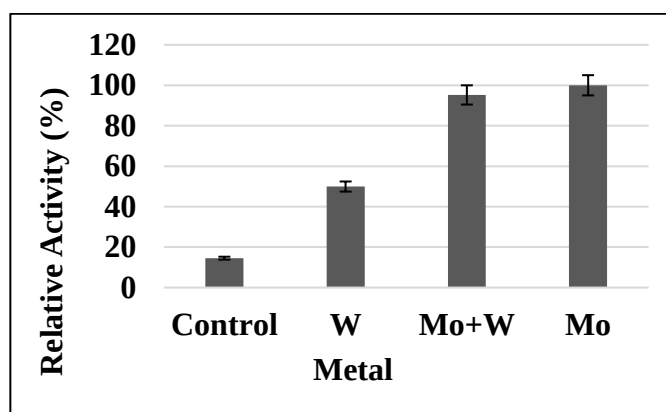


Figure 4.5: Effects of different metals in the growth media on the activity of C/FDH. Control: Overnight culture without any metal added, W: $(\text{WO}_4)^{2-}$, Mo + W: $(\text{MoO}_4)^{2-} + (\text{WO}_4)^{2-}$ and Mo: $(\text{MoO}_4)^{2-}$: (in the presence 10 μM final concentration of each metal).

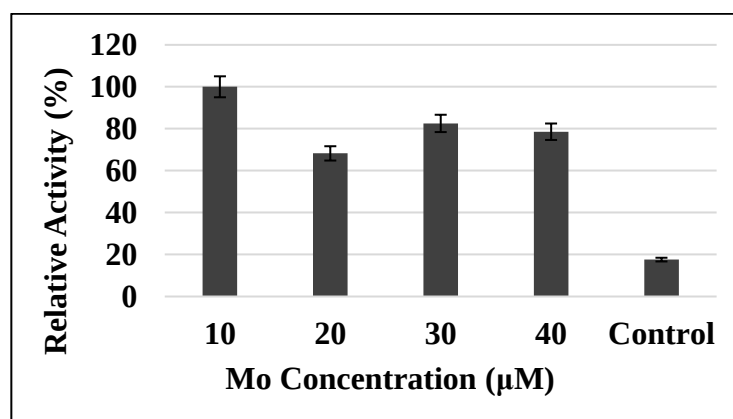


Figure 4.6: Effect of different $(\text{MoO}_4)^{2-}$ concentrations on the activity of C/FDH.

Ci FDH is a molybdate dependent FDH rather than a tungstate dependent FDH. While the enzyme shows some activity with W present, it is not fully active unless Mo is present. Additionally, it is likely that only a low concentration of Mo is required because the enzyme has a low K_M .

Although molybdenum and tungsten have very similar chemical and catalytic properties, reported many studies about the effect of substituting Mo and W for related enzymes indicated that this substitution leads either lose or no enzyme activity. It is difficult to understand mechanism of how enzyme discriminate between the two. However, last advances showed that this discrimination may be developed because of metal composition of environment and selective transporters of the origin cell of enzyme. But, there is no enough information effect of presence of two metals on the intracellular regulation of protein [27].

4.5. Effect of the Different Storage Temperatures on the Activity of *Ci*FDH

*Ci*FDH was stored at different temperatures to determine the effects of time and temperature on its activity. At least 50% activity for formate oxidation remained after storage for one week at 4, -20 and -80 °C, with 4 °C being the most beneficial (Figure 4.7), as the enzyme appears to not tolerate a freeze/thaw cycle. More than 80% of activity was lost after storage at 22 °C for one week. After two weeks, all samples retained only 20% activity or below.

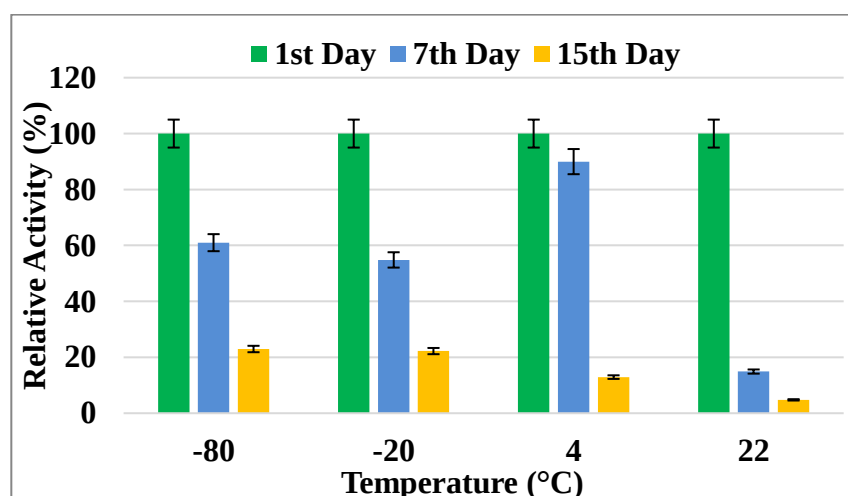


Figure 4.7: Effect of the different storage temperatures on the activity of *Ci*FDH.

4.6. Kinetic Analysis

Kinetic parameters were determined for *C*/FDH activity toward formate, CO₂ and NAD⁺, whereas measurements for NADH could not be calculated due to low activity (Table 3.1). As expected, catalytic efficiency (k_{cat}/K_M) is much greater for formate oxidation than CO₂ reduction, reflecting the natural direction of enzyme activity.

Table 4.1: The kinetic parameters of *C*/FDH

	Formate Oxidation	NAD⁺	CO₂ Reduction*	NADH
k_{cat} (min⁻¹)	14.77 ± 0.93	29.95 ± 1.61	0.73 ± 0.05	nd
K_M(mM)	1.40 ± 0.25	0.85 ± 0.18	7.27 ± 1.14	nd
k_{cat}/K_M (min⁻¹mM⁻¹)	10.55	35.24	0.10	nd
nd: not determined, *: HCO ₃ as a substrate				

K_m of *C*/FDH against formate is better than almost all reported FDHs. However, K_m of the *C*/FDH against NAD⁺ is not promising. Catalytic efficiency of *C*/FDH for formate oxidation and NAD⁺ reduction is quite well. Activity of *C*/FDH in reverse reaction, CO₂ reduction, is the lowest rate in literature [6].

4.7. Bioinformatic Analysis and Molecular Modeling *C*/FDH

An alignment of NAD⁺-dependent Mo-FDH sequences is shown in Figure 4.8, highlighting the molybdenum ligand residue. Cn: *Cupriavidus necator*, Des_v: *Desulfovibrio vulgaris*, Rc: *Rhodobacter capsulatus*, E. coli: *Escherichia coli* strain K12, Ac_w: *Acetobacterium woodii*, Cl: *Clostridium ljungdahlii*, Des_d: *Desulfovibrio desulfuricans*, Mf: *Methanobacterium formicicum*. The amino acid is generally Cys in the aligned FDHs while it is Ser in Cn and SeCys in Des_v *E. coli* and Des_d FDH.

Cn	SGAVLVTSRA---SYEMVLKTAAGVLA-----VSAPTALA--V-----RL-
Des_v	SIFMMGTSHA-SNEECAVIHQAMR-GLGVVHMDHQA RVUHSPTVAALAESFGRGAMTNHW
Rc	ALGVITSSRC-TNEETLVQKLARAVFGTNNNTDTCARVCHSPTGYGLKQTFGTSAGTQDF
E.coli	AIQTTGSSRG TGNETNYVMQKFARAVIGTNNVDCCARVUHGPSVAGLHQSVNGAMSNAI
Ac_w	SIIGTGCARGSGNEANYVMQKFMRAVIGTNNVDHCA RVCHAPSVSGLAYSLGSGAMSLSI
C1	SIMGTGCARGSGNEANYIMQKFMRAVIGTNNVDHCA RVCHAPSVA GLAYVLNGAMSNGI
Des_d	AVAGFSSARC-TNEENYLMQKFMRAAVGTNNVDHCA RLCHAPT VAGLAAAFGSGAMTNSI
Mf	EIGFFCCARS-PNENIYV NQKFARIVVGTHNIDHCA RLCHGPTVAGLAASFSGAMTNSY

Figure 4.8: Partial alignment of Mo-dependent FDHs with the predicted molybdenum coordinated residue shown in box.

A homology model of *C1*FDH was generated through SWISS-MODEL [24] using *E. coli* Mo-dependent FDH (PDBID: 1FDO) as the template [23]. Despite *C1*FDH and *E. coli* FDH having 77% sequence similarity, the two enzymes have conserved first shell active site residues (Figure 4.9).

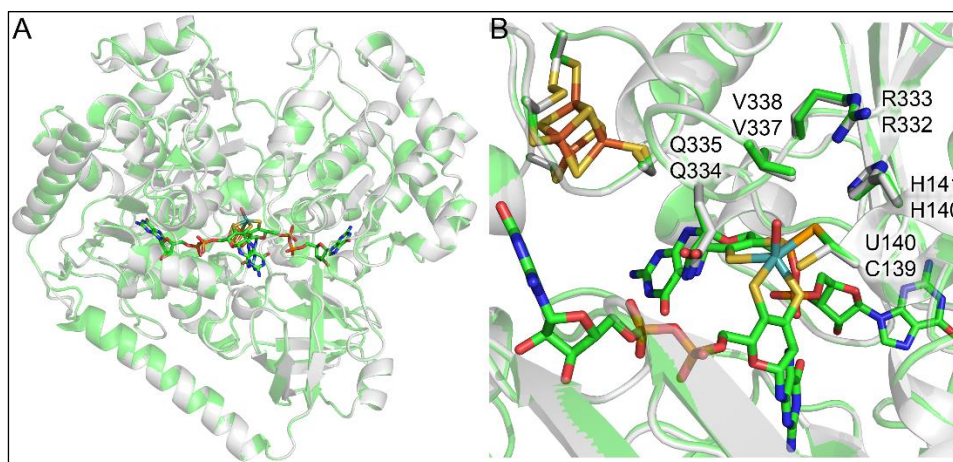


Figure 4.9: Homology model of *C1*FDH overlaid with the structure of *E. coli* FDH a) Full enzyme structure and model of both homologues, including the molybdopterin and Fe_4S_4 cluster from *E. coli* FDH (PDB ID: 1FDO) b) Active site comparison of both enzymes, showing highly similar active site architecture and identical amino acid residues near the molybdate ion (upper number for *E. coli* residue, lower number for *C1*FDH residue). Some backbone structure is excluded from panel B for clarity. *E. coli* enzyme and molybdopterin cofactor are shown in green (with molybdenum ion in aqua with a coordinated red water molecule, iron is shown in brown and the selenium in orange), and *C1*FDH homology model shown in grey.

Metal-dependent FDHs are structurally very diverse [4] although the active site of all currently known metal-dependent FDHs is well conserved. The catalytic site is formed by the molybdopterin cofactor, ligand amino acid position (SeCys140), His141 and Arg333 (numbers from *E. coli* FDH-H) [17]. These active site arginine and histidine residues are strictly conserved in all known FDHs, making important mechanistic and/or substrate binding interactions [23, 28–30]. The bound and unbound ligand amino acid reaction mechanisms were proposed for the oxidation of formate to carbon dioxide by NAD⁺-dependent Mo-FDH. In these mechanisms, His141 and Arg333 play either direct or indirect roles through interactions with the residue and/or formate. There are limited number of modelled Mo dependent FDHs. A well modelled Mo dependent-FDH will have potential for further studies. The presented model could suggest potential targets for engineering at specific sites via an accurate model although no major differences in the active site with existing FDHs were found.

Figure 4.9 highlights the predicted Mo residue, which is predominantly Cys (*Rhodobacter capsulatus*, *Acetobacterium woodii*, and *Methanobacterium formicicum* as well as the modified *Clostridium ljungdahlii* construct characterized here), or SeCys (*Desulfovibrio vulgaris*, *Escherichia coli*, and *Desulfovibrio desulfuricans*). Only, CnFDH varies from this with a predicted Ser at this position. This residue contacts the active site metal ion (Mo or W) and participates in the catalytic mechanism, and thus is critical for activity [31]. The presence of activity for CO₂ reduction is not linked with residue identity (Table 2), as the k_{cat} is widely variable among homologues (28 s⁻¹ for AwFDH (Cys) [32, 33], 47 s⁻¹ DdFDH (SeCys) [34, 35], 1.5 s⁻¹ for RcFDH (Cys) (Table 3.2.) [36–38].

Table 4.2: The features of Mo dependent FDHs.

FDH Source	Ligand Amino Acid	NAD(P) ⁺ -Dependent	CO ₂ Reduction (k _{cat})(s ⁻¹)	Reference
<i>Delsulfovibrio desulfuricans</i>	SeCys	No	46.6	[34, 35]
<i>Desulfovibrio vulgaris</i>	SeCys	No	3.4	[27, 39–42]
<i>Acetobacterium woodi</i>	Cys	No	28	[32, 33]
<i>Rhodobacter capsulatus</i>	Cys	Yes	1.5	[36–38]
<i>Methanobacterium formicicum</i>	Cys	No	nd	[43–47]
<i>Methylosinus trichosporium</i>	Cys	Yes	nd	[48, 49]
<i>Cupriavidus necator</i>	Ser	Yes	nd	[50–52]
<i>Clostridium ljungdahlii</i>	Cys	Yes	0.01	This study
nd: Not determined				

Instead, several other factors such as polarization of active site residues by second shell residues, enzyme flexibility, and the presence of oxygen, salts and other stabilizing or destabilizing compounds likely give rise to the high variation in activity. It is additionally possible that fully optimized reaction conditions have not been identified for CO₂ reduction by many of these homologues.

C/FDH has a good catalytic efficiency for formate oxidation but has the lower K_{cat} value for the CO₂ reduction among the other Mo-dependent FDHs. Therefore, *C/FDH* is a good template to engineer to improve the enzymatic activity resulting in an increased CO₂ reduction.

5. CONCLUSION

In the present work, a new recombinant form of NAD⁺ and Mo-dependent FDH from *Clostridium ljungdahlii* was characterized biochemically, kinetically, and structurally through a homology model. Expression was carried out aerobically, and the enzyme retained the activity under normal conditions. C/FDH activity strongly depends on the addition of molybdate to generate the molybdopterin cofactor, where the active site Cys ligand to the molybdenum cofactor is critical for activity. While C/FDH was more efficient in formate oxidation, the wild type enzyme also shows some activity for CO₂ reduction. Although this activity is presumably at a level too low to be harnessed for CO₂ utilization, beneficial characteristics such as the retention of activity over a relatively broad pH range, C/FDH may be an interesting template to engineer the enzyme for increased CO₂ reduction activity.

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BIOGRAPHY

After completing his high school degree at Mardin Anatolian High School, Mervan continued on to his undergrad degree in Molecular Biology and Genetics at Istanbul Arel University. Mervan completed his internship at Marmara University Industrial Biotechnology and Systems Biology Laboratory. After graduation from Bachelor's degree Mervan started his MSc. program at Yıldız Technical University in Molecular Biology and Genetics. After two semesters at YTU, Mervan changed his MSc. program to Chemistry in Graduate School of Natural and Applied Sciences at GTU. He went to Institute of Biotechnology, Manchester, UK as a visiting academic under the supervision of Prof. Dr. Nicholas J. TURNER to complete his MSc. studies.

APPENDICES

Appendix A: Publications related with the thesis

Çakar M. M., Aslan A. S., Ordu E. B., Turunen O., Binay B., (2016) “Identification a New NAD⁺-Dependent Formate Dehydrogenase from Anaerobic *Clostridium Ljungdahlii*”, 11th International Protein Stabilisation Conference, ProtStab2016, 1-14, İstanbul, Turkey, 09-11 May.

Çakar M. M., Mangas-Sanchez J., Birmingham W. R., Turner N. J., Binay B., (2017) “Discovery of New Formate Dehydrogenases for CO₂ Reduction”, BioTrans 2017 13th International Symposium on Biocatalysis and Biotransformations, 274, Budapest, Hungary, 9-13 July.

Çakar M. M. and Yelmazer B., Pala U., Çorbacioğlu M., Aslan S. A., Binay B., (2017), “Problem of The Century: The Emission of Carbon Dioxide (CO₂): An Enzymatic Solution”, BioTrans 2017 13th International Symposium on Biocatalysis and Biotransformations, 270, Budapest, Hungary, 09-13 July.

Çakar M. M., Mangas-Sanchez J., Birmingham W. R., Turner N. J., Binay B., (2018) “Discovery of a new metal and NAD⁺-dependent formate dehydrogenase from *Clostridium ljungdahlii*”, *Preparative Biochemistry and Biotechnology*.

Appendix B: Michaelis-Menten Plots for Kinetic Study

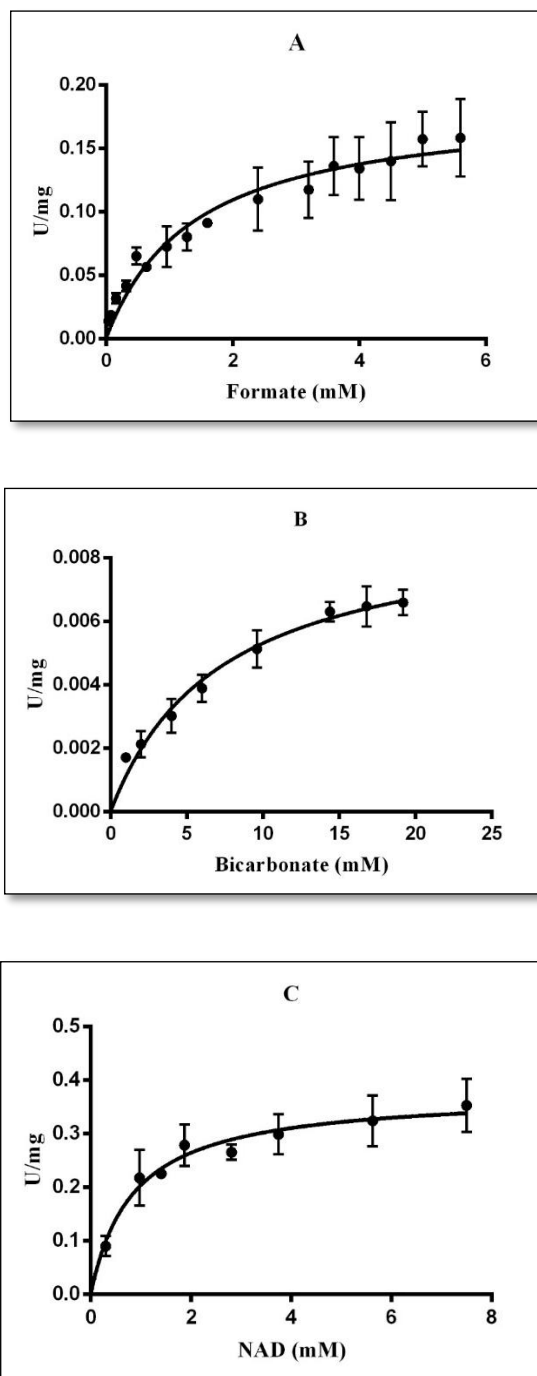


Figure B1.1: Michaelis-Menten plots of C/FDH-catalyzed a) formate oxidation and b) CO₂ reduction c) NAD⁺ reduction

Appendix C: Vector Map of Recombinant *clfdh* Gene

The image was created with SnapGene software.

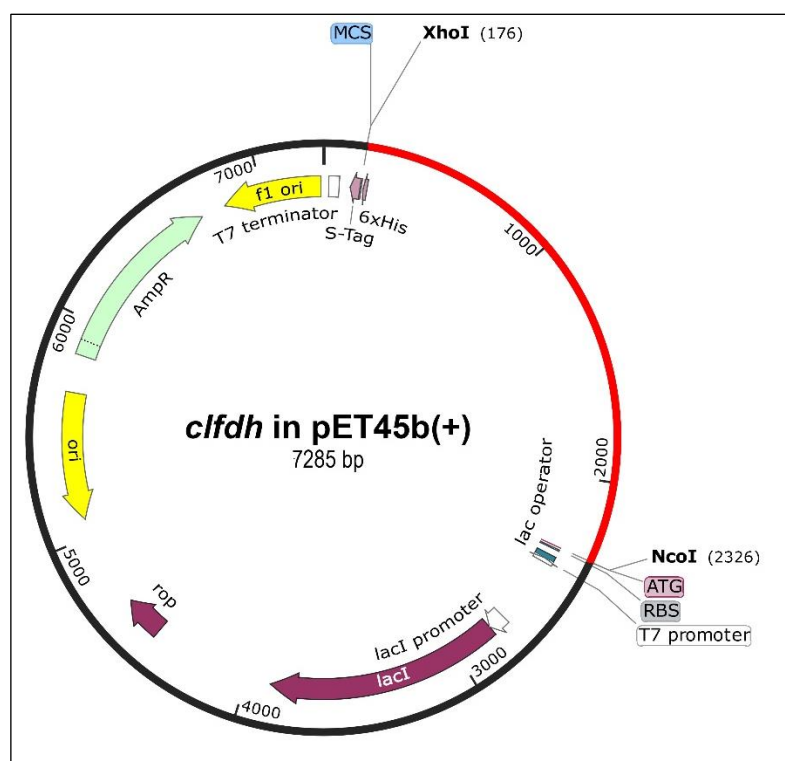


Figure C1.1: Cloned *clfdh* gene (red DNA fragment) into pET45b(+) vector.