ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INCREASING SPECIFIC ACTIVITY OF NAD^+-DEPENDENT Q105R MUTANT OF *Candida methylica* FORMATE DEHYDROGENASE

M.Sc. THESIS

Soner TORUN

Department of Advanced Technologies

Molecular Biology and Genetics-Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

NAD^+-BAĞIMLI MUTANT Q105R *Candida methylica* FORMAT DEHİDROGENAZIN SPESİFİK AKTİVİTESİNİN ARTIRILMASI

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HAZİRAN 2016

Soner Torun, a M.Sc. student of ITU Molecular Biology and Genetics-Biotechnology student ID 521121152, successfully defended the thesis entitled "**INCREASING SPECIFIC ACTIVITY OF NAD$^+$-DEPENDENT Q105R MUTANT OF *Candida methylica* FORMATE DEHYDROGENASE**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 02 May 2016
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To my family,

FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

APS : Ammonium Persulfate
BSA : Bovine Serum Albumin
CBB : Coomassie Brillant Blue
***cb*FDH** : *Candida boidinii* Formate Dehydrogenase
***cm*FDH** : *Candida methylica* Formate Dehydrogenase
CO_2 : Carbon Dioxide
DNA : Deoxyribonucleic Acid
DTT : Dithiothreitol
FAD : Flavin Adenine Dinucleotide
FDH : Formate Dehydrogenase
FMN : Flavin mononucleotide
HCl : Hydrochloric Acid
IPTG : Isopropyl-Beta-D-Thiogalactopyranoside
LB : Luria-Bertani
$MgCl_2$: Magnesium Chloride
NAD : Nicotinamide Adenine Dinucleotide
NADP : Nicotinamide Adenine Dinucleotide Phosphate
NaOH : Sodium Hydroxide
OD : Optic Density
PCR : Polymerase Chain Reaction
SDS-PAGE : Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOC : Super Optimal Broth with Catabolite Repression
TAE : Tris Acetate EDTA Buffer
TEMED : Tetramethylethylenediamine
UV : Ultraviolet

LIST OF TABLES

Page

LIST OF FIGURES

Page

INCREASING SPECIFIC ACTIVITY OF NAD^+ DEPENDENT Q105R MUTANT OF *CANDIDA METHYLICA* FORMAT DEHYDROGENASE

SUMMARY

NAD^+-dependent FDHs (EC 1.2.1.2) use sodium formate as the substrate and catalyze convertion of sodium formate ion into CO_2. At the same time, NAD^+ is reduced into NADH form. Because of low redox potential, NAD^+-dependent FDHs are mostly used enzymes in the industrial area and they provide NADH regeneration for the synthesiz of optical chiral compounds. NAD^+-dependent FDHs have some disadvantages such as low specific activity and they can be inactivated at high temperatures especially when they are used in the industrial area. Recently, protein engineering studies are aimed to overcome these disadvantages.

In this study, we used site-directed mutagenesis method for increasing specific activity of Q105R mutant *Candida methylica* FDH whose thermostability was increased. In the previous studies, K328V mutation has been introduced into the Q105R mutant of *Candida methylica* FDH due to the specific activity of K328V mutation was increased at *Candida boidinii* FDH. First we have designed primer that codes valine residue at 328^{th} position. Site directed mutagenesis method has been performed by using the designed primers. Then PCR products have been digested by *Dpn*I enzyme which cuts methylated adenine site. Digested DNA fragments carrying the required double mutant (K328V on Q105R) have been cloned into pQE-2 vector then transformed into BL-21 chemical competent cells. Three random colonies have been chosen and purified at the end of the transformation. Purified plasmids have been sent for DNA sequence analysis. The accuracy of mutation has been checked by comparing with DNA sequence. Mutant K328V cells have been cultivated for kinetic measurement and then protein purification has been carried out for cultivation of the cells. Purified protein has been run on SDS-PAGE and chosen fractions have been collected for kinetic measurement analysis. The kinetic measurement has been carried out at 25 ^oC at 340 nm wavelength with constant NAD^+ concentration (4 mM) and changing substrate concentrations (0-80 mM). To calculate Km and kcat value, Hyper32 programme has been used. Calculated Km and kcat value reveals that K328V mutation decreases specific activity and Km value.

NAD^+-BAĞIMLI MUTANT Q105R *CANDIDA METHYLICA* FORMAT DEHİDROGENAZIN SPESİFİK AKTİVİTESİNİN ARTIRILMASI

ÖZET

NAD^+-bağımlı format dehidrogenazlar (EC 1.2.1.2) sodyum formatı substrat olarak kullanır ve sodyum formatın CO_2'e dönüşmesini katalizler. Aynı zamanda, NAD^+'i NADH formuna indirger. NAD^+-bağımlı FDH'ler, düşük redoks potansiyelinden dolayı, saf kiral bileşiklerin sentezlenmesinde gerekli olan NADH rejenerasyonunu sağlamak için endüstriyel alanda en çok kullanılan enzimlerden biridir. NAD^+-bağımlı FDH'lerin düşük aktivite göstermesi ve yüksek sıcaklıkta inaktif olması, bu enzimlerin endüstriyel alanda kullanımı açısından bir dezavantaj yaratmaktadır. Son zamanlarda yapılan protein mühendisliği çalışmaları ile bu dezavantajları en aza indirgemek amaçlanmıştır.

Oksidoredüktaz enziminin alt sınıfında yer alan dehidrogenaz enzimleri optikçe aktif maddelerin sentezlenmesinde etkilidirler. Dehidrogenaz sınıfı içerisinde yer alan format dehidrogenazlar (FDH) koenzimlerin rejenerasyonu açısından -özellikle $NAD(P)^+$ rejenerasyonu- endüstriyel ve kimyasal alanda tercih edilen bir enzim haline gelmiştir. Özellikle format dehidrogenazın katalizlediği reaksiyonlarda substrat olarak kullanılan sodyum format bileşiğinin çok ucuz maliyetinin olması ve kolayca elde edilebilir olması, katalizlenen reaksiyon sonucu oluşan karbon dioksitin (CO_2) reaksiyon ortamından kolayca uzaklaştırılabilmesi, format dehidrogenaz enziminin çok geniş bir pH aralığında (6.0-9.0) etkinlik gösterebilme kapasitesi ve reaksiyon sonucu oluşan ürün veriminin fazla olması, format dehidrogenazın endüstriyel alanda daha fazla tercih edilmesini sağlamaktadır. Aynı zamanda endüstriyel uygulamalarda format dehidrogenaz enziminin yüksek sıcaklıklarda kullanılması enzimin aktivitesinde büyük dezavantaj teşkil etmektedir (50-60°C arasında enzim aktivitesi yarıya düşmekte, daha yüksek sıcaklıklarda enzim inaktif olmaktadır). Bu nedenden dolayı, son zamanlarda endüstriyel alanda sıcaklığa dayanıklı enzimler geliştirme ihtiyacı ortaya çıkmıştır. Bu ihtiyacı karşılamak üzere son yıllarda protein mühendisliği çalışmaları büyük ölçüde önem kazanmıştır.

Geçmiş çalışmalarda FDH'in aktivitesini artırmak üzere birçok organizmada çalışmalar yapılmış ve elde edilmiş olan mutant FDH'ler yabanıl tipteki FDH'ler ile karşılaştırılmıştır. *Candida boidini*'de (*Cb*)`den izole edilen FDH`in 23. pozisyonunda bulunan sistein aminoasiti serin aminoasitine (C23S), 285. pozisyondaki fenilalanin aminoasiti serin aminoasitine (F285S) dönüştürülerek spesifik aktivitenin artırılmasına çalışılmıştır. Sodyum format ve NAD^+ ya karşı kinetik ölçümler yapılmış ve spesifik aktivitede yaklaşık 1.7 kat artış olduğu gözlemlenmiştir. *Candida boidini* için yapılan bir diğer çalışmada ise 195., 196., 356. ve 379. pozisyonlarda bulunan aminoasitlere odaklanılmış ve NAD^+ ye karşı aktif mutant FDH'ler elde edilmiştir fakat sodyum format ve koenzimler için herhangi bir Km değeri belirtilmemiştir. *Saccharomyces cerevisiae* FDH'i (*Sc*FDH) için 196. ve 197. pozisyonlardaki aminoasitler üzerine çalışma yapılmış olup, yapılan çalışmalar sonucu koenzim

seçiciliğinin NAD^+ dan $NADP^+$ ya değiştiği gözlemlenmiştir. Yine spesifik aktiviteyi artırmak amacıyla *Pseudomonas* sp.101. FDH'e yönelik (*Pse*FDH) birçok çalışma yapılmış olup özellikle 131., 160., 168., 184. ve 228. pozisyonunda bulunan serin aminoasitlerinin alanin aminoasitine dönüştürülerek elde edilmiş olan mutantlar yabanıl tipteki *Pse*FDH'ler ile karşılaştırılmış ve kinetik değerlerde değişme olmadığı saptanmıştır.

Candida methylica FDH (*Cm*FDH) için yapılmış olan geçmiş çalışmalarda 195. ve 221. pozisyondaki asparajin aminoasiti serin aminoasiti ile değiştirilmiş olup elde edilmiş olan mutantların yabanıl tipe oranla NAD^+ koenzimi için spesifik aktivitesinin daha düşük olduğu gözlemlenmiştir. Yine aynı organizma için 169. ve 226. pozisyonundaki treonin aminoasitleri valin aminoasitine dönüştürülmüş, 169. pozisyonda yapılmış olan mutasyonun kinetik ölçümleri sonucu Kcat değerinde yaklaşık 4 kat azalma olduğu, bu mutasyonun 226. pozisyondaki mutasyonla beraber gerçekleştirildiğinde ise kinetik değerinde herhangi bir değişim gözlemlenmemiştir. Daha çok elektrostatik etkileşim elde etmek amacıyla *Candida methylica* FDH'sinde 13., 105., 147., 160., 187. ve 302. pozisyonlarda mutasyonlar yapılmış olup, elde edilmiş bu mutantlar tek tek ve ikili veya daha fazla kombinasyonları denenmiş olup sodyum formata karşı aktivitesi artırılamamıştır.

Bu çalışmada, daha önceden termal stabilitesi artırılmış olan *Candida methylica* FDH'nin (Q105R) spesifik aktivitesinin artırılması için protein mühendisliği yöntemlerinden birisi olan yönlendirilmiş-bölge mutagenez yöntemi kullanılmıştır. Geçmiş çalışmalarda, *Candida boidinii* FDH'inde (*cb*FDH) K328V mutasyonunun spesfik aktivitesinin artırılmasından dolayı Q105R mutant *Candida methylica* FDH'inin (*cm*FDH) üzerine K328V mutasyonu uygulanmıştır. İlk olarak, 328. pozisyonda lizin aminoasidini kodlayan (K328V) AAA kodonunun yerine, valin aminoasidini kodlayan GTT kodonunu içeren primer dizayn edilmiştir. Dizayn edilen primerler ile yönlendirilmiş-bölge mutagenez polimeraz zincir reaksiyonu (PZR) yapılmıştır. PZR sonucunda elde edilen PZR ürünleri agaroz jel elektroforezinde yürütülüp, yürütülen jel UV altında görüntülenerek yaklaşık 6000 baz çiftine (bp) denk gelen bir bant gözlemlenmiş olup PZR ürünlerinin doğruluğu kontrol edilmiştir. Daha sonra PZR ürünleri *Dpn*I enzimi ile metillenmiş adenin kısmından kesilmiştir ve mutasyon içermeyen DNA'ların uzaklaştırılması sağlanmıştır. Kesilmiş DNA fragmentlerini içeren çift mutant (Q105R/K328V) taşıyan PZR ürünleri pQE-2 vektörü içerisine klonlanıp, daha sonra BL-21 kimyasal kompetent hücreleri içerisine transforme edilmiş ve transforme olmuş hücreler ampisilin içeren katı besiyerine ekim yapılarak 16-20 saat boyunca 37 ^oC de büyütülmeye bırakılmıştır. Transformasyon sonucunda büyüyen kolonilerden üç tanesi rastgele seçilerek plazmid izolasyon işlemi yapılmıştır. İzole edilmiş olan plazmitler, öncelikle agaroz jelde kontrol edilmiş daha sonra mutasyonun doğruluğunu saptamak amacıyla DNA dizi analizi için gönderilmiştir. Mutasyonun doğruluğunu tespit etmek amacıyla BioEdit programı kullanılmış, elde edilmiş olan mutantlar *cm*FDH DNA dizisi ile karşılaştırılarak mutasyonun doğruluğu kontrol edilmiştir. Mutant Q105R/K328V kinetik enzim ölçümleri yapılmak üzere, mutant hücreler transforme edildikten sonra ampisilin içeren Luria-Bertani (LB) katı besiyerinde 16-20 saat boyunca 37 ^oC de büyütülmüş ve büyütülmüş olan hücrelerden protein saflaştırması yapılmak üzere tek koloni alınarak ampisilin içeren 50 ml LB sıvı besiyerine ekimi yapılıp 1 gece boyunca bekletilmiştir. Bekletilmiş olan kültür ampisilin içeren 1 L lik sıvı besiyerine transfer edilmiş olup OD_{600} değeri yaklaşık 0.6 ya ulaştığında kültür içeresine IPTG eklenerek protein ekpresyonu indüklenmesi sağlanmıştır. İndüklenmiş proteinler HisTrap

yöntemi kullanılarak saflaştırılmıştır. Saflaştırılan proteinler SDS-PAGE'de yürütülmüş ve belirlenen protein fraksiyonları kinetik ölçümlerde kullanılması için toplanmıştır. Kinetik ölçümler, sabit NAD^+ konsantrasyonunda (4 mM) ve değişen sodyum format konsantrasyonlarında (0-80 mM) 25 ^oC 'de 340 nm dalga boyunda gerçekleştirilmiştir. Km ve kcat değerlerinin hesaplanmasında Hyper32 programı kullanılmıştır. Hesaplanmış Km ve kcat değerleri Q105R/K328V mutasyonunun spesifik aktiviteyi ve Km değerini düşürdüğü göstermiştir.

1. INTRODUCTION

1.1 Enzymes

Enzymes are catalysers in the biological mechanisms and they are responsible for the all metabolic pathways in living organisms. They increase specificity and velocity of metabolic chemical reactions about 10^{12}-fold. Although most of the enzymes are in protein structure, a small group of them are catalytic RNA like ribozymes [1]. The conformation of enzymes in protein structure which include primary, secondary, tertiary, quaternary structure affect their catalytic activity. Some factors can affect the catalytic activity of the enzymes. For example: if enzymes are denatured by temperature, pH etc. or separated to their subunits, they can lose their function. In addition, when enzymes are degraded into amino acids which built them, their activity can also be negatively affected [1].

While some enzymes that do not require any side group can work alone, others require one or more inorganic group which are called cofactor like Fe^{2+}, Mg^{2+}, Zn^{2+} or need organic molecules which are called coenzymes such as biyositine, coenzyme A. Coenzymes, and cofactors can be tightly bound to enzymes or form covalent bonds. An enzyme which is catalytically active when it is bound to its metal ion and coenzyme together is called holoenzyme. Protein structures found in holoenzymes are called apoenzyme or apoprotein.

Two models explain how enzymes bind their substrate. First one is" lock and key" model which claims that enzyme and substrate bind together by the help of their complementary regions. This model was suggested by Emil Fischer in 1894. However, nowadays, "induction-fit model" which was discovered by Daniel E. Koshland in 1958 is much more preferred rather than "lock and key" model. This model suggests that when a substrate binds specific active-site of enzymes, the conformation of active site will change and then enzyme will cover the substrate. [1].

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

Figure 1.1 : A simple enzymatic reaction [1].

1.1.1 Classification of Enzymes

At first, enzymes were randomly named, then they were named according to the type of the reactions they catalyze or their binding molecules. In 1961, The International Union of Biochemistry determined new norms for denotation and classification of enzymes consisting six sub-classes which are given in Table 1.

i. Oxidoreductases: Catalyse oxidation and reduction reaction between two substrates with transferring hydride ions or H atoms.

ii. Transferases: Catalyse transfer of functional group between two substrates.

iii. Hydrolases: Catalyse hydrolyzation of biological bonds such ether, ester, peptide, glycoside etc.

iv. Lysases: Catalyse addition of groups on double bonds or change groups to form double bonds.

v. Isomerases: Provides isomeration of a substrate by group transfer to the molecule.

vi. Ligases: Catalyse condensation of two metabolites by using ATP or similar cofactor to synthesize a new molecule [2].

Table 1.1 : Classification of enzymes [1].

Class no.	Class name	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor

1.2 Formate Dehydrogenases

Formate dehydrogenase is an enzyme which belongs to the family of oxidoreductase (EC 1.2.1.2, FDH). Formate dehydrogenases catalyse the convertion of formate ions into carbon dioxide meanwhile $NAD(P)^+$ is reduced to NADH(P).

1.2.1 Dehydrogenases

Dehydrogenases are sub-class of the oxidoreductase which catalyses oxidation-reduction mechanism in the organism. These types of enzymes use cofactor as NAD^+, $NADP^+$, FAD, FMN (Flavin mononucleotide) in the enzymatic reactions.

1.2.2 General properties of FDH

D-specific 2-hydroxy acid dehydrogenases have belonged to FDHs. In nature, three types of FDH is founded. The first type of FDHs consists from archaea and anaerobic microorganism which contain heterooligomers with a complex quaternary structure. This type of FDHs are also possessed high molecular weight and can be described by existence of some prosthetic group such as iron-sulphur clusters, molybdenum, tungsten ions in the active site of them. In addition, they show too much susceptibility to oxygen [3]. Complex NAD^+-dependent formate dehydrogenases (EC 1.2.1.2, FDH) are the second type of FDHs and also require heavy metals such as molybdenium, iron and selenium. The third one is the simplest one and called as NAD^+-dependent FDH. This group of enzyme have two active sites and also they do not contain metal ions or prosthetic groups in their structure[3]. NAD^+-dependent FDHs are so important in terms of the catalysing of oxidation of formate anion into carbon dioxide, at the same time they are realized to the reduction of NAD^+ to NADH which occur in the terminal step of the catabolism of C1 compounds in yeast and bacteria cells [4].

At 1921, formate dehydrogenase was found firstly in a plant which is called as *Phaseolus vulgaris*. [5]. Firstly in 1951, Davison studied NADH formation by ethanol, succinate and glutamate in pea and bean seeds [6]. In early 1970s, FDH enzymes were studied in microorganisms like yeast, bacteria, and fungi. FDHs have an important role in the microorganisms and in plants. While at plants FDHs which biosynthesis was gone up the under stressful conditions are founding in mitochondria, at microorganism FDHs can show different properties such as in methanol-utilizing bacteria and yeast,

FDH uses the supply of energy for a cell, whereas in pathogenic bacteria and fungi FDH is found as stress protein [4].

An important advantage of the FDH is to catalyse the reactions which are not returned back which provide to get 100% yield. FDHs can be worked at different pH range (between 4 to 10) which most efficient range of FDH is between 6 to 9 [7].

1.2.3 Structural features of NAD^+-dependent FDH

The molecular mass of NAD^+-dependent FDHs can change especially in some methylotrophic organisms. In the eukaryotic and some methylotrophic organisms it can be found between 70 to 100 kDa. It composes of two identical subunits which consist of "NAD binding domain" and "catalytic domain" [7] and neither prosthetic groups nor metal ions are not contained at the FDHs. In the *Mycobacterium vaccae* 10 and *Pseudomonas sp.* 101, which is methylotrophic bacteria, are the presence of molybdenum their molecular mass range can be reached to nearly 450 kDa whereas the presence of tungsten in the growth media their molecular mass range can be between 80-93 kDa [4]. Generally, homodimers are formed by NAD^+-dependent FDHs and they are considerably specific to both formate and NAD^+.

Primary structure of FDH is compared and observed greatly similarities in different organisms. 71 amino acid residues that consist of nearly 20% of all residues are highly conserved during the evolutionary period in the FDH. Moreover, they include active site in which occurs catalytic activity and sites, which are linked coenzyme.

Some amino acid residues are important in the different organisms in terms of catalytic activity which are formed of the active side of FDH. Pro97, Phe98, Ile122, Gly123, Ser124, Asp125, Asn146, Thr282, Ala283, Arg284, Asp308, Gln313, and His332 are important amino acid residues in the *Pseudomonas* sp. 101 active sites that are shown at figure 2 [9]. Pro77, Phe78, Ile102, Asn118, Gly171, Gly173, Gly176, Arg267, Gln278 and His310 are important for *Candida methylica* FDH (*cm*FDH) and also Phe69, Asn119, Ile175, Arg258, Gln287, Pro288 and His311 position too in *Candida boidinii* FDH(*cb*FDH). These residues are situated in and around the catalytic site and have proposed binding or catalytic functions (Figure 3) [10].

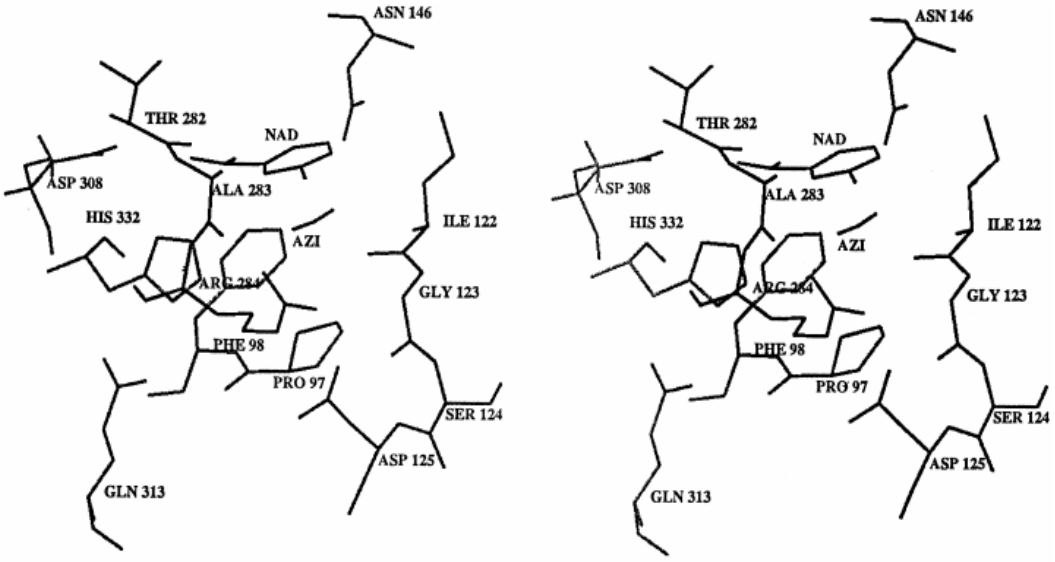

Figure 1.2 : Active centre of FDH from *Pseudomonas sp.* 101. [9].

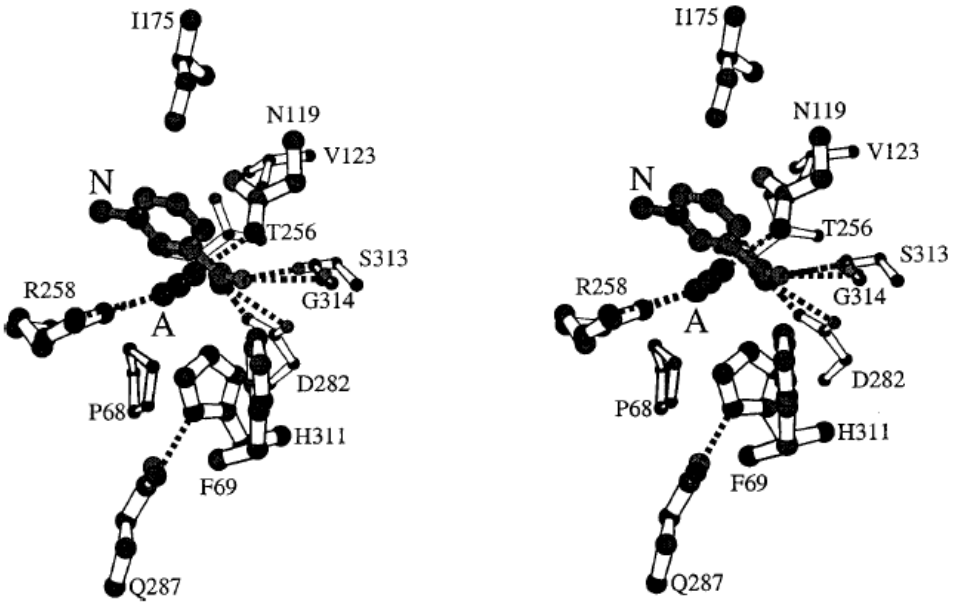

Figure 1.3 : Active centre of FDH from *Candida boidinii* [10].

1.2.4 Catalytic properties of NAD^+-dependent FDH

Bi-Bi reaction was observed in the NAD^+-dependent FDHs. In this type of reaction, NAD^+ is used as a first substrate and an active site functions independent of other active sites . A substrate which is bound to an enzyme is increased to the affinity of other substrate 3.5-fold. FDH catalyse the reaction by the hydride ion transfer from the substrate onto NAD^+. This hydride ion transfers to the fourth carbon of the nicotinamide. This reaction is also seen in other related dehydrogenases. FDHs can work at the best pH values between 6.0 to 9.0 [11, 12] and activity of FDHs are

decreased half between 50-60 °C temperature. While FDHs can work at the wide pH range, they do not work at high temperatures conditions. They are inactive at the extreme conditions [8]. Catalytic properties of FDH at the some organism was given at Table 2.

Table 1.2 : Catalytic properties of FDH at the different organism [4].

Source	Subunits (kDa) [pl]	Activity (units/mg)	Thermal stability	pH optimum	K_m^{NAD+} (μM)	K_m^{format} (mM)
Bacteria						
Pseudomonas oxalaticus	2x100, 2x59			7.5	105	0.14
Methylomonas methylica (x32)		3.1 37		7.7-8.0	160	0.40
Methylomonas extorquens AM1	2x44	0.42		8.4	90	0.25
Pseudomonas sp.		16.0	55	6.0-9.0	110	15
Moraxella sp. C-1	2x48	6.0	55	6.0-9.0	68	13
Paracoccus sp. 12-A	2x49	11.6	50-55	6.5-7.5	36	5
Mycobacterium vaccae 10	2x44	6.0	57	6.0-9.0	200	20
Yeast						
Candida boidinii	2x36	2.4	55	6.5-8.5	90	13
Candida methylica	2x46	10.0	50	6.0-9.0	100	13
Candida methanolica	2x43	7.5	50	6.5-9.5	110	3
Kloeckera sp. 2201		0.14	50	7.0-8.0	100	22
Pichia pastoris NRRL-Y-7556	2x47	8.2	20-25	6.5-7.5	140	16
Pichia pastoris IFP206	2x34	2.8	47	7.5	270	15
Hansenulla polymorpha (x16)	2x40	2.8	60	7.0	70	40

1.2.5 Practical applications of FDH

Optically active compounds are necessary for cellular activities. Optically chiral compounds contain a chiral center, which consists of an asymmetric carbon which is bound to four different groups. Optically chiral compounds have two forms (D- and L-), whose mirror image does not overlap with each other, which are called enantiomers or optical isomers. Different enantiomers of the same compounds can bring about diseases due to their adverse physiological effects. According to prescriptions of Food and Drug Administration of USA, the optical purity of all chiral compounds used as drugs has to be no less than 99%. For that reason, many of researcher is attracted considerable attention on enzyme applications in pharmaceutical industry and studies were increased on pharmaceutic in the past years. Dehydrogenases show extreme stereospesificity when transferring the hydride ion between the substrate and coenzyme. Because of this, nonchiral products can be turned into optically active ones using dehydrogenases. These enzymes can produce optically active compounds with very high optical purity (around 99.9-99.99%). Using

dehydrogenases is also cheap when compared to NADPH and NADH (>USD 12/kg). To solve this problem, another reaction has been suggested to decreased of the price of coenzymes which is converted of $NAD(P)^+$ to NAD(P)H which was explained at Figure 4 [13].

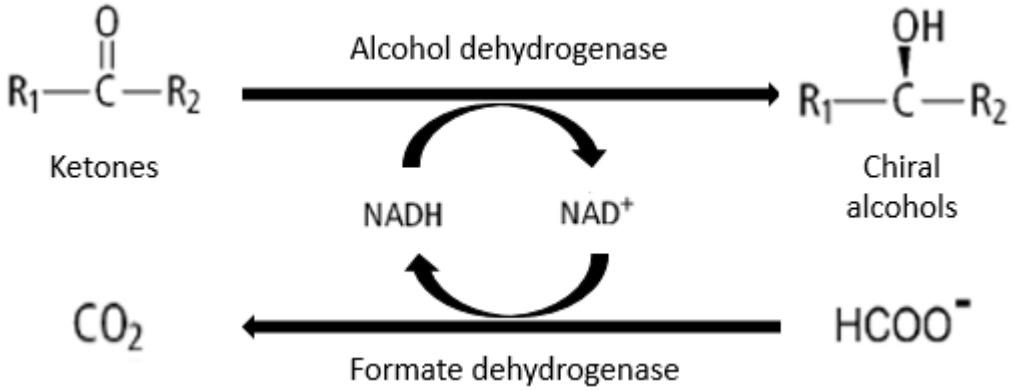

Figure 1.4 : General scheme of conversion of $NAD(P)^+$ to NAD(P)H [4, 8].

Regeneration of NAD(P)H (figure 5) can be realized with FDHs and these reactions are important for composed of chiral compounds in chemistry.

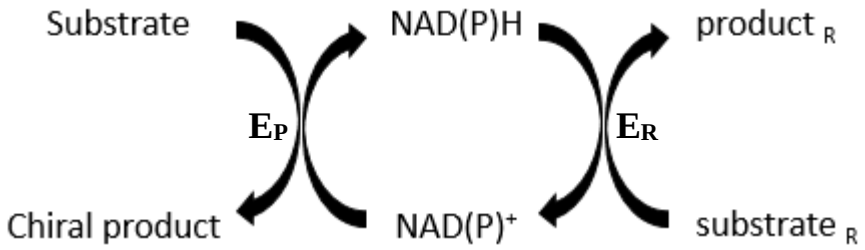

Figure 1.5 : General scheme of NAD(P)H regeneration [12].

Using of FDH in the NAD(P)H regeneration reaction can be listed below;

i. The reaction catalysed by FDHs are commonly irreversible and shifting the equilibrium of the main reaction by regeneration results in 99-100% yield of final product.

ii. Formate is a cheap substrate which can be used in catalysed of FDHs reaction and comprised of CO_2 in the result of reaction can be easily removed the reaction mixture.

iii. FDHs can work at the best pH values between 6.0 to 9.0 [11].

iv. FDHs, especially isolated from yeast and bacteria, are stable enzymes and they are usually used in the system for a long time.

v. Using the methanol-utilizing yeast and bacteria can decrease the cost of production and they supply to produce enough enzyme in large scale. [12].

NAD(P)H regeneration systems which are catalysed with FDH are so important and appropriate in chemistry. On the other hand, FDHs which are constituted naturally are specific NAD^+ as well. Because of the fact that, the aim of altering coenzyme specificity of FDH by protein engineering ($NADP^+$ versus NAD^+) has importance [7, 8, 12, 14, 15, 16]. In recent years, various experiments were performed to alter the specificity of isolated from different organisms of FDH towards $NADP^+$ have yielded promising results but the activity of NAD^+ is so high [16, 17, 18].

1.3 Protein Engineering

Protein engineering is an important tool for creating new and improved protein. It provides knowledge about protein structure and function. Protein engineering has become a great potential and enables using various industrial applications such as chemical, food, textile and medical. Protein engineering is necessary for protein expression studies and understanding protein function. To make changes in protein structure, first, characteristics of wild-type proteins should be identified by analytic methods. New properties are introduced to protein by creating changes in the protein functional region of proteins. Proteins can be modified to make changes in their catalytic activity, for receptor binding and alteration of specificity [19].

Protein engineering is also used in enzymology. Especially, in the industrial process, temperature, pressure and pH could affect reactions. However, natural forms of enzymes could possess some limitations (limited substrate and coenzyme specificity and low kcat) in terms of stability and activity in industrial processes when they are subjected to extreme conditions. To solve the limitation problems, nanotechnology, metabolic engineering, cellular membrane engineering and protein engineering can be utilized [20, 22, 23]. Thanks to these alterations, especially the ones made by protein engineering, a gene which encodes the enzyme structure can be changed and the desired enzyme can be highly expressed [24]. Protein engineering can be classified into threemain categories: rational design, directed evolution and semirational design. They provide researchers to increase the stability or activity of enzyme, coenzyme specificity of enzyme and overcome the substrate limitation [25].

The rational design gives information about structure and function of known proteins. Site-directed mutagenesis is the most important technique for rational design. In directed evolution, random mutagenesis is used and the knowledge of protein structure is not required. This approach makes use of mutant libraries and screening studies to verify desired property of enzymes. Both the strategies have a lot of advantages in the industrial applications for optimization of proteins while they have some limitations such as complexity of enzyme structure or function. To overcome the limitation, rational design and directed evolution were combined which is called semi-rational design. This strategy could be effective to solve the limitations of these strategies and the properties of an enzyme is developed [25, 26, 27].

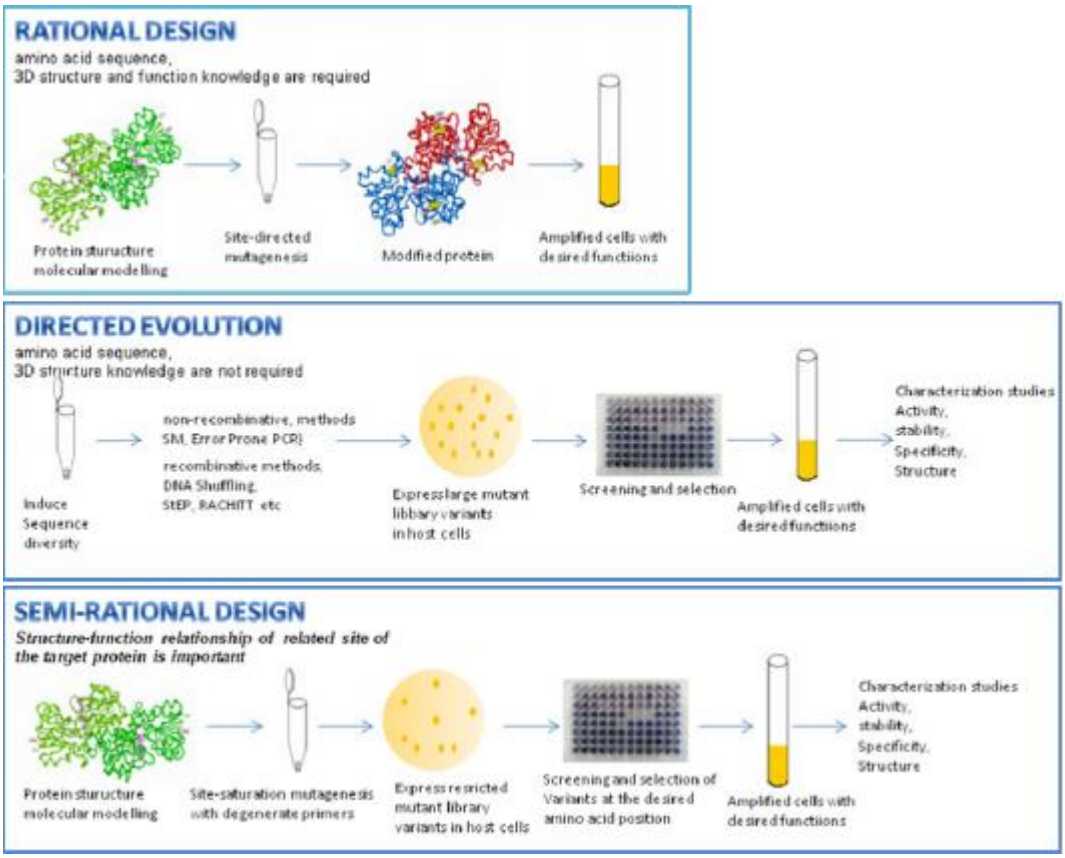

Figure 1.6 : General scheme of the protein engineering strategies [28].

1.3.1 Rational Design

1.3.1.1 Site-directed mutagenesis

Site-directed mutagenesis is a technique which is mostly used in protein engineering approach. This method helps understanding how a sequence of interest affects the gene and its endpoint protein product [29]. The purpose of this technique is to convert an

amino acid into another one at a defined site to identify the effect of the mutation on conformation and structure of the protein. Changing enzyme properties strongly requires manipulation of the primary structure of the protein. Structural and functional properties can be altered by single point mutations which make differences in electrostatic interactions of the enzyme, in disulfide bridges, core packing, condensing surface loops. In site-directed mutagenesis method, polymerases chain reaction (PCR) is used to modulate the gene sequence with designed primers for the specific region [30].

Advances in protein modeling tools and knowing the three dimensional structure of a protein will help directed evolution studies [25, 26].

1.3.2 Directed Evolution

In contrast to rational design, in this technique, information of the 3D structure of the protein is not required and natural evolution process is mimicked and a mutant library of the desired enzyme is created, selected and screened. [25, 26, 31, 32]. Directed evolution technique is also used for developing enzyme activity and new metabolic pathway. Different strategies can be used for directed evolution. DNA shuffling and error prone PCR are the most preferred in the directed evolution.

i. In error prone PCR reaction, $MgCl_2$ is used at high concentration and therefore stability of non-pairing PCR product is increased. The error of margin is increased by $MgCl_2$ which is used. Mutation frequency and nucleotide ratio can be determined by changing amount of $MgCl_2$. The amount of template DNA which is used at the beginning can be altered by changing number of cycles used in the PCR reaction. These conditions provide alternation in a number of mutated genes [33].

ii. DNA shuffling is the strongest and most efficient technique used in the directed evolution method. This technique provides the increase in mutation number causing variability in DNA libraries. In addition, it contributes to recombination between different DNA species giving rise to different mutations. In this technique, DNA molecules that are created with the other methods are separated into small pieces by Dnaz. Then, these pieces are assembled in PCR reaction. In this PCR process, resulting DNA fragments are used as a primer instead of the conventional primers. At the end of PCR, several DNA molecules are obtained which includes different genes [34].

1.3.3 Semi-rational Design

1.3.3.1 Site-saturation mutagenesis

The rational design gives information about structure and function of known proteins; in the directed evolution, the 3D structure of a protein is not required to know and natural evolution process is mimicked and a mutant library of the desired enzyme is created, selected and screened with tools [25, 31, 32]. Both of these techniques are useful for protein engineering but they have still a limitation in some aspect. Therefore, these two methods were combined to overcome the limitations. This method is called as semi-rational.

In semi-rational approaches, pre-determined specific residues through the basic structural or functional knowledge are randomized using directed evolution tools, especially saturation mutagenesis, create "smarter" libraries that give positive results [20]. For using the site-saturation technique, which is usually used for creating mutant library, knowledge about the protein structure should be known. In this technique, degenerate primers are used to form different for each amino acid residues. As a result, all possible amino acid types are formed at the mutant library.

1.4 Protein Engineering of Formate Dehydrogenase

However, FDH enzyme is used in cofactor regeneration processes. Native FDHs do not have much operational stability, their cofactor is not $NADP^+$, they are not thermostable and cost for production is high. Because of this FDH is a good candidate to engineer by using protein engineering strategies [21].

FDHs are inactive above 55-60°C [4] so thermostability is one of the most important properties of the engineered FDH. This could be done by measurement of the enzyme's residual activity upon incubation at a fixed temperature by introducing the Tm value [12]. There are several studies about improving the thermostability of FDH by either rational design or directed evolution.

Studies about thermostable FDH showed different results. Rojkova et al. (1999) reported that hydrophobization of α-helices within the protein results in higher thermostability with either single or different combinations of the mutations to the enzyme from *Pseudomonas sp.*101. This enzyme is the most thermostable FDH.

Slusarczyk et al. (2004) used directed evolution method and got about 10-fold increase in thermal stability as a result. According to the research, mutating Glu151 to Asp and Arg178 to Ser increases thermostability. Moreover, Fedorchuck et al. (2002) confirmed that the interaction between the residues 43 and 61 is important. Their results show that electrostatic repulsion between Asp43 and Glu61 gives the FDH from *Mycobacterium vaccae* by substitution of Glu61 with a non-negatively charged amino acid.

1.5 Aim of the study

As mentioned above, because of the some features such as stability and comparatively production of good yield, FDH can be used at NADH regeneration system in the many applications. *Candida methylica* FDH (*cm*FDH) was cloned and overproduced at the University of Bristol and purification processes have been improved at Department of Molecular Biology and Genetics of Istanbul Technical University (ITU) giving a much better yield.

In the previous study, 105th amino acid position was converted glutamin (CAA) to arginin (AGA) for increasing Tm value and for getting to more thermostabil enzyme.

In this study, we have designed K328V mutation for increasing the specific activity of *Candida methylica* FDH (*cm*FDH) mutant (Q105R) which has increased Tm value by site-directed mutagenesis. For this purpose, the double mutant (Q105R/K328V) of *cm*FDH have been constructed, purified and compared the activity with Q105R mutant.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipments

Laboratory equipments which are used in this work are given in Table 2.1.

Table 2.1 : Laboratory Equipments.

Equipments	**Trade Mark/Model**
Vortex	Scientific Industries
pH Meter	InoLab
Autoclaves	Tuttnauer 2540ml (Switzerland)
Magnetic Stirrer	Heidolph
Micropipettes	Eppendorf,Gilson
Orbital Shaker Incubator	Biolab-Certomat (Germany)
Microplate Shaker Incubator	Ika
Microfuge	Microfuge 18 Beckman
Centrifuge	Allegra 25R Centrifuge Beckman
UV-Visible Spectrophometer	Shimadzu UV-1601 (Japan)
Microplate Reader	Perkin Elmer
Thermocycler	Biometra
Cycle Sequencer	ABI 3130 Avanti
Water-Bath	Eppendorf
Thermomixer	Eppendorf
Deep freezers (-80 °C)	Ultra Low Sanyo
Freezer (-20 °C)	Biomedical Freezer Sanyo
Refrigerator (+4 °C)	Arçelik (Turkey)

2.1.2 Enzymes, kits and chemicals

During this study, used enzymes, kits and chemicals are given in Table 2.2.

Table 2.2 : Chemicals, enzymes and used kits.

	Materials	Trademark
Chemicals	Tris-Base	Carlo Erba
	Boric Acid	Merck
	EDTA	BDH Laboratory
	Imidazole	Merck
	$NaH_2PO_4.2H_2O$	Sigma
	Glycose	Sigma
	NADP	Roche
	Formate	Aldrich
	IPTG	Applichem
	Sodium Asetate	Fluka
	Glyserol	Carlo Erba
	Ampicillin	Roche
	Ethanol	Merck
	Agarose	Applichem
	EZ-vision	Fluka
	dNTP	Roche
	10X Pfu Buffer	Fermentas
	Tango Buffer	Fermentas
	Marker 3 “Lamda DNA”	Fermentas
Enzymes	Pfu Taq Polymerase	Fermentas
	PstI	Fermentas
	SacI	Fermentas
	DpnI	Roche
Kits	High Pure Plasmid Isolation Kit	Agilent

2.1.3 Preparation of media for culture and transformation: Luria-Bertani (LB) and Super Optimal Broth with Catabolite repression (SOC)

Luria-Bertani (LB) liquid and Luria-Bertani (LB) agar medium were used to grow the cell. The usage before, antibiotic which is called ampicillin was added to each LB media at the rate of 1:1000 for growing to culture. In addition, Super Optimal Broth with Catabolite repression (SOC) medium was used for transformation. The component of LB, LB with agar and SOC media are given at below.

Luria-Bertani (LB) media preparation : 10 g NaCl, 10 g tryptone, 5 g yeast extract were weighed and dissolved about 950 mL distilled water (dH_2O) and final volume was completed 1000 mL with distilled water (dH_2O).

Luria-Bertani (LB) media with agar : 10 g NaCl, 10 g tryptone, 5 g yeast extract were weighed and dissolved about 900 mL distilled water (dH_2O). Then, 15 g agar was weighed and added to the media mixture. Finally, the volume of media mixture was completed 1000 mL with distilled water (dH_2O).

Super Optimal Broth with Catabolite repression (SOC) preparation : 20 g tryptone, 5 g yeast extract, 0.58 g NaCl (final concentration 10 mM), 0.186 g KCl (final concentration 2.5 mM) were weighed and dissolved about 950 mL distilled water (dH_2O) and final volume was completed 1000 mL with distilled water (dH_2O). Then, medium was autoclaved at 121 ^oC and under 1 atm pressure for 15 minutes. Finally, 10 ml filtered $MgSO_4$ solution (final concentration 10 mM) and 10 ml filtered glycose solution were added to the medium.

2.1.4 Buffers and solutions

2.1.4.1 TAE buffer (50X)

50x TAE buffer was prepared for using in agarose gel electrophoresis and 50x TAE buffer was diluted with dH_2O to 1x TAE for using to prepare agarose gel and running in the agarose gel tank. Preparation of 50x TAE is given in Table 2.3.

Table 2.3 : Preparation of 50x TAE buffer.

Content	Amount
Tris Base	242 g
Glacial Acetic acid	57.1 mL
EDTA	14.6 g
dH_2O	1 L

2.1.4.2 Protein purification buffers

Protein purification buffers were used for purifying proteins from bacterial pellet by HisTrap purification method metal affinity chromatography and ion-exchange chromatography.

Preparation of protein purification buffers which were used in this study are given at below:

Buffer A: 3.12 g $NaH_2PO_4.2H_2O$ (20mM), 29.22 g NaCl (500 mM) and 2.04 g imidazole (30 mM) were weighed and dissolved in 950 mL dH_2O and final volume was completed 1000 mL with distilled water (dH_2O). Finally, pH was adjusted to 7.4 using NaOH.

Buffer B: 3.12 g $NaH_2PO_4.2H_2O$ (20mM), 29.22 g NaCl (500 mM) and 34.04 g imidazole (500 mM) were weighed and dissolved in 950 mL dH_2O and final volume was completed 1000 mL with distilled water (dH_2O). Finally, pH was adjusted to 7.4 using NaOH.

50 mL Buffer A + 100 mM imidazole: 42.55 mL Buffer A and 7.45 mL Buffer B is mixed.

50 mL Buffer A + 200 mM imidazole: 31.9 mL Buffer A and 18.1 mL Buffer B is mixed.

50 mL Buffer A + 400 mM imidazole: 10.6 mL Buffer A and 39.4 mL Buffer B is mixed.

2.1.4.3 Buffers and solutions for SDS – PAGE analysis

2.1.4.3.1 6x sample buffer

6x sample buffer was used to denature protein samples. This buffer was mixed with our protein sample and was loaded on SDS- polyacrylamide gel. Preparation of 6x sample buffer is given at Table 2.4.

Table 2.4 : Preparation of 6x sample buffer.

Content	Concentration	Amount
Tris-HCl pH: 6.8	0.3 M	1.25 mL (of 1 M)
SDS	6%	6 mL (of 10%)
Glycerol	20%	2 mL (of 100%)
Bromophenol Blue	0.05%	5 mg
DTT	0.15 M	231 mg
dH_2O		Up to 10 mL

2.1.4.3.2 Tris – Tricine running buffer (10X)

Tris-Tricine SDS Buffer (10x) was used as the electrophoresis running buffer at the stacking and resolve process of SDS-PAGE. Preparation of Tris-Tricine buffer is given at below (Table 2.5).

Table 2.5 : Preparation of Tris-Tricine buffer.

Content	Concentration	Amount
Tris Base	1 M	60.55 g
Tricine	1 M	89.60 g
SDS	1%	5 g
dH_2O		Up to 500 mL

2.1.4.3.3 Coomassie Brilliant Blue (CBB) stain solution

CBB stain solution was used to visualize separated protein bands on SDS polyacrylamide gel. Preparation of CBB stain solution is given at Table 2.6.

Table 2.6 : Preparation of CBB stain solution.

Content	Concentration	Amount
CBB R-250	0.1%	0.5 g
Methanol	45%	450 mL
Acetic Acid	10%	100 mL
dH_2O	45%	450 mL

2.1.4.3.4 Destain solution

Destain solution was used to remove the background on SDS polyacrylamide gel and protein bands is become too visible. Preparations of CBB destain solution is given at Table 2.7.

Table 2.7 : Preparations of CBB destain solution.

Content	Concentration	Amount
Methanol	45%	450 mL
Acetic Acid	10%	100 mL
dH_2O	45%	450 mL

2.1.5 pQE – 2 expression vector

TAGZyme pQE-2 expression vector® which is produced by Qiagen was used for expression of mutant *cm*FDH. The vector comprises of 4758 bp in length and includes ampicillin resistance gene as the selective marker. And also, it includes lac operator and lacIq repressor gene for gene expression regulation, uses T5 promoter. In addition, it has also 6xHis tag sequence, which facilitates the protein purification. The wild-type FDH coding sequence was inserted into the multiple cloning sites from *Sac*I and *Pst*I cleavage sites (Figure 2.1).

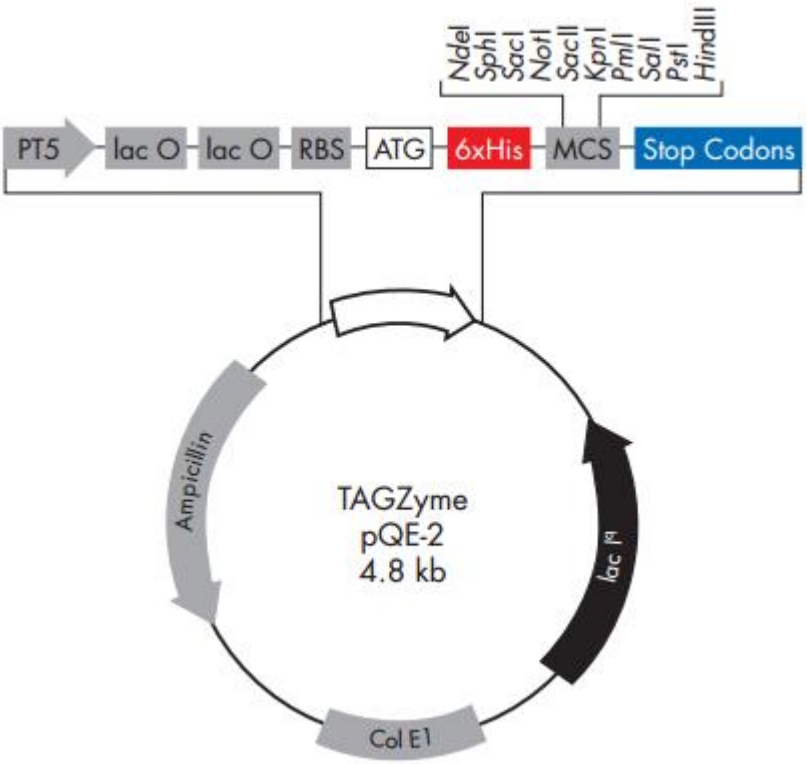

Figure 2.1 : pQE-2 vector [36].

2.1.6 BL – 21 cells

Competent BL-21(DE3) cells which were prepared from commercial BL-21 (DE3) (New England Biolabs) were used for the mutagenesis production at the transformation process. BL-21(DE3) is used *Eschericchia coli* strain for protein expression. It includes λDE3 lysogen phage gene for the expression of T7 polymerase providing high levels of protein expression. Preparation of competent BL-21(DE3) cell is given at section 2.2.5.

2.2 Methods

2.2.1 Site – directed mutagenesis

Site-directed mutagenesis is a rational design technique.. For the increase our knowledge, structure of protein and function associations of the enzyme should be known. The aim of this technique, the desired amino acid at a defined site is altered into another amino acid based on conformational and structural knowledge about the protein.

First step of the design is the development of the model of the desired molecule. This model needs an appropriate algorithm. Experimental studies for the properties of the protein follows after. Rational protein design can be applied to improving the thermostability as it can be applied in other properties of a protein.

Primary structure of the protein should be changed when changing the property of an enzyme. Even the change of a single residue will result in a drastic change of the structure and function of an enzyme. Electrostatic interactions would also be optimized with the change in the structure. Site directed mutagenesis is done by changing the desired sites which is defined with the computational study using polymerase chain reaction with spesific primers carrying the desired mutation.

Primer Design

In this study, we designed one set of primer for target residue (K328V). OligoEvaluator^{TM} programme (http://www.oligoevaluator.com/OligoEvaluator) was used for design primers whose sequences were given at table 2.8.

Table 2.8 : Designed primers for K328V.

Primers	Sequences
K328V-F	**5'**- GATACGCTGAAGGTACTGTTAATATTTTGGAATC-**3'**
K328V-R	**5'**- GATTCCAAAATATTAACAGTACCTTCAGCGTATC -**3'**

PCR conditions

Site-directed mutagenesis PCR was performed to amplify for using *cm*FDH plasmid. PCR mixture and PCR condition were given at table 2.9, 2.10 and 2.11.

Table 2.9 : Site-directed PCR mixture (HF).

Ingredient	Stock Concentration	Volume	Final Concentration
HF buffer	5x	5 µl	0.5x
Primer mix	10 ng/µl	1 µl	1 ng/µl
dNTP mix	10 mM	1 µl	200 µM
dH_2O	-	42.25 µl	-

Table 2.9 (continued): Site-directed PCR mixture (HF).

Ingredient	Stock Concentration	Volume	Final Concentration
Template DNA	203.5 ng/µl	0.25 µl	50 ng/µl
Pfu polymerase	2 U/µl	0.5 µl	0.02 U/µl
TOTAL		50 µl	

Table 2.10 : Site-directed PCR mixture (GC).

Ingredient	Stock Concentration	Volume	Final Concentration
GC buffer	5x	5 µl	0.5x
Primer mix	10 ng/µl	1 µl	1 ng/µl
dNTP mix	10 mM	1 µl	200 µM
dH_2O	-	41.25 µl	-
Template DNA	203.5 ng/µl	0.25 µl	50 ng/µl
DMSO	-	1 µl	-
Pfu polymerase	2 U/µl	0.5 µl	0.02 U/µl
TOTAL		50 µl	

Table 2.11 : Site-directed PCR conditions.

Cycle Number	Degree	Time	Phase
1	98°C	30 sec	Initial Denaturation
18	98°C	30 sec	Denaturation
	55°C	1 min	Annealing
	72°C	6 min	Elongation
1	72°C	5 min	Final Extension
1	4°C	∞	Final Hold

2.2.2 Control of PCR products

After the PCR reaction, PCR products are controlled in agarose gel electrophoresis. The size of used PCR product (plasmid) consists of about 6 kb (4758 bp pQE-2 vector + 1094 bp *cm*FDH gene). This control is performed at 1% agarose gel (prepared of 1% agarose gel is given below). 5 µl of PCR products were mixed with 1 µl EZ-vision one DNA dye & buffer and then mixing samples are loaded into the wells. In addition, for determining of the size of PCR product, 3 µl DNA marker (Lambda DNA/*Eco*RI+*Hind*III, Marker 3® by Fermantas) was used and added into wells. Loaded samples were run at 120 V for 30-35 minutes and 1% agarose gel observed under UV light.

2.2.3 *Dpn*I treatment

After controlled of PCR products at the 1% agarose gel electrophoresis, PCR products were digested with *Dpn*I enzyme, which identifies the methylated adenine sites, the methylated DNA and dam+ strains, which is given restriction site at Figure 8. Therefore, the vector, which carries to original DNA sequence, was removed from the PCR product that includes dam+ strain. In this study, we treated to 40 µl of PCR product with added on 1 µl *Dpn*I enzyme. The reaction was performed reaction at 37°C for 2 hours.

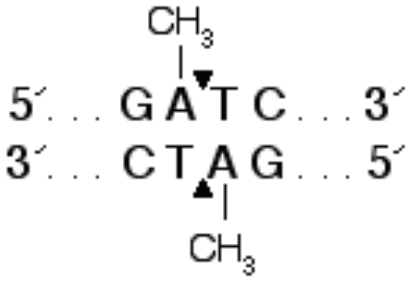

Figure 2.2 : Restriction site of *Dpn*I.

2.2.4 Competent cell preparation

In this study, we prepared BL-21 chemical competent cells from using commercial BL-21 cells (New England Biolabs) for transforming of mutagenesis PCR product.

The preparation of BL-21 competent cells are given at below:

- Cells are taken from the glycerol stock and cultivated into LB-Amp plate with a needle. Cultivated plates were incubated at orbital shaker at 37 °C for 16-20 hours.
- A single colony was picked up from plates and it was cultivated into 5 µl LB-liquid and again was incubated at orbital shaker at 37 °C for 16-20 hours.
- 1 mL of incubated cells were taken and cultivated into 50 mL LB-liquid media and were incubated at orbital shaker at 37 °C until OD_{600} reach to 0.4-0.6.
- Incubated cells were transferred into 50 mL falcon tubes and waited on ice for 10 minutes.
- Waited falcon tubes were centrifuged at 5000 rpm for 10 minutes.
- The supernatant was discarded and the pellet was resuspended with 30 mL cold 80 mM $MgCl_2$ + 20 mM $CaCl_2$ and waited on ice for 5 minutes (This step is repeated for 2 times).
- Falcon tubes were centrifuged at 5000 rpm for 10 minutes.
- Pellets were resuspended with 100 mM $CaCl_2$ + 15% glycerol solution. This mixture was aliquoted into 50-100 µl eppendorf tubes and was kept at -80°C.

2.2.5 Transformation

PCR products that are digested with *Dpn*I were transformed into BL-21 chemical competent cells. On this purpose, 0.5 of PCR product was added into 50 µl BL21 chemical competent cell and tube was waited on ice for 30 minutes. Then, tubes was waited into water-bath at 42°C for 30-45 seconds, immediately tube was put on the ice and was waited for 1-2 minutes. After this step, 250 µl S.O.C medium was added into tube and tube was shaken at orbital shaker at 37 °C for 1 hour. Finally, 50-100 µl of transformation product was taken and spread on LB-Agar-Amp plate. The plate was incubated overnight at 37 °C.

2.2.6 Mutation confirmation

For the mutation confirmation, 4 colonies were picked randomly and cultured through overnight incubation at 37°C, then plasmid DNA was isolated as mentioned in section 2.2.8.

2.2.7 *Pst*I / *Sac*I restriction

For the mutation confirmation, isolated plasmid DNA was digested by *Sac*I/*Pst*I restriction enzymes, in order to accuracy of *cm*FDH gene if inserted into *Sac*I/*Pst*I restriction sites of the PQE-2 vector. In this study, 2.5 µl of plasmid DNA was digested with *Sac*I: *Pst*I (1:1 units) for 2 hours at 37°C.

2.2.8 Plasmid isolation

This process was performed with StrataPrep Plasmid Miniprep Kit by Agilent.

A single colony was taken from LB-Agar-Amp plate and each colony was inoculated into the 5 ml LB liquid medium containing 100 µg/ml ampicillin. Cells were incubated at 37°C for 16 hours. 1.5 ml of cell cultures are aliquoted into a 1.5 mL eppendorf tube. Tubes are centrifuged at 13000 rpm for 1 minute. After centrifugation, supernatant phase is removed and discarded. 100 µ-l of solution is added to the eppendorf tube. Tube is vortexing or pipeting to resuspend and completely disperse the cells. 100 µl of solution-2 is added to the eppendorf tube. Tube is mixed gently until homogenous by inverting the tube several times. 125 µl of solution-3 is added to the eppendorf tube. The tube is mixed by inverting the tube several times. The tube is centrifuged at 13000 rpm for 5 minutes. Then supernatant is transferred into high pure filter which is placed in a 2-ml receptacle tube. Tube is centrifuged at 13000 rpm for 30 seconds for passing of liquid and our plasmid DNA is clinged to filter. The liquid that passed through the collection tube is discarded. 750 µl of Nuclease Removal Buffer is added onto filter tube and is centrifuged at 13000 rpm for 30 seconds. The liquid that passed through the collection tube is discarded again. 750 µl of 1x Wash Buffer is added onto filter tube for washing of our plasmid. Then, tube is centrifuged at 13000 rpm for 30 seconds, liquid phase is removed and again tube is centrifuged at 13000 rpm for 30 seconds. Finally, 50 µl of Elution Buffer is loaded onto filter tube for eluting to plasmid and is waited at room temperature for 5 minutes. Tube is centrifuged at 13000 rpm for 1 minute. The eluted plasmid DNA was measured at nanodrop for controlling of concentration. Content of the solution-1-2, elution and 1x Wash Buffer is given at Table 2.12.

Table 2.12 : Content of plasmid purification kit of solutions.

Solution 1	Solution 2	Elution Buffer	2xWash Buffer
50 mM Tris-HCl (pH: 7.5)	0.2 M NaOH	10 mM Tris Base Adjust pH: 8.5 with HCl	10 mM Tris-HCl (pH: 7.5)
10 mM EDTA 50 µg/mL RNaseA	1% (w/v) SDS		100 mM NaCl 2.5 mM EDTA

2.2.9 Sequence analysis

Sequence analysis was performed from isolated plasmid DNA which was purified from the transformed colonies which were picked randomly. Sequence of the mutantion was controlled and compared with native *cm*FDH by BioEdit programme.

2.2.10 Expression of *cm*FDH protein

pQE-2 vector with cloned *cm*FDH gene was transformed into BL21 competent cells. A single colony was chosen from transformed mutant and native *cm*FDH. Native and mutant *cm*FDH were inoculated into 50 ml LB-liquid media in flasks which contain 50 µl ampicillin. Then, flasks were incubated at orbital shaker for overnight at 37ºC. The next day, growing pre-cultures were transferred into 1 L LB-liquid media (this media contains 1 mL ampicillin) and culture was incubated OD_{600} value of cultures reaches 0.6. Then, 1 M IPTG was added into culture (final concentration of IPTG is 0.1 mM) and waited at 37ºC for 4 hours due to induce protein expression. Finally, expressed culture was centrifuged at 5000 rpm for 15 minutes and pellets were stored at -20 ºC.

2.2.11 HisTrap purification of 6xHis tagged *cm*FDH protein

After precipitation of cells containing mutant FDH proteins, purification protocol of proteins were performed as follows; precipitated cells treated with Lysis buffer which contains 5 mL Buffer A+5mg lysozyme and cells was resuspended completely. Resuspended cells were waited on ice for 1 hour. Then resuspended cells were disrupted by sonication (20 sec pulse on/20 sec pulse off). Sonicated cells were centrifuged at 5000 rpm for 20 minutes and supernatant which is called clarified lysate was transferred into new falcon tubes. Clarified lysate was filtered with 0.45 µl for 2 times. Filtered lysate was loaded to HisTrap column which is equilibrated with 10 mL dH_2O and 5 mL Buffer A and lysate was passed from column (2 times). Column was washed with 5 mL Buffer A and passed fraction from column was collected into a new

tube. Then, column was washed respectively, 3 mL Buffer A + 100 mM imidazole, 5 mL Buffer A + 200 mM imidazole, 3 mL Buffer A + 400 mM imidazole. Finally, column was washed with 10 mL Buffer B and fraction was collected into a new falcon tube.

2.2.12 SDS-polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

Tricine/polyacrylamide gel with 12% acrylamide concentration was prepared for expressed *cm*FDH which was expected to be around 32-45 kDa. Contents of each part of gels are shown in Table 2.13 and 2.14.

Table 2.13 : 12% separating gel solution (5 ml).

Contents	Volume
40% acrylamide-bisacrylamide	1.5 mL
Tris-HCl (pH: 8.8, 1.5 M)	1.3 mL
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl
dH_2O	2 ml

Table 2.14: 5% separating gel solution (2 ml).

Contents	Volume
40% acrylamide-bisacrylamide	0.25 mL
Tris-HCl (pH: 6.8, 1 M)	0.25 mL
10% SDS	20 µl
10% APS	20 µl
TEMED	5 µl
dH_2O	1.4 ml

The separating gel solution was performed into the gel cassette up to ± 6.5 cm, and, the last ± 2.5 cm of the cassette was filled with isopropanol for upper part of gel is smooth, immediately. After the gel was polymerized, the isopropanol was removed by use of filter papers. The stacking gel solution was directly poured into the gel cassette, and the gel comb was placed to form the slots. The stacking gel was waited to polymerized.

The samples were denaturated at 95ºC for 10 min and were loaded on the SDS-PAGE gel. As molecular weight marker, Unstained Protein Molecular Weight Marker (Thermo Scientific) which contains 7 unstained protein bands in the range of 14.4-116

kDa was used and was loaded on the gel. Samples was run at SDS-PAGE firstly 110 V until samples reach to stacking gel, then, gel was run at 180 V.

After electrophoresis, SDS-PAGE was stained in CBB stain solution and gel was waited overnight on shaker for dying completely. Finally, it was destained in destain solution about 30 minutes and gel was kept into dH_2O.

2.2.13 Bradford protein assay

Quantification of *cm*FDH was performed by Bradford protein assay according to manufacturers (BioRad) protocol as follows : 195 µl of 1x dye reagent was put into each 10 well. 5 µl of bovine serum albumin (BSA) was pipetted onto each 8 well in certain concentrations between 0 to 2 mg/ml. 5 µl of protein samples that contain unknown concentrations were added into the last 2 well. Plate was kept in dark for 5 minutes and colorimetric mesaurement was performed at 595 nm for 10 seconds by manufacturers device (Perkin Elmer).

2.2.14 *cm*FDH protein activity assay

Activity of *cm*FDH protein was measured by using NAD^+ and formate coupled assay. For this purpose, we measured towards changing substrate concentration for determined *cm*FDH enzyme activity. Reaction was prepared as 200 µl which contains 50 µl *cm*FDH enzyme, 50 µl NAD^+ mixture (final concentration: 1 mM) and 100 µl substrate mixture (final concentration: 0-40 mM). Reaction was performed at the room temperature (25 ^oC) with varying formate concentrations by the spectrophotometer under 340 nm wavelength. Preparation of the NAD^+ mixture and substrate mixture were given at below:

NAD^+ mixture: 27.42 mg NAD^+ was weighed and dissolved in 10 ml Tris-HCl pH: 8.0 (4 mM)

Substrate mixture: 272 g sodium formate was weighed and dissolved in 50 ml Tris-HCl pH: 8.0 (80 mM). Then, 80 mM solution was diluted to used concentrations with Tris-HCl pH: 8.0.

3. RESULTS

3.1 Mutagenesis PCR Control

In order to obtain mutant *cm*FDH, we carried out the PCR. After that, agarose gel electrophoresis was performed to verify the product of site-directed mutagenesis PCR. Our product consists of nearly 6000 bp (4758 bp pQE-2 vector + 1094 bp *cm*FDH gene) which was monitored under the UV light. Agarose gel image of mutant PCR product is given in Figure 3.1.

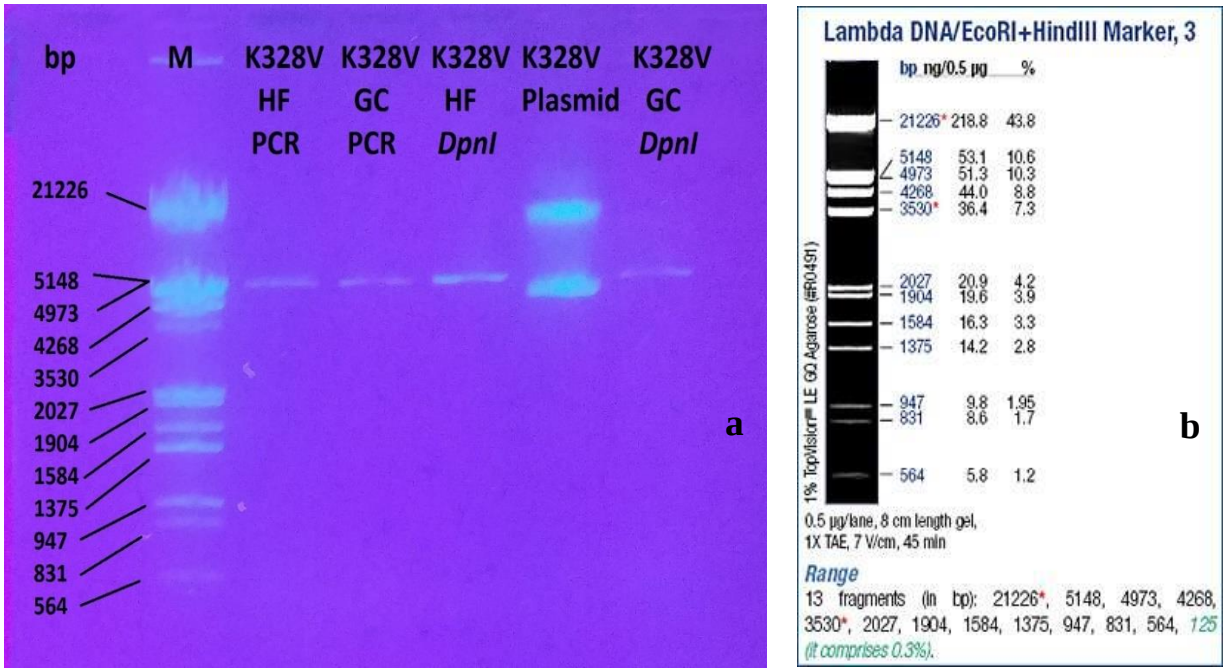

Figure 3.1 : Agarose gel image of mutagenesis PCR product. (a) Agarose gel image of K328V. M: Marker 3 (λ DNA / *Eco*RI + *Hind* III (Fermentas)); 21226 bp, 5184 bp, 4973 bp, 4268bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp, 564 bp. HF and GC are the PCR products of mutant K328V. HF *DpnI* and GC *DpnI* are the PCR products of mutant K328V which were treated with *DpnI* enzyme. (b) Marker 3 (λ DNA / *Eco*RI + *Hind* III (Fermentas)).

3.2 Mutation Confirmation with *Sac*I/*Pst*I restriction enzyme

Confirmation of mutation, which contains desired positions' alteration, PCR products were restricted by *Sac*I/*Pst*I restriction enzymes. Agarose gel electrophoresis was performed with *Sac*I/*Pst*I restriction enzymes for determination of insert and vector (Figure 3.2).

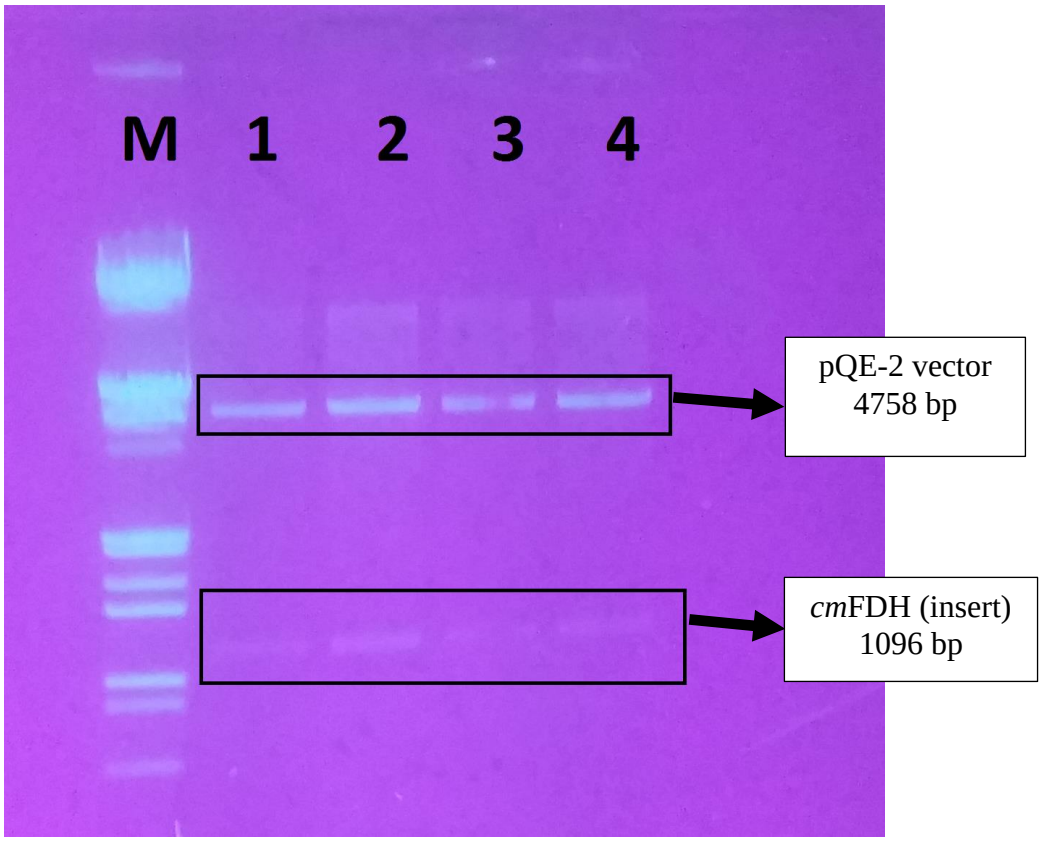

Figure 3.2 : Restriction of the Q105R/K328V plasmids with *Sac*I/*Pst*I enzyme. M: Marker 3 (λ DNA / *EcoR*I + *Hind* III (Fermentas)); 21226 bp, 5184 bp, 4973 bp, 4268bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp, 564 bp. 1, 2, 3, 4: Q105R/K328V plasmids.

3.3 Transformation

After the PCR which their products were checked on agarose gel electrophoresis, PCR product that includes mutation Q105R and Q105R/K328V was digested with *Dpn*I enzyme. Digested samples were transformed into chemically competent BL-21 cells. Image of colonies obtained after transformation are shown at Figure 3.3.

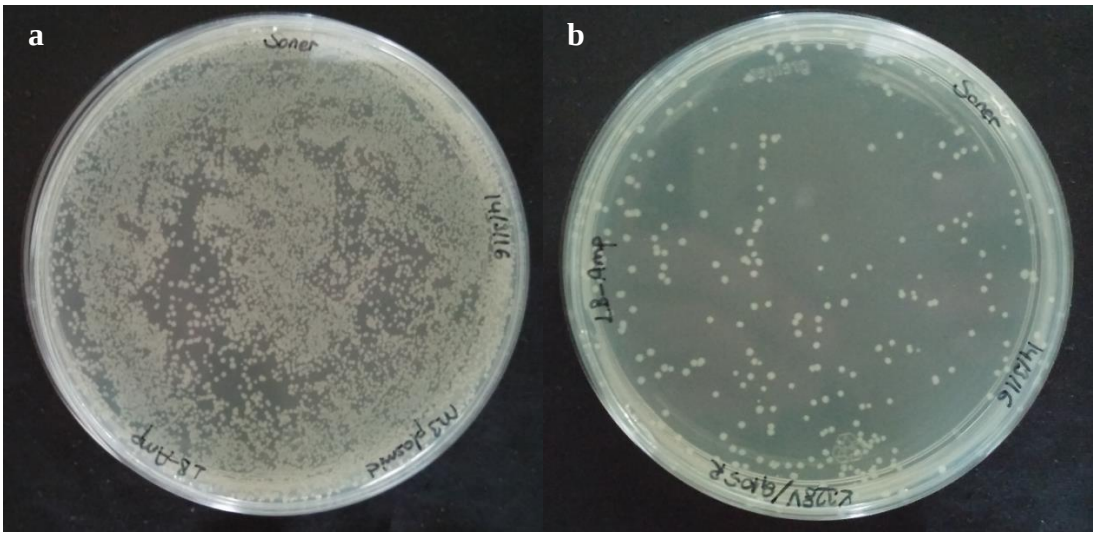

Figure 3.3 : Transformation of *Cm*FDH cells **(a)**Q105R cell colonies as result of transformation. **(b)** Q105R/K328V colonies as a result of transformation.

3.4 Plasmid isolation

Subsequently the transformation, three random colonies were chosen on Q105R/K328V plate. Plasmid isolation was done for these three colonies by using StrataPrep Plasmid Miniprep Kit-Agilent. Accuracy of isolated plasmids were checked by agarose gel electrophoresis (Figure 3.4).

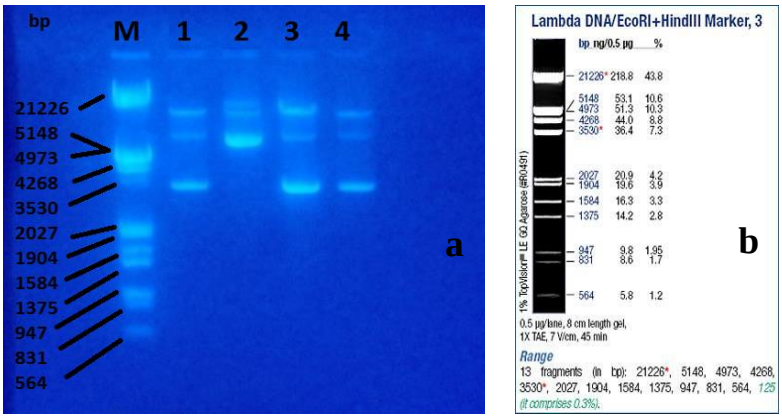

Figure 3.4 : Agarose gel image of *Cm*FDH plasmids. **(a)** Agarose gel image of Q105R and Q105R/K328V plasmids. M: Marker 3 (λ DNA / *EcoR*I + *Hind* III (Fermentas)); 21226 bp, 5184 bp, 4973 bp, 4268bp, 3530 bp, 2027 bp, 1904 bp, 1584 bp, 1375 bp, 947 bp, 831 bp, 564 bp. 1, 2, 3: Q105R/K328V plasmid, 4: Q105R plasmid. **(b)** Marker 3 (λ DNA / *EcoR*I + *Hind* III (Fermentas)).

3.5 Accuracy of DNA sequencing

To determine K328V mutation, Q105R and Q105R/K328V plasmids were sequenced with pQE-2 promoter and reverse primer. BioEdit programme was used to compare the base changes with sequence of Q105R and Q105R/K328V plasmids are shown at Figure 3.5 and 3.6.

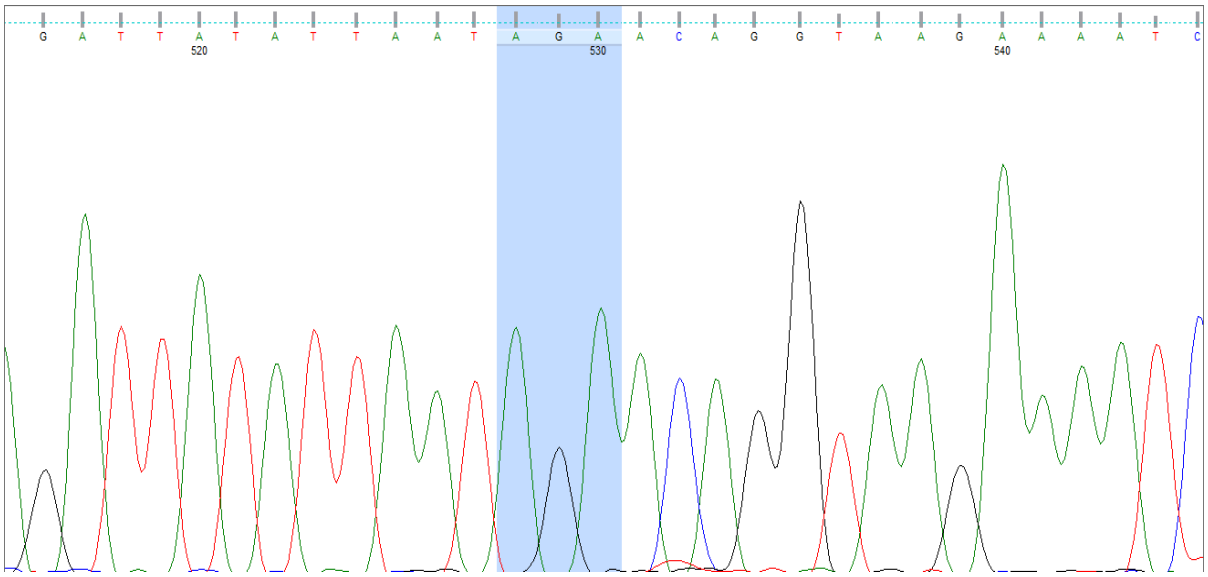

Figure 3.5. Sequences alignment of the Q105R.

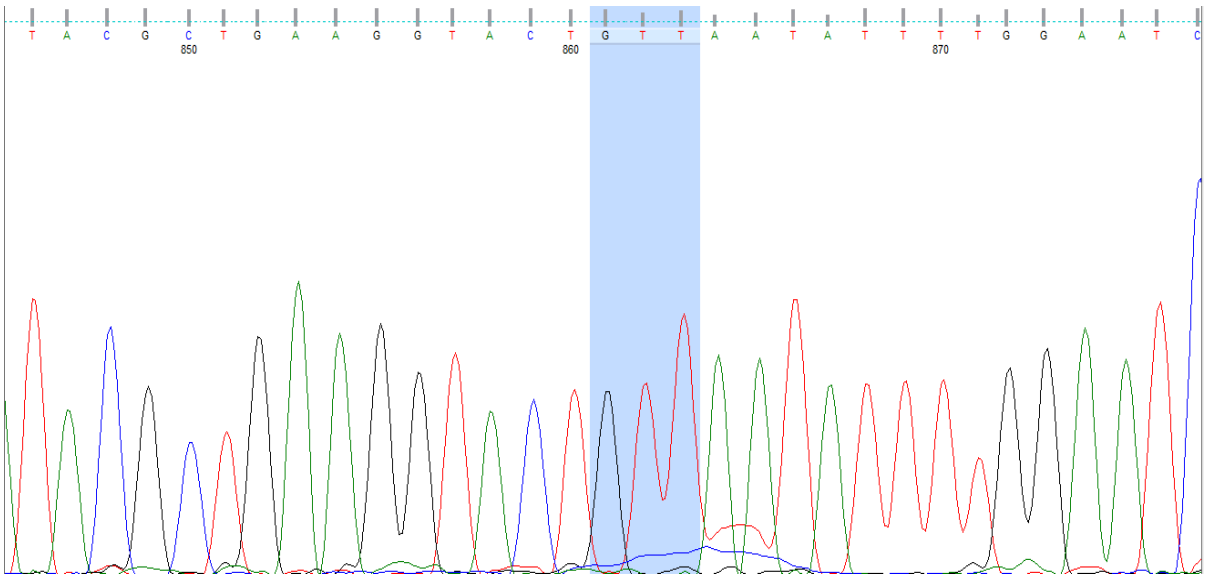

Figure 3.6. Sequences alignment of the Q105R/K328V.

3.6 Protein Purification Control (SDS-PAGE)

After the accuracy of mutation, Q105R and Q105R/K328V plasmids were transformed into BL-21 cells and proteins were overexpressed by inducing with IPTG, which provides overexpression of desired protein. Protein purification was done by using Ni-NTA HisTrap column. Protein samples were ckecked by SDS-PAGE for determination of protein purity. According to SDS-PAGE result, the pure bands were collected and were diluted bu using ultrafilteration. These ultrafiltered enzymes will be used for steady-state kinetic experiments. Protein bands were observed around 45 kDa seen at Figure 3.7.

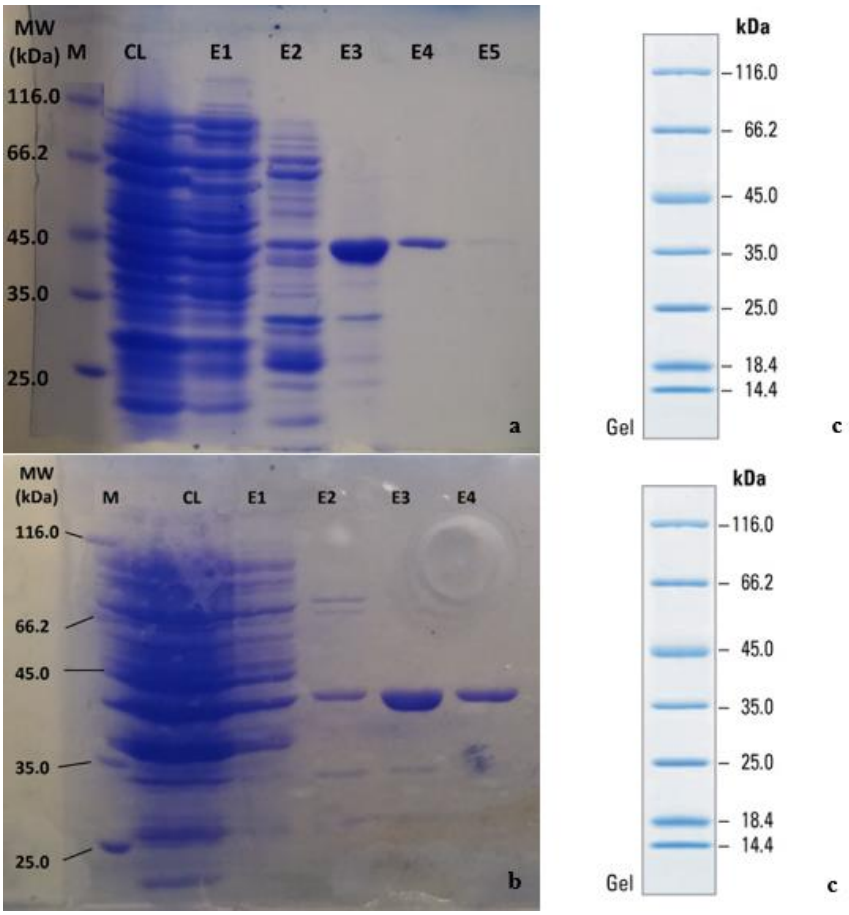

Figure 3.7 : SDS-PAGE analysis of *Cm*FDH protein samples. **(a)**SDS-PAGE analysis of Q105R/K328V protein samples. M: Marker 116.0 kDa, 66.2 kDa, 45.0 kDa, 35.0 kDa, 25.0 kDa, CL: clarified lysate, E1-E5: Elution 1-5. **(b)** SDS-PAGE analysis of Q105R protein samples. M: Marker 116.0 kDa, 66.2 kDa, 45.0 kDa, 35.0 kDa, 25.0 kDa, CL: clarified lysate, E1-E4: Elution 1-4. **(c)** Unstained Protein Ladder (Thermo Scientific Fisher).

3.7 Steady-State Kinetics

After the SDS-PAGE analysis, E3 and E4 fractions were collected and both samples were combined. Until final volume (50 ml), 50 mM Tris-HCl pH: 8.0 solution was added into collected fractions. The enzymes were centrifuged to use ultrafiltration tubes for several times (Until obtaining nearly 5 ml enzyme). According to Bradford protein assay, we measured Q105R and Q105R/K328V separately in the result of 0.731-0.337.After that, kinetic measurements were performed at 25 ^{o}C for collected enzymes. Reaction mixture which includes 50 µl enzyme, 50 µl 1 mM NAD^{+} and 100 µl substrate mixture (0-40 mM) was prepared from sodium formate. All measurements for the kinetic activity was made as twice. Hyper32 programme was used to analyse the Km and kcat value and Michelis-Menten graphs which are given at Figure 3.8 and 3.9 were graphed for Q105R and Q105R/K328V enzymes. Km and kcat values and specific activity of Q105R and Q105R/K328V enzymes are shown at Table 3.1, 3.2 and 3.3.

Table 3.1 : Specific activity of Q105R.

Final Concentrations of Substrate (formate) (mM)	Slope	Specific Activity (µmol/sec.mg)
0	**0**	**0**
1	**0,0833**	**0,00222783**
2	**0,1288**	**0,003444711**
3	**0,1643**	**0,004394147**
5	**0,2245**	**0,006004175**
7,5	**0,2697**	**0,007213033**
10	**0,29755**	**0,007957872**
12,5	**0,28525**	**0,007628913**
15	**0,2921**	**0,007812113**
17,5	**0,2955**	**0,007903045**
20	**0,3027**	**0,008095607**
22,5	**0,30495**	**0,008155782**
25	**0,3101**	**0,008293517**
30	**0,3148**	**0,008419217**
40	**0,3192**	**0,008536894**

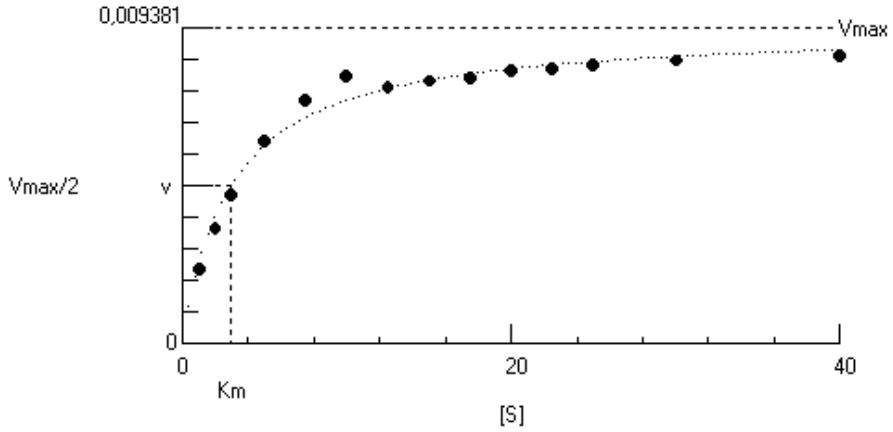

Figure 3.8 : Michelis-Menten graph of Q105R.

Table 3.2 : Specific activity of Q105R/K328V.

Final Concentrations of Substrate (formate) (mM)	Slope	Specific Activity (µmol/sec.mg)
0	**0**	**0**
1	**0,02385**	**0,001383607**
2	**0,0416**	**0,002413336**
3	**0,058**	**0,003364747**
5	**0,0778**	**0,004513402**
7,5	**0,10405**	**0,006036241**
10	**0,11775**	**0,006831017**
12,5	**0,1269**	**0,007361835**
15	**0,13205**	**0,007660601**
17,5	**0,1467**	**0,00851049**
20	**0,1594**	**0,009247254**
22,5	**0,16085**	**0,009331372**
25	**0,17275**	**0,010021726**
30	**0,1957**	**0,011353122**
40	**0,20905**	**0,012127594**

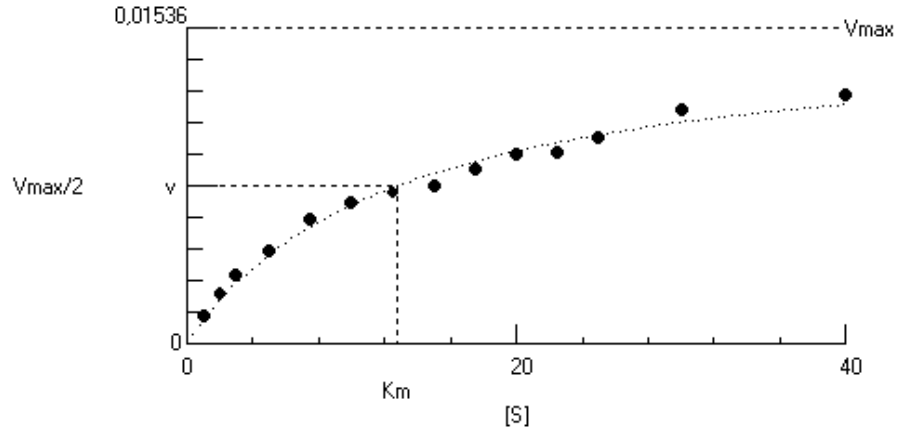

Figure 3.9 : Michelis-Menten graph of Q105R/K328V.

Table 3.3 : K_m and k_{cat} values of studied enzymes.

Enzyme	Km (mM)	k_{cat} (sn^{-1})	kcat/Km (sn^{-1} mM^{-1})
Q105R	2.963±0.5959	0,384621	0,129808
K328V + Q105R	12.77±3.068	0,62976	0,049316

4. DISCUSSION

In this study, we aimed to increase specific activity of NAD^+-dependent mutant Q105R *Candida methylica* formate dehydrogenase (*cm*FDH) which has increased thermostability [35].

For this purpose, 328th amino acid position was determined from *Candida boidinii* organism that shows nearly 98% similarity with *Candida methylica* according to Schirwitz's article [7]. We expected that Km value was increased when compared to that study. However, unexpectedly, specific activity of *cm*FDH carrying double mutant (Q105R/K328V) did not increase. In K328V mutation, we converted lysine to valine amino acid. As known, lysine is a positively charged amino acid and valine is a neutral charged amino acid residue. Therefore, K328V position changes could have been negatively affect affinity of our mutant enzyme. The other reason of decrasing the activity of enzymes could be occured because of the amino acid size since valine has too small size when compared to lysine. This situtation can also affect interaction of substrate with the enzyme's active site.

To overcome the low specific activity of *Candida methylica* formate dehydrogenase, alternative mutants can be applied on Q105R thermostabile mutant.

5. CONCLUSIONS

NAD^+-dependent formate dehydrogenase (EC 1.2.1.2) belongs to oxidoreductase family. NAD^+ and sodium formate are reduced to NADH and CO_2 by formate dehydrogenase in the organisms. Formate dehydrogenase is used in various application such as chemistry and industrial area because of their great potential. However, in industrial process, some extreme conditions can affect activity of these enzymes such as high temperature, pressure and pH. Therefore, they have limited activity when used in reactions. Recently, protein engineering studies have helped for solving limitation problem and improving the enzyme functionality.

In this study, we used site-directed mutagenesis technique for increasing specific activity of Q105R mutant which is accepted as the most thermostabile *cm*FDH [35]. Firstly, we designed a primer set for the alteration of lysine into valine at 328^{th} position (K328V). After that, we performed PCR in order to amplify the mutant gene. Agarose gel electrophoresis is performed to visualize the PCR products. Then, mutant cells were incubated and overexpressed for producing more protein. SDS-PAGE was applied to the protein samples. Finally, enzymatic activity of Q105R/K328V mutant was measured at constant NAD^+ and various substrate concentrations. As a result of the study, while Km value of Q105R/K328V was decreased, kcat value was increased.

In the further studies, alternative mutants will be constructed by using the Q105R mutant as a template to increase the specific activity of thermostabile *cm*FDH. Thermostability experiments of double mutant will also be analysed.

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