İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

ATTEMPTS TO CHANGE COENZYME SPECIFICITY OF NAD^+ DEPENDENT FORMATE DEHYDROGENASE FROM *Candida methylica* BY RATIONAL DESIGN

M.Sc. Thesis by

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Department: **Advanced Technologies**

Programme: **Molecular Biology-Genetics and Biotechnology**

JUNE 2008

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Candida methylica KAYNAKLI NAD^+- BAĞIMLI FORMAT DEHİDROJENAZ ENZİMİNİN KOENZİM ÖZELLİĞİNİN RASYONEL DİZAYN İLE DEĞİŞTİRİLME DENEMELERİ

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ABBREVIATIONS

***cm*FDH**	**:** *Candida Methylica* Formate Dehydrogenase
DNA	**:** Deoxyribonucleic acid
FDH	**:** Formate Dehydrogenase
FIDH,	**:** Formaldehyde dehydrogenase
GTCHO	**:** Sformylglutathione
$GTCH_2HO$	**:** Shydroxymethylglutathione
MO	**:** Methanol Oxidase
NAD	**:** Nicotinamide Adenine Dinucleotide
NaCI	**:** Sodium Chloride
NADP	**:** Nicotinamide Adenine Dinucleotide Phosphate
LB	**:** Luria-Bertani
***pse*FDH**	**:** *Pseudomonas sp.* 101 Formate Dehydrogenase
PCR	: Polimerase Chain Reaction
SDS-PAGE	**:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
***sec*FDH**	**:** *Saccharomyces cerevisiae* Formate Dehydrogenas

ATTEMPTS TO CHANGE COENZYME SPECIFICITY OF NAD^+ DEPENDENT FORMATE DEHYDROGENASE FROM *Candida methylica* BY RATIONAL DESIGN

SUMMARY

NAD^+ dependent formate dehydrogenase (EC 1.2.1.2, FDH) belongs to the super family of D-specific 2-hydroxy acid dehydrogenase. Highly evolutionarily conserved FDH gene is expressed in mostly methylotrophic yeasts and several plants. NAD^+ dependent FDH is the last enzyme in the metabolism of methanol and catalyzes the oxidation of formate anion into carbon dioxide concomitant with the reduction of NAD^+ to NADH, which has a crucial importance in industrial redox chemistry.

In the coenzyme regeneration system, it functions as a regenerator of reduced coenzyme-NADH and enables to produce optically active chiral compound by irriversible reaction. Due to all naturally available NAD^+ dependent FDHs exhibit a high preference for NAD^+ over $NADP^+$ in the redox reactions and thus produce only NADH in the reaction, it would desirable to make FDH enable to bind $NADP^+$ and reduce into NADPH.

However, the molecular basis of the coenzyme specificity of FDHs enzyme is the fundamental challenging for molecular biologist. So far, general approach for changing the coenzyme specificity of FDHs has not been defined clearly. Today, rational design, namely site directed mutagenesis, which is the scope of this study is commonly applied method for this approach.

In this study, it is aimed to introduce single and double mutations to change the substrate specifity of *candida methylica* formate dehdrogenase (*cm*FDH) to remove the absolute requirement for NAD^+ over $NADP^+$ shown by the wild type enzyme. *Cm*FDH with its coenzyme specificty has been tried to change by indroducing a single Aspartic acid195→Serine (D195S) and double Aspartic acid195→Serine plus Tyrosine196→Arginine (D195S+Y196R) point mutations respectively.

These candidate aminoacids were detected based on the previous results of homology remodeling of a NAD^+ specific FDH from *saccharomyces cerevisiae* (*sce*FDH), *candida boidinii* (*cb*FDH) and *pseudomonas sp.* 101 (*ps*FDH). Thus, by this study aspartic acid and tyrosine positioned at 195^{th} and 196^{th} aminoacid sequence of *cm*FDH respectively for the first time were replaced together with serine and arginine to alter the coenzyme specificity of $NADP^+$.

Candida methylica KAYNAKLI NAD^+- BAĞIMLI FORMAT DEHİDROJENAZ ENZİMİNİN KOENZİM ÖZELLİĞİNİN RASYONEL DİZAYN İLE DEĞİŞTİRİLME DENEMELERİ

ÖZET

NAD^+ bağımlı format dehidrojenaz (EC 1.2.1.2, FDH), D-spesifik 2-hidroksi asid dehidrojenaz superfamilyasına ait bir enzimdir. Evrimsel olarak yüksek oranda korunmuş gen yapısına sahip olan bu enzim çoğunlukla metilotrofik mayalarda ve bazı bitkilerle eksprese edilir. FDH enzimi metanol metabolizmasında ki son enzimdir ve format anyonunun oksidazyonununu katalize ederek karbondiokside dönüştürürken endüstriyel redoks kimyasında çok önemli olan NAD^+'' nın indirgenerek NADH' a dönüşmesini de gerçekleştirir.

Koenzim rejenerasyon sisteminde, FDH enzimi indirgenmiş koenzim NADH rejenerasyonun gerçekleştirirken tek yönlü reaksiyonla optik olarak aktiv olan kiral moleküllerin üretilmesine de olanak sağlar. Redoks reaksiyonlarında doğada bulunan tüm FDH enzimlerinin koenzim olarak NAD^+'' ı $NADP^+$ ye tercih etmesi, FDH'ın $NADP^+$ bağlanabilmesinin ve NADPH' a indirgeyebilmesinin gereklililiğini ortaya çıkarmıştır.

Bununla beraber, günümüzde, halan FDH enziminin koenzim spesifikliğinin moleküler temelleri tam olarak açıklanamamıştır. Şimdiye kadar yapılan denemeler ise de FDH'in koenzim spesifikliğini değiştirmeye yönelik genel bir yaklaşım ortaya koyamamıştır. Rasyonel dizayn, bir başka ifadeyle bu çalışmanında konusu olan yönlendirilmiş mutasyon analizi, bahsi gecen amaç için sıklıkla kullanılan yöntemlerden biridir.

Bu çalışmada, *cm*FDH' enziminin NAD^+ 'olan koenzim spesifikliği, *cm*FDH geninde yapılacak tek ve çift mutasyonlar ile değiştirilerek $NADP^+$'' ye özel hale getirilmeye çalışılmaktadır. *cm*FDH enzimini koenzim spesifikliğini tek Aspartik asit195→Serin(D195S) ve çift Aspartik asit195 → Serin + Tirosin196 → Arjinin (D195S+Y196R) mutasyonlarını ile değiştirilirek $NADP^+$ koenzimini kullanabilir hale getirilmesi amaçlandı.

Bu çalışmada aday amino asitler, *saccharomyces cerevisiae* (*sce*FDH), *candida boidinii* (*cb*FDH) and *pseudomonas sp.* 101 (*ps*FDH) kaynaklı format dehidrejenaz enzimlerinin yapılmış olan homoloji modellerinden faydalınarak tespit edildi. Bu çalışma ile ilk kez 195. ve 196. pozisyonda bulunan aspartik asit ve tirosin, serin ve arjinin ile yerdeğiştirilmiş oldu.

1. AIM OF THE THESIS

The purpose of this thesis is by introducing a single and double mutations, regarding the rational design princibles, to change the substrate specifity of *candida methylica* formate dehdrogenase (*cm*FDH) to remove the absolute requirement for NAD^+ over $NADP^+$ shown by the wild type enzyme.

2. INTRODUCTION

2.1 Environmental Biocatalysis and Green Process

Nowadays, reducing the environmental pollutant chemical processes and petroleum-based technologies is the agenda of scientists from all disciplines and obviously, advances in both chemical catalysis and biocatalysis are the key determinant from this aspect.

Long term effects of end procucts of dirty fuels and chemical based processes are very clear today. Everbody are being exposed to the risk of health problems, global warming, dramatic changes in weather patterns, droughts and dirty water. All these effects have traditionally been associated with unfriendly chemical based techologies.

On the hand, biocatalysis, which is also termed as white technologies is chance to change the faith of living world, since bioenzyme based technologies are environmentally friendly processes and be able to remediate many compounds unfriendly or even toxic to the environment.

Green chemistry is a concept defined as the desing, development and application of processess and products to reduce or eliminate the use and generation of substances hazardous to human health and the environment (Armor, 1999).

Biocatalysts, which are either enzymes or whole microorganism can be a greener alternative to still applied organic synthesis. This replacement offers appropriate reaction for the industrial transformation of natural or synthetic materials under the mild reaction conditions.

Moreover, it enable to solve major problems of traditional methodology by lowering higher energy requirements and minimizing the problems of isomerization, racemization and rearrangement (Alcalde et al. 2006).

Biocatalysts are biodegradable and have chemoselectivity and diastereoselectivity and enantioselectivity. In addition bioenzymes can have function in relatively lower temperature, higher pH range and pressure as well (Johannes and Zhao, 2006).

Enzymes are chiral molecules, since almost all enzymes are made from L-amino acids. Thus, if the chirality present in the substrate molecule, then molecule can be recognized by enzyme upon the formation of the enzyme-substrate complex (Schmidt-Dannert, 2001; Schoemaker et al. 2003).

As a consequence of this reaction, prochiral substrate may be transformed into commercially important and optically active product, since they are mostly used in pharmacy and agrochemistry as an ingredients of most best seller medicines.

Today, 500-bestseller medicines are produced by bioenzymes (Schoemaker et al. 2003). These properties of biocatalyts decrease by-product formation and avoiding the need for functional-group activation, protection or deprotection.

Currently, although, there are good examples of replacement of traditional organic processes by a white or greener biocatalysis technology in various industries, in general, bioenzymes isolated from organisms may not be used directly in industrial processes.

The characteristics, such as activity, stability, specificity, solubility, optimum pH and temperature ranges of candidate enzymes should be optimized usually based on the types industrial processess (Burton et al. 2002).

In the last ten years, promising advances in protein engineering technologies make possible to design or modify enzymes to function in a proper way (Dalby, 2003; Hibbert et al. 2005).

2.2 Enzyme Engineering

As it is mentioned above, naturally available many enzymes lack high stability under industrial manufacture process conditions, such as high temperature, extremes of pH, the presence of organic solvents, detergents, and oxidants and limited substrate and coenzyme specificty. Thus, enzyme should be improved or adapted to function under the harsh conditions often required for industrial processes (Table 2.2.1). These modifications are the scope of enzyme engineering. Currently, two complementary strategies are available: rational redesign (Chen, 1996; Hurley, 1996) and directed evolution (Chen, 1999; Stemmer, 1994).

Rational redesigns which are required precise changes in amino acid sequence are based on a detailed knowledge of protein structure, function and mechanism. Then, these knowledge are put into practive by using site-directed mutagenesis (Chen, R).

Site directed mutagenesis is a molecular biology techniques in which mutation is introduced at a defined site in a DNA (Figure 2.2.1). In general, site-directed mutagenesis requires that the wild-type gene sequence be known.

Table 2.2.1: Enzymes engineered for synthesis and degradation (Nixon and Firestine, 2000)

Scaffold	Purpose	Method[a]	Reference
Subtilisin E	Enhance stability in DMF	A	13
Subtilisin E	Enhanced thermostability	A	72
Kanamycin resistance protein	Enhanced thermostability	A	70
p-Nitrobenzyl esterase	Increased rate of catalysis	A	14
Atrazine chlorohydrolase	Increased rate of catalysis	B	18
Fungal peroxidase	Increased stability	B	64
β-Glucuronidase	Resistance to glutaraldehyde modification	B	68
Esterase	Increased enantiomeric excess	C	71
Pseudomonas aeruginosa lipase	Increased enantiomeric excess	A	16
P. fluorescens esterase	Increased enantiomeric excess	A,C	15
Cytochrome P450 monooxygenase	Change in substrate specificity	A	17
Biphenyl dioxygenase	Change in substrate specificity	B	19
Butyrylcholinesterase	Change in substrate specificity	E	20
P450	Change in substrate specificity	E	66
Aspartate aminotransferase	Change in substrate specificity	B	67
P. fluorescens esterase	Change in substrate specificity and enantiomeric excess	C	69
Myoglobin	Enhanced peroxidase activity	E	65
Nuclear transport factor-2	Conversion to an enzyme	E	2
Cyclophilin	Creation of proline-specific peptidase	E	22

[a] A, error-prone polymerase chain reaction; B, DNA shuffling; C, in vivo mutagenesis; D, rational mutagenesis

This promising technique allows to optimize enzyme, so having the desired properties for commercial applications. In addition, rational redesing gives information clearly about enzyme binding and catalytic mechanisms, which enhances the success of functional prediction of new protein sequences in databases.

The rational redesign is based on the confirmation of the mutation by sequencing and then by purification of the mutant enzymes. Following of mutagenesis reaction kinetic and functional properties of new enzyme can be determined. This cycle should be repeated until the desired function is obtained. For this reason, this

approach is tedious and expensive, and might be impractical for multiple cycles of mutagenesis.

In contrast to rational redesing, directed evolution does not require information about how enzyme structure relates to function (Stemmer, 1994; Kuchner, 1997). This technique requires random process in which error-prone PCR is used to create a library of mutagenized genes (Figure 2.2.2).

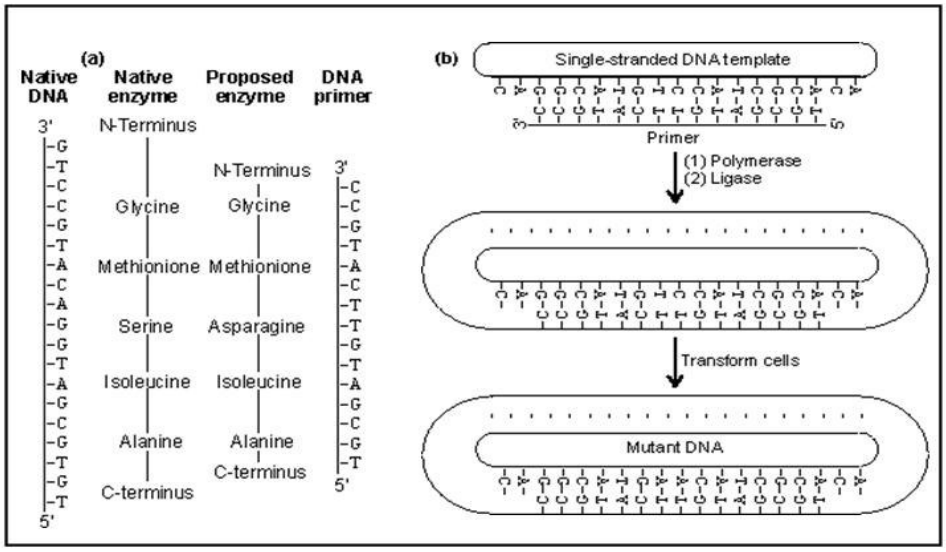

Figure 2.2.1: An outline of the process of site-directed mutagenesis, using a hypothetical example. (a) The primary structure of the enzyme is derived from the DNA sequence. A putative enzyme primary structure is proposed with an asparagine residue replacing the serine present in the native enzyme. A short piece of DNA (the primer), complementary to a section of the gene apart from the base mismatch, is synthesised. (b) The oligonucleotide primer is annealed to a single-stranded copy of the gene and is extended with enzymes and nucleotide triphosphates to give a double-stranded gene. On reproduction, the gene gives rise to both mutant and wild-type clones. The mutant DNA may be identified by hybridisation with radioactively labelled oligonucleotides of complementary structure (Enzyme engineering, http://www.lsbu.ac.uk/biology/enztech/engineering.html, 25.08.2006)

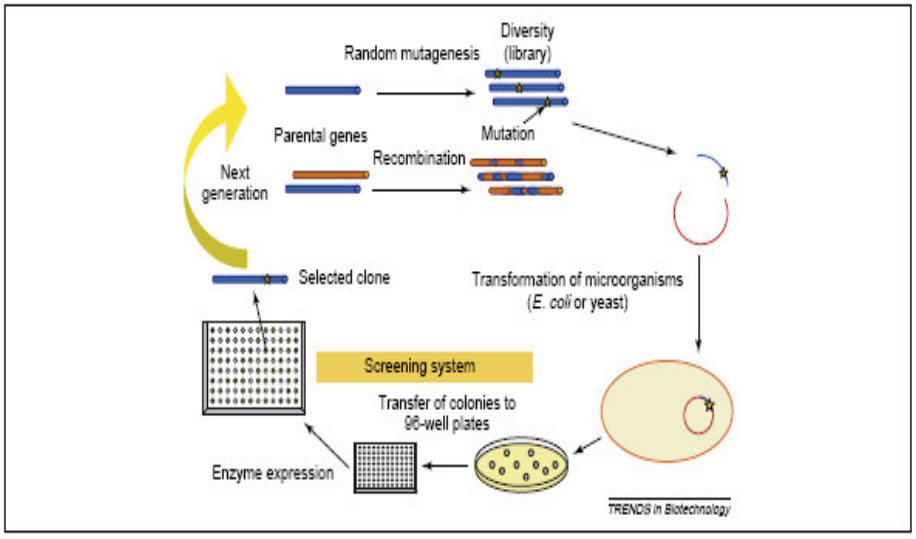

Figure 2.2.2: Typical experiment of laboratory evolution (Alcalde, 2006).

Genetic selection provides the mutants that possess desired properties. By applying further cycles of mutation and screening, the sorted genes are subjected to improve the original beneficial mutation.

Today, molecular biologists combine in vitro recombination or DNA shuffling with directed evolution, which enhance the success rate of mutagenesis and lower the time (Stemmer, 1994).

These methods rapidly combine beneficial mutations that arise from random mutagenesis and significantly expand the sequence diversity derived from small pools of homologous genes.

For the last years, directed evolution has been widely used by industry and is practical for improving enzymes and evolving new metabolic pathways (Kuchner, 1997). Although these techniques have been relatively successful in improving enzyme catalytic activity and physical properties, engineering substrate specificity appears to be more challenging.

In the majority of cases, the enzymes had low catalytic activity and modest substrate specificity and enzymes with new functions were rarely demonstrated (Chen, 2001).

Evolutionary analysis of enzyme families suggests that drastic changes in enzyme function might require considerable changes in polypeptide backbones. Such changes will probably not occur during the current in vitro evolution process. Thus, development of novel enzyme assays suitable for high-throughput screening is needed to extend the applicability of directed evolution to many more reactions of industrial interest (Chen, 2001).

2.3 Formate Dehyrogenase

NAD^+ dependent formate dehydrogenase was first discovered in 1950-1951 (Mathews and Vennesland, 1950). At first its discovery did not attract great attention in chemistry (Davidson, 1951). However, in the middle of seventies of last century when the importance of cofactor regeneration in the synthesis of high value added organic chemicals was realized, its function was being more understood.

Today, it is well-known that FDH is promising model enzyme for determining the general mechanisms of catalysis involving hydride ion transfer. Simply, the function

of FDH is catalysing cleavage of carbon-hydrogen bond in the substrate and inducing formation of a single one in the product. This reaction is devoid of proton release or abstraction steps (Popov and Lazmin, 1994).

This intrinsic catalytic and enzymatic properties of NAD^+ dependent FDH makes it very well known and more extensively studied enzyme among various sectors of life such as biotechnology and chemistry.

NAD^+ dependent FDH catalyzes the oxidation of formate ion to carbon dioxide in the coupled reaction of NAD^+ to NADH:

$$HCOO^- + NAD^+ \rightarrow NADH + CO_2 \qquad \textbf{(2.1)}$$

FDH belongs to the superamily of D-specific 2-hydroxy acid dehydrogenase (Vinals *et al.,* 1993). FDH genes are encoded by all methylotrophic microorganisms and plants. Among these organisms FDH shows 40-50% amino acid similarites, which deduces high structural conservation during the evolution.

For the methylotrophic microorganisms the oxidation of formate to CO_2 by NAD^+ dependent FDH is one of the main energy sources. All the methanol utilizing yeasts *Candida, Pichia and Hansenula* genera synthesize NAD^+ dependent FDH (Figure 2.3.1).

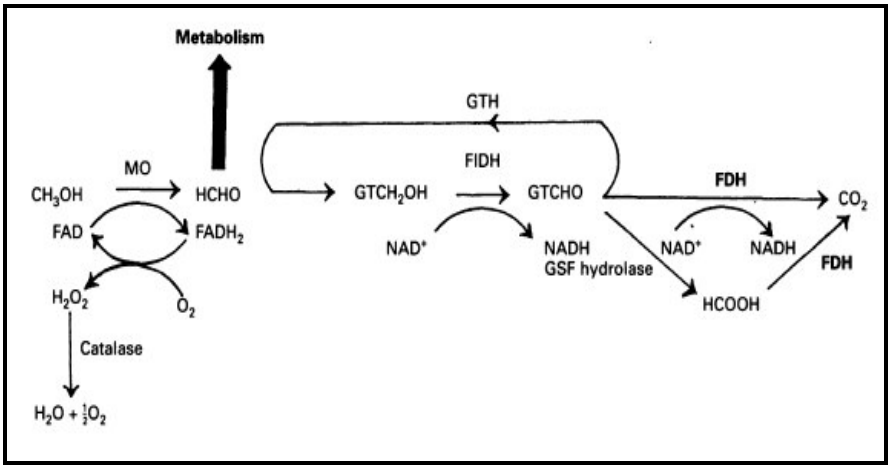

Figure 2.3.1: General scheme of methanol metabolism in yeasts (Popov and Lazmin, 1994).

So far, it has been isolated, sequenced and characterized from several strains belonging the methonal utilizing yeasts. However, FDH is not so widely distributed among bacteria. FDH from plants was first described in 1951 (Davidson, 1951). Until now, from pea seeds and string bean FDH could be isolated.

On the other hand, the function of FDH in plant is quite clear (Tishkov and Popov, 2004). FDH genes in higher eukaryotes are not described yet.

2.4 Structure of FDH

All NAD^+-dependent FDHs, except the enzyme from *Ps. oxalaticus*, are stable in air (Table 2.4.1) (Popov and Lazmin, 1994). The majority of NAD^+-dependent FDHs have closely similar thermostabilities and are rapidly inactivated at 55-60 °C. FDH has also large pH range stability.

FDH is composed of two identical subunits each including two domains: a coenzyme binding domain and substrate binding domain. The active center is located at the domain interface and does not include metal ions or other proshetic groups (Figure2.4.1)(Popov and Lazmin, 1994). They have molecular masses ranging from 70 to 100 kDa.

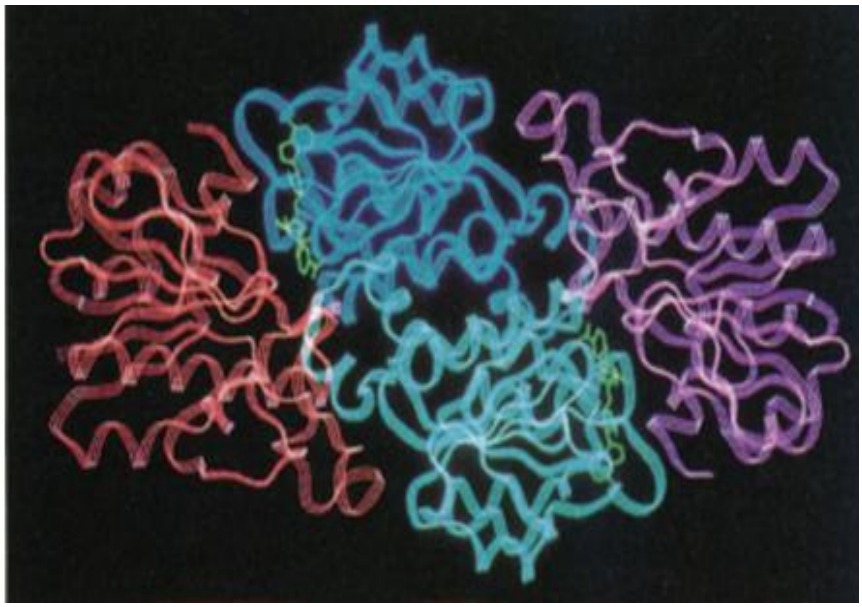

Figure 2.4.1: Ribbon plot of FDH ternary complex with NAD^+ in green. Catalytic domains are coloured in red and magenta, and coenzyme binding domains are depicted in blue and cyan.

Table 2.4.1: Physico-chemical properties of NAD^+ dependent FDH(Popov and Lazmin, 1994).

Source*	Subunits (kDa) [pI]	Activity (units/mg)†	Temp. stability (°C)	pH optimum	K_m^{NAD} (μM)	K_m^{form} (mM)	Inhibitors	Comments	Refs.
Bacteria									
Pseudomonas oxalaticus	2 × 100, 2 × 59			7.5	105	0.14	N_3^-, CN^-, NO_3^-, Hg^{2+}, PCMB	Light- and oxygen-labile; contains Fe, S^{2-} and FMN	27
Methylomonas methylica (× 32)		3.1 (37 °C		7.7–8.0	160	0.40			28
Methylomonas extorquens AM1 (× 3)		0.42 (22 °C)		8.4	90	0.25	CN^-, Cu^{2+}, Fe^{3+}, iodoacetamide	Temperature-labile	25
Pseudomonas sp. 101‡§	2 × 44 [4.6–5.2]	16.0 (37 °C)	55	6.0–9.0	110	15	N_3^-, CN^-, NO_3^-, Hg^{2+}, PCMB, DTNB	Active with $NADP^+$; uses GSF; random Bi-Bi kinetic scheme	21, 50, 67
Moraxella sp. C-1	2 × 48 [3.9]	6.0 (25 °C)	55	6.0–9.0	68	13	N_3^-, CN^-, Ag^+, Hg^{2+}, DTNB, hydroxylamine		22
Paracoccus sp. 12-A	2 × 49 [5.4]	11.6	50–55	6.5–7.5	36	5	N_3^-, CN^-, Ag^+, Hg^{2+}, PCMB		23
Mycobacterium vaccae 10	2 × 44 [4.6]	6.0 (37 °C)	57	6.0–9.0	200	20	N_3^-, SCN^-, Cu^{2+}, Hg^{2+}, DTNB		24
Yeasts									
Candida boidinii	2 × 36 [5.4]	2.4 (30 °C)	55	6.5–8.5	90	13	N_3^-, CN^-, SCN^-, NO_3^-, Ag^+, Hg^{2+}, PCMB	Ordered Bi-Bi kinetic scheme	14, 64
Candida methylica	2 × 46 [4.6–4.8]	10.0 (37 °C)	50	6.0–9.0	100	13	N_3^-, CN^-, Hg^{2+} DTNB	Ordered Bi-Bi kinetic scheme	18, 65
Candida methanolica	2 × 43 [5.5]	7.5 (30 °C)	50	6.5–9.5	110	3	N_3^-, CN^-, Hg^{2+}, Ni^{2+}, PCMB	Ordered Bi-Bi kinetic scheme	20
Kloeckera sp. 2201		0.14 (30 °C)	50	7.0–8.0	100	22	N_3^-, CN^-, Hg^{2+}, Cu^{2+}, PCMB		15
Pichia pastoris NRRL-Y-7556	2 × 47	8.2	20–25	6.5–7.5	140	16	CN^-, Hg^{2+}, Cu^{2+}, PCMB, DTNB		16
Pichia pastoris IFP 206	2 × 34	2.8 (37 °C)	47	7.5	270	15	N_3^-, CN^-, Hg^{2+}, Cu^{2+}, PCMB		17
Hansenula polymorpha‡ (× 16)	2 × 40	2.8 (37 °C)	60	7.0	70	40		Uses GSF	19, 85
Plants									
Phasoleus aureus	2 × 46‖				7.2	1.6		Ordered Bi-Bi kinetic scheme	38
Pisum sativum	2 × 42	3.7 (25 °C)		6.0–8.0	43	1.7	DTNB, PCMB		39
Pisum sativum sp. Onwards	2 × 42	4.1 (25 °C)			23	2.1		Uses GSF	40
Glycine soja var. Beeson	2 × 47	1.4 (25 °C)		6.0	5.7	0.6		Uses GSF; ordered Bi–Bi kinetic scheme	41
Solanum tuberosum¶	2 × 42 [6.8]								42

* For partially purified preparations, purification (fold) is shown in parentheses.
† 'Units/mg' are μmol/min per mg of protein; the temperature at which the activity was measured is given in parentheses.
‡ Gene sequenced and expressed.
§ Three-dimensional structure available.
‖ Date on subunit composition not available; the molecular mass of the whole protein was divided by two.
¶ Gene sequenced.

The coenzyme binding domain is responsible for recognition and binding of the NAD^+ molecule in a productive conformation and has an evolutionarily conserved structure, comprise the central part of the polypeptide chain (amino acid residues 147-333). Catalytic domain is specific to each protein and comprises amino acid residues essential for catalysis, comprise polypeptide fragments (residues 1-146 and 334-400). These domains located apart and connected via two long alpha helix (Popov and Lazmin, 1994).

FDH is a highly conservative enzyme. The absolute homology is approximately 80-85% between enzymes of the same group and 50-55% and more between two enzymes from the different groups.

At present, 36 complete and 25 partial sequences of FDH from various sources are known, and their comparison reveals that 71 conservative residues, and, because the calculated mean length of the enzyme includes about 365 residues (neglecting the length of the loop in bacterial FDHs), this is nearly 20% of all residues (Tishkov and Popv, 2004). Analysis of spatial location of conservative residues in the structure of the FDH from *Pseudomonas sp.* 101 has shown that the functions of these residues are different. Some of the residues provide stability of the subunit structure (e.g., the ion pair Lys2_Asp89), others are involved in intersubunit interactions (Arg163, Asn164, Trp177, Ala180, Asp188), and only a few of them are involved in catalysis.

In Figure 2.4.2 regions of the polypeptide chain which directly form the active center of the enzyme are shown with gray color and asterisks indicate catalytically important amino acid residues: Pro97, Phe98, Ile122, Asn146, (Ala/Gly)198, Gly200, Gly203, Arg284, Gln313, and His332.

Moreover, the asterisk indicates the position 255 (in the majority of FDHs it is occupied by Cys) because the residue in this position is responsible for interaction with the adenine moiety of NAD^+. Among conservative residues in FDH, the residue Asp308 should be also noted because its carboxyl group forms a hydrogen bond with the amide group of the nicotinamide moiety of NAD^+.

The major advantage of using FDH for cofactor recycling is the almost irreversible catalytic reaction that provides approximately 100% product yield at certain conditions. FDHs are active over a wide pH range (pH 5.5–11.0) and by this way can be coupled with almost any other dehydrogenase .

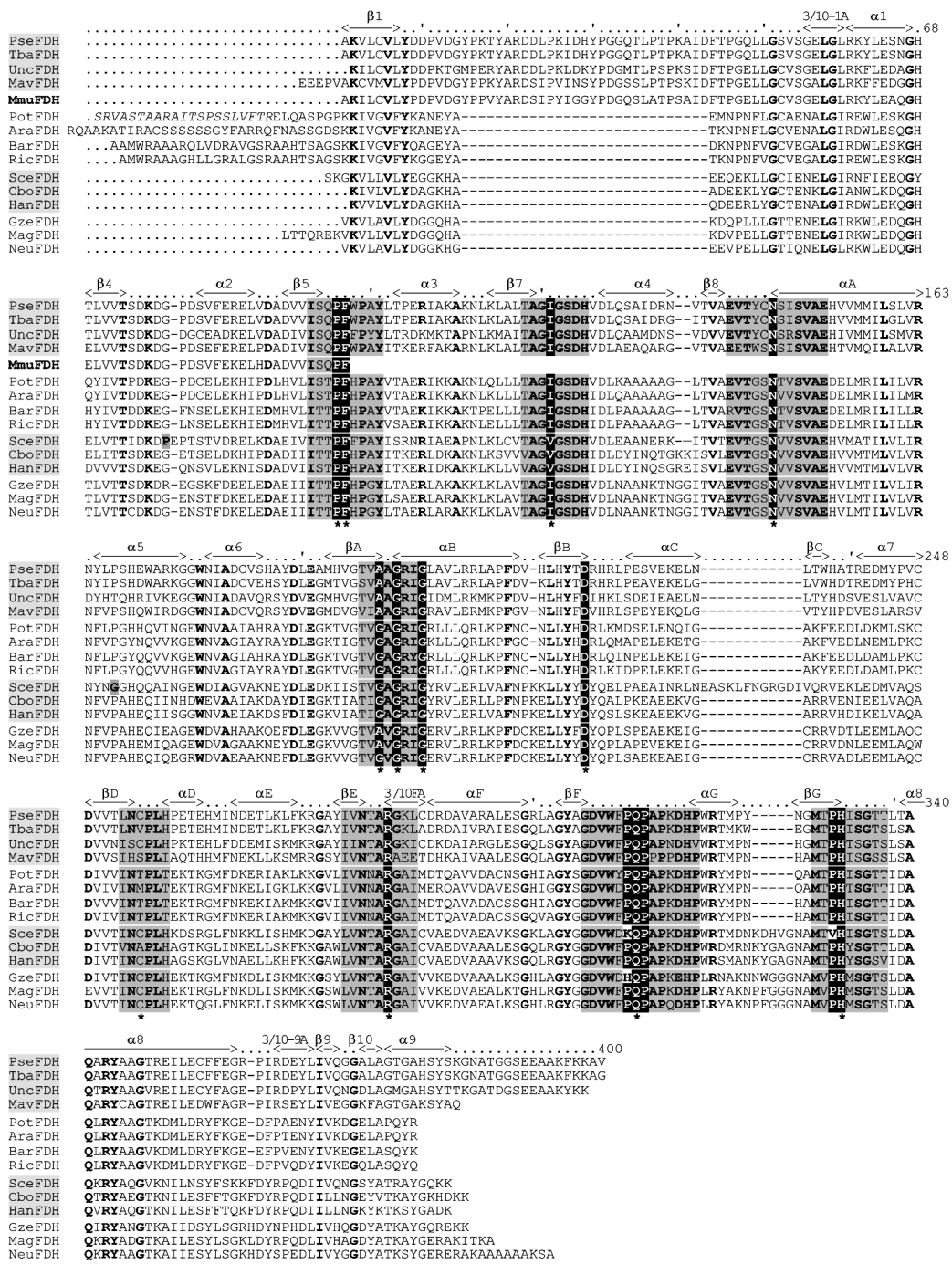

Figure 2.4.2: Amino acid sequences of FDH; *Pse*FDH, *Thiobacillus sp*.(*Tba*FDH), uncultured *γ-proteobacterium* EBAC31A08 (*Unc*FDH), *Mycobacterium avium* subsp. paratuberculosis strain k10 (MavFDH), the N-terminal region of the enzyme from mouse *Mus musculus* (*Mmu*FDH, EMBL AI505623); FDHs from higher plants: potato (PotFDH) [41], *Arabidopsis thaliana* (*Ara*FDH), barley (BarFDH), and rice Oryza sativa (RicFDH); yeasts: *Saccharomyces cerevisiae* (*Sce*FDH) *Candida boidinii* (*Cbo*FDH), and *Pichia angusta* (*Han*FDH); fungi: *Gibberella zeae* PH-1 (*Cze*FDH), *Magnaporthe grisea* (*Mag*FDH), and *Neurospora crassa* (*Neu*FDH). Amino acid residues are numerated and the structural elements are placed in reference to FDH from Pseudomonas sp. 101. Catalytically essential residues are indicated with asterisks, and the gray background shows the sequence regions that form the active center of the enzyme (Tishkov and Popov, 2004).

Examples include the reduction of acetophenone where NADPH is regenerated with the mutant $NADP^+$-specific FDH from *Pseudomonas sp.* 101 (*Ps*FDH) (Seelbach et al. 1996), the production of optically active a-haloalcohols with the NADH regeneration carried out by site-specific *Mycobacterium vaccae* mutant *Mc*FDH-26 with improved productivity (Yamamoto et al., 2005), and the largest scale industrial process, where native enzyme from yeast *Candida boidinii* (*Cb*FDH) is used in the production of an unnatural amino acid, tert-L-leucine, a component of some HIV protease and matrix metalloprotease inhibitors.

FDH's two identical subunits each comprising two domains, a coenzyme binding domain and a substrate binding domain based on Rossmann folds are depicted in Figure 2.4.3 (Popov and Tishkov et al. 2003). The two domains are connected via two long a-helices, αA and α8. The active center is situated at the domain interface and is formed by residues from only one subunit.

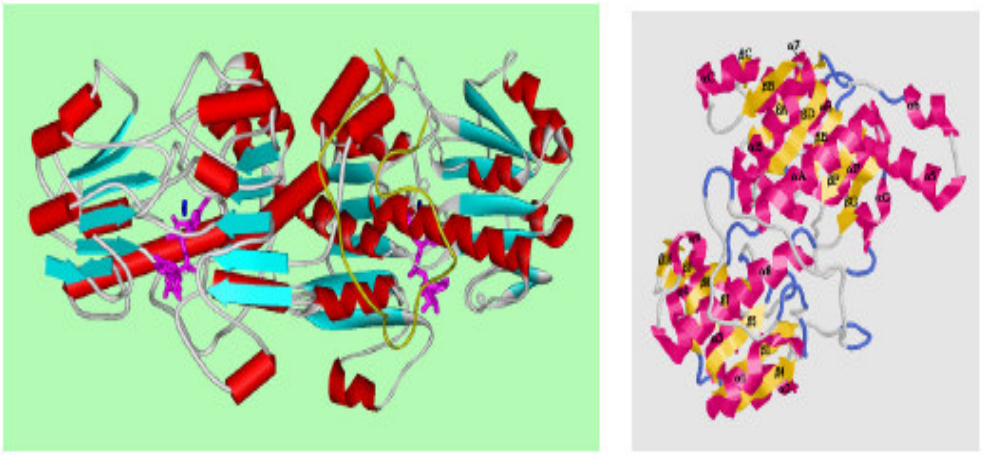

Figure 2.4.3: Structure of PseFDH. A) FDH ternary complex with NAD+ (magenta) and formate (blue) occupying azide binding site. α-helices are depicted as red cylinders (left subunit) or helices (right subunit) while β-strands as cyan arrows (left) or strips (right). A long loop comprising a/a residues 12-47 present in bacterial FDHs but absent in the enzymes from other species is shown in yellow. B) Representation of the structure of the FDH subunit.

FDH undergoes considerable conformational change on cofactor binding, as revealed by a structure of the FDH-NAD^+-azide ternary complex (Popov and Tishkov et al. 2003). The conformational transition is accomplished via a rotation of a peripheral

catalytic domains at an angle of 7.5° around hinges connecting residues 146-147 and 340-341 located in the αA and βB helices respectively.

2.5 Site-Directed Mutagenesis of FDH

The molecular basis of the coenzyme specificity of enzyme is still the fundamental question for molecular biologist. For the last few decades molecular biologist by applying protein engineering methods have been trying to understand mechanisms underlying special features of biological enzymes and their intrinsic interactions with cofactors, coenzymes and substrates.

In particular, information getting from these researches is being aimed to either improve the thermal stability or to change coenzyme specificity of biological enzymes as well as to probe the enzyme catalytic mechanisms. Moreover, genetic engineering methods are especially performed to develop highly effective process of culture and purification. To change coenzyme specificity of NAD^+ dependent FDH and apply more effective and simple purification method are the scope of this project.

So far, site directed mutagenesis experiments were carried out on FDH from various species for above mentioned purposes. Despite the structural similarity of NAD^+ and $NADP^+$ all FDHs are highly specific to NAD^+ and virtually fail to catalyze the reaction with $NADP^+$ (Serov et al., 2002). Dehydrogenases exhibit strong specificity either one of these enzymes (Popov and Tishkov, 2003). This situation for $NADP^+$ can be explained by strict steric and electrostatic requirements for the binding of the 2'phosphate group of $NADP^+$ within the active site of these enzymes (Serov et al., 2002). Thus, the directed change in coenzyme specificity of dehydrogenase by applying universal approach has still more difficulties that should be overcomed.

Despite, there have been attempts to overcome more challenging task. Over the last decades more than 200 attempts to change the coenzyme specificity of $NAD(P)^+$ specific enzymes have been published, but only a few of them can achieve this aim (Serov et al., 2002). However, in these successful trials more than one mutation were introduced in dehydrogenases.

Considering the commercial importance of NAD(P)H, because of its widely used as a coenzyme in the synthesis and biotransformation of valuable chiral compounds and

needs to construct more cheaper and effective recovery systems, finding the solution of this pronounced task has been taken into consideration not only by molecular biologist but also various field of sectors, especially pharmacy and biotechnology (Popov and Tishkov, 2003). Unfortunately, there is no $NADP^+$ specific FDH found in nature so far. So, inevitably solution should be found by performing further experiments.

By now, three successful examples for changing the coenzyme specificity of FDH from NAD^+ to $NADP^+$ had been published (Table 2.5.1) (Tishkov and Popov, 2004).

Table 2.5.1: Kinetic properties of mutant formate dehydrogenases and recombinant wild type enzymes from the yeast *saccharomyces cerevisiae* (*sce*FDH), *candida methylica* (*cm*FDH), and the bacterium *pseudomonas* sp. 101(*pse*FDH) (Tishkov and Popov, 2004).

Enzyme	K_m for NAD^+, μM	k_{cat} with NAD^+, sec^{-1}	K_m for $NADP^+$, μM	k_{cat} with $NADP^+$, sec^{-1}	K_m for formate, mM	$\frac{(k_{cat}/K_m)^{NADP^+}}{(k_{cat}/K_m)^{NAD^+}}$
CmeFDH, wild type [63]	55 ± 4 (0.2 M formate)	1.4 ± 0.1	n.d.**	n.d.	n.p.***	$< 4 \cdot 10^{-6}$
Mutant CmeFDH D195S [63]	4700 ± 300 (0.2 M formate)	1.6 ± 0.1	n.d. (>0.4 M****)	n.d.	n.p.	$2.4 \cdot 10^{-2}$ (0.2 M formate)
SceFDH, wild type [64]	36 ± 5 (0.25 M formate)	6.5 ± 0.4	n.d.	n.d.	5.5 ± 0.3 (15 mM NAD^+)	$< 3.3 \cdot 10^{-10}$
Mutant SceFDH D196A/Y197R [64]	7600 ± 800 (0.25 M formate) 8400 ± 900 (0.5 M formate)	0.095 ± 0.01 (0.25 M formate) 0.12 ± 0.02 (0.5 M formate)	4500 ± 500 (0.25 M formate) 7600 ± 900 (0.5 M formate)	0.13 ± 0.01 (0.25 M formate) 0.16 ± 0.02 (0.5 M formate)	1000 ± 200 (40 mM $NADP^+$)	2.3 (0.25 M formate) 1.5 (0.5 M formate)
PseFDH, wild type [64]	60 ± 5 (0.3 M formate)	10.0 ± 0.6	>0.4 M	n.d.	7.0 ± 0.8 (15 mM NAD^+)	$4.2 \cdot 10^{-4}$ (0.3 M formate)
Mutant $NADP^+$-dependent PseFDH [64]	1000 ± 150 (0.3 M formate)	5.0 ± 0.4	150 ± 25 (0.3 M formate)	2.5 ± 0.15	9.0 ± 3.0 (25 mM $NADP^+$)	3.5 (0.3 M formate)

In the first trial, the (k_{cat}/K_m) $NADP^+$ value of mutant pseFDH was threefold less than the (k_{cat}/K_m) NAD^+ (Tishkov et al., 1993). The D195S replacement in *cm*FDH increased 10.000 fold affinity of the enzyme for $NADP^+$ (Karagüler et al., 2000). However, *cm*FDH was still much more specific to NAD^+ than to $NADP^+$. On the other hand, double replacement D196A/T197R in scaFDH makes enzyme 2.4 fold more specific to $NADP^+$ (Serov et al., 2002). Thus, the change of coenzyme specificity of FDH from NAD^+ to $NADP^+$ needs constructing new further experiments, introducing novel mutations in FDH.

2.6 Industrial Importance and Applications of FDH

As it is mentioned before, optically active compounds are the most selective and efficient regulators of vital activity of the cell because of chirality of the living world. Among the 500 drug best sellers all over the world more than 58% are chiral compounds, and among new preparations, their fraction achieves 70%.

It is well known that different optical isomers of the same compound can have certain opposite physiological effects and probably, resulting in disease. Therefore, the optical purity of all chiral compounds used in drugs has to be no less than 99%. Thus, concerning the application of biological enzymes in pharmacy there is increase interest on bioenzyme dependent studies for the last years. Although, all classes of enzymes can be used for synthesis of optically active compounds, today, various hydrolases are used in general. On the other hand, these enzymes can be used only for separation of racemates, and 50% of the initial substance represented by the other isomer remains as a byproduct. Consequently, additional procedures are needed to increase the yield of the interest product. Thus, the remaining enantiomer can be converted into a mixture of racemates with a corresponding racemase.

Unlike hydrolases, oxidoreductases, and in particular dehydrogenases can be used to produce optically active compounds from nonchiral ones. Also, in present there are successfull examples of this convertion by using dehydrogenase, such as L-and D-lactate preparation from pyruvate by using L-and D-lactate dehydrogenases.

All dehydrogenases are extremely stereospecific in the transfer of hydride ion between the substrate and coenzyme. Moreover, as differentiated from hydrolases, the rate of nonenzymatic process in reactions catalyzed by dehydrogenases virtually equals zero. Therefore, these enzymes are promising for production of optically active compounds with very high optical purity, approximately 100%.

Beside the high effectivity of dehydrogenase for chiral compound production, process involving only dehydrogenase is costly because of the high price of NADH and especially of NADPH. Thus, to decrease the costs, scientists proposed another reaction, in which $NAD(P)^+$ is converted back to NAD(P)H (Vinals et al. 1993; Wichmann et al. 1981). This new proposal included catalyzing of this reaction by both alcohol dehydrogenase and formate dehydrogenase. Excess isoproponal, alcohol dehydrogenase and other additional enzymes were involved in this reaction. In

Figure 2.6.1, the general scheme of process, in which FDH was added as a second enzyme, is depicted.

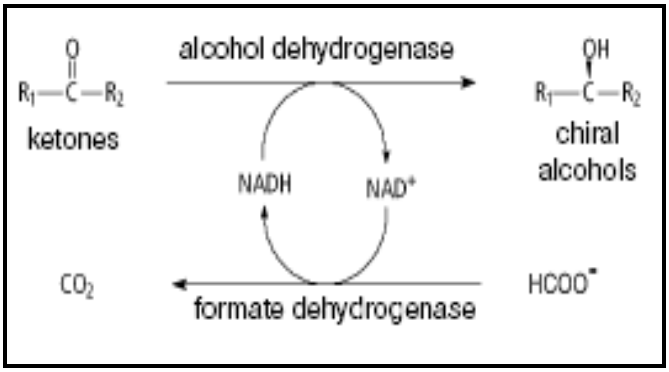

Figure 2.6.1: A scheme for synthesis of optically active compounds using dehydrogenases and formate dehydrogenase for regeneration of NADH (Tishkov and Popov, 2004).

Industrial production of *tert*-L leucine by combination of leucine dehydrogenase and FDH from *C.boidinii* is certain example of above depicted scheme. Today, in an industry tert-L leucine synthesis by using dehydrogenase is still the largest scale industrial production (Bommarius et al.1995).

The reaction catalyzed by FDH is the most proper enzyme for the system of NADH regeneration (Tishkov, 2002). The reaction of formate oxidation to carbon dioxide is irreversible, and this allows us to use thermodynamic pressure on the major process and obtain 99-100% yield of the desired product.

As it is clearly shown in table 4.2.1, another advantage of FDH is continuous activity of the FDH in the pH range from 5.5 to 11.0 (Table 2.4.1). In addition, values of Michaelis constants for NAD^+ and formate are unchanged in the pH range from 6.0 to 9.5 (Mesentsev et al. 1997), therefore, FDH can be used in combination with any dehydrogenase that has activity optimum in this range.

Formate salts, which are used in carbon dioxide and NADH regeneration, are very low cost and available substrates. Formate ion does not influence activities of dehydrogenase enzymes used in the proposed process. At present, only one enzyme is known xylitol reductase that is inhibited by formate, and the inhibition constant is comparable with formate concentrations used in practice (Neuhauser, 1997).

Carbon dioxide, which is the product of the FDH catalyzed reaction, also does not inhibit the majority of dehydrogenases, does not interfere with purification of the

desired compound, and can be easily removed from the reaction mixture at decreased pressure (Weusterbotz, 1995).

In addition, FDHs from bacteria and yeasts are highly stable enzymes and can work in systems for weeks and months. FDHs are inexpensive and available, and the price for the production of the enzyme is low. The content of FDH in the starting strains of methylotrophic bacteria and yeasts can be up to 10-18% of the total cell protein and technology of recombinant DNA provides for obtaining of still higher expression of the desired product (Mesentsev, 1997; Neubauser, 1998).

Although, above factors demonstrate that FDH is almost an ideal candidate for the regeneration of the reduced cofactor, FDHs have some disadvantages, such lower specific activities, 6-7 and 10 units/mg protein for FDHs from yeasts and bacteria, respectively (Table 2.4.1) (Labrou et al. 2000; Sakai et al. 1997). Another drawback of FDH is its limited coenzyme specificity to $NADP^+$. Since FDH having specificity to $NADP^+$ is not present in nature, enzyme engineering helps scientist to overcome this problem. One of the good examples of this approach was change in coenzyme specificity of pseFDH from NAD^+ to $NADP^+$. This was the first step in FDH regeneration and so far, since continuing interest on this subject is still present, several enzyme engineering applications on FDH have been performed (Seelbach et al. 1996).

Regeneration of NADH is costly process and still needs improvements. Some proposed approaches for reducing the price of FDHs as biocatalysts for regeneration of NADH are obvious, such as increase in the operational stability of FDH, decrease in the price of production and storage, and improvement of kinetic properties. These three factors are closely interrelated. Thus, increase in the thermal stability will allow us to increase the temperature of the process, which will also increase the specific activity of the enzyme, resulting in the improvement of kinetic properties of FDH (Tishkov and Popov, 2004).

3. MATERIALS AND METHODS

3.1 Bacterial Strain

Two different bacterial strains were used in this project, *JM*105 [F'traD36 proA+ proB+ laclq lacZΔM15/Δ (lac-pro) X1.11 thi rpsL (Strr) endA sbcB supE hsdR9] as a host cell and DH5αTM-T1R competent cells [F-ø80lacZΔM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 tonA], that is supplied with the Invitrogen GeneTailor™ Site-Directed Mutagenesis System. These strains have no antibiotic resistance and transformation by PQE-2 vector bring them in resistant to amphiciline antibiotic.

3.2 Overnight Inoculation of *JM*105 Strain

*JM*105 cells are long term cryopreserved in the mixture of 85% glycero and 15% nutrient broth at -80°C. Cells were thaw on ice first and then 10ul of thawed *JM*105 cells were taken from the 85% glycerol stock and inoculated in 3ml LB with 100ug/ul ampicillin at 37°C in shaker at 200 rpm for overnight (Table 3.2.1).

Table 3.2.1: Preparation of LB broth

Name of Ingredients	Amount (gram)
Tryptone	10
Yeast extract	5
NaCl	10
Dissolve in 1L distilled water and autoclaved for 20-30 min	

After the end of overnight inoculation overgrowth cells in 15ml centrifuge tubes were centrifuged at 13000rpm for 5min and the supernatant was discarded. The cell pellets were used for plasmid isolation directly or stored -20°C for later plasmid isolation. Plasmid isolation was performed by using QIAGEN's QIAprep Miniprep Kit (cat no: 27106). The principle of plasmid isolation is simply follwing the bind–wash–elute procedures. First, bacterial cultures are lysed and the lysates are cleared

by centrifugation. The cleared lysates are then applied to the QIAprep module where plasmid DNA adsorbs to the silicagel membrane. Impurities are washed away and pure DNA is eluted in a small volume of elution buffer or water. All the steps were followed from the written instructions in manual of QIAGEN's QIAprep Miniprep Kit **.** No any modifications were applied during plasmid isolation**.**

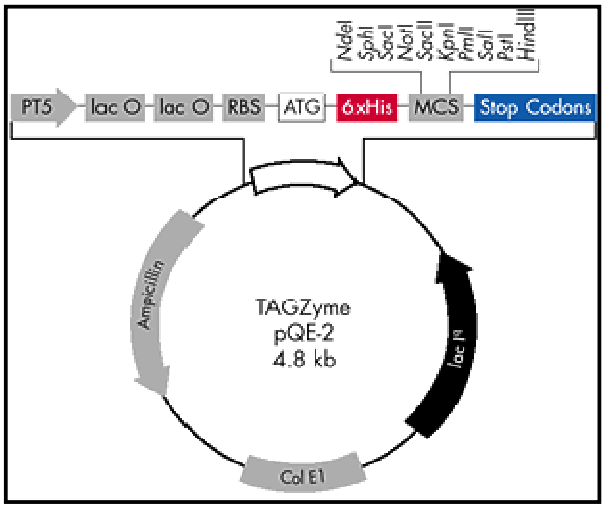

Figure 3.1: TAGZyme pQE-2 vector; **PT5**: T5 promoter; **lac O:** lac operator, RBS: Ribosome-binding site; **ATG:** Start codon, **6xHis:** 6xHis tag sequence, **MCS:** Multiple cloning site; **Stop Codons:** In all 3 reading frames; **Col E1:** Col E1 origin of replication; **Ampicillin:** Ampicillin resistance gene; **laclq:** laclq repressor gene (QIAGEN-Handbooks & Protocols, http://www1.qiagen.com/literature/hanbooks/literature.aspx?id=100031, 17.04.2005)

After the plasmid isolations, polymerase chain reaction (PCR) and enzyme restriction reactions, agarose ge electrophoresis were applied, Briefly, end product DNA samples were loaded into 1% agorose gel and runned under the 120mW to control whether the reactions were successful and concentration of DNA.

3.3 Designing Site Directed Mutagenesis Primers

Firstly, based on to the aminoacid types, triplet codons, which are seen almost in every organisms were selected (Table 3.3.1). The plasmids were designed based on the directions of the Invitogen Gene Tailor^{TM} Site Directed Mutagenesis system.

Table 3.3.1: Candidate aminoacids for replacement. Triplet codons depicted in red were used for primer design

Name of Aminoacids	Most Frequent Triplet Codons
Aspartic Acid (Asp-D)	**GAU** / GAC
Serine (Ser-S)	**AGU** / AGC / UCU / UCC / UCA / UCG
Tyrosine (Try-Y)	**UAU** / UAC
Argine (Arg-R)	**CGU** / CGC / CGA / CGG / AGA / AGG

For the substitution of aspartic acid positioned as a 195th aminoacids with serine (D195S) forward **5' TGGTAAAGCTTGATAAGAGTAGTATAATAA3'** and reverse **3'GTCGAAGAAAACCATTTCGAACTAT5'** were used. For replacement of both aspartic acid residue to serine and tyrosine residue to argine at the 195th and 196th positions together in the aminoacid sequence of NAD^+ dependent FDH (D195S+Y196R), forward primer **5'AAGAATTATTATACTACAGTCGTC AA GC TTTAC3'**and reverse primer **3'AGGGTAAATTAGGTTTTCTTAA TAATA TG ATG 5'.**

3.4 Application of Invitrogen Gene Tailor^{TM} Site Directed Mutagenesis System

All procedures applied for methylation, mutagenesis, and transformation were applied following the written instructions in Invitrogen Gene Tailor^{TM} Site Directed Mutagenesis System manual (Figure 3.4.1).

3.5 Methylation Reaction

All reagents and amounts used for methylation reaction depicted in Table 3.5.1

Table 3.5.1: Reagents and amounts used in methylation reaction

Reagents	Volume
Plasmid DNA	100ng (0.7ul)
Methylation Buffer	1.6ul
10x SAM	1.6ul
DNA methylase(4U/ul)	1.0ul
Sterile Distilled water	up to final volume 16ul

Accoring to the Invitrogen Gene Tailor™ Site Directed Mutagenesis System manual the amount of plasmid in this reaction should be 100ng. So, the concentration of isolated plasmid was measured depending on the absorbance value at 260nm by spectrophotometry. Methylation Reaction was carried out 37 °C for 1 hour.

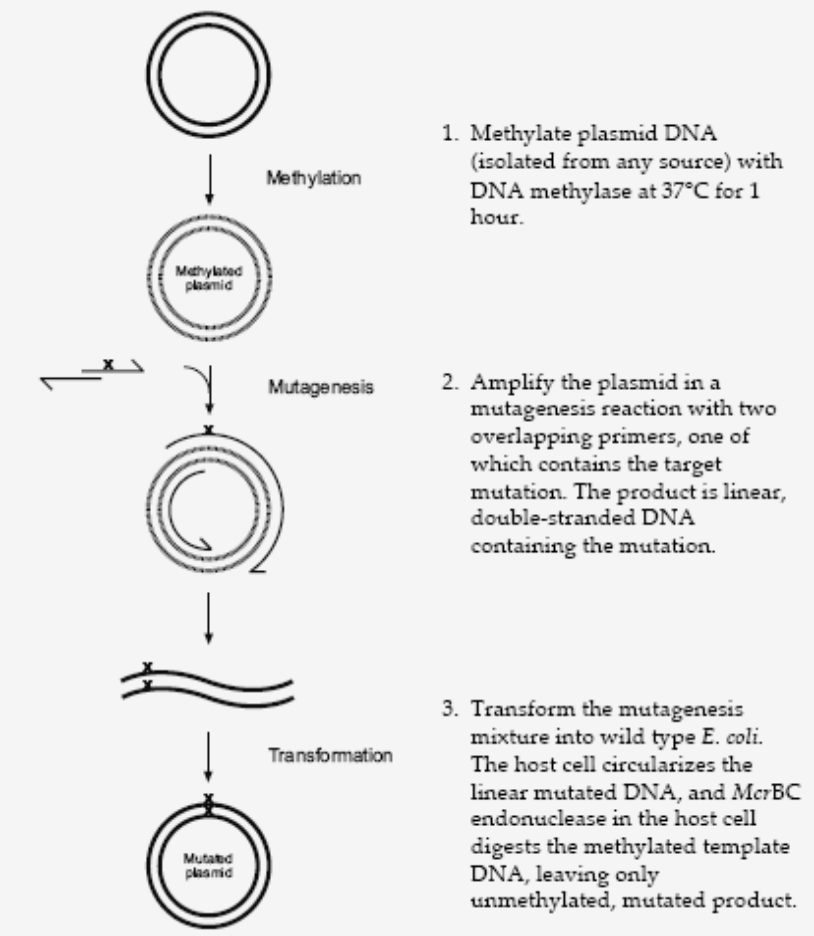

Figure 3.4.1: Work flow of The GeneTailor™ Site-Directed Mutagenesis System (Invitrogen Online Ordering: Site Directed Mutagenesis Using Gene TailorTM Site Directed Mutagenesis,https://commerce.invitrogen.com/index.cfm?fuseaction=iProtocol.unitSectionTree&treeNodeId=3DCD743998C153D43546D2D34A1E86A6, 23.10.2005).

3.6 Mutagenesis Reaction

After addition of reagents in certain amounts, shown in Table 3.6.1, mutagenesis reaction was introduced under the cycling conditions written briefly,1 denaturation cycle at 94°C for 1min, 20 annealing cycles at 94°C for 30s, at 55°C for 30s and at 68°C for 3min and 1 final extention cycle at 68°C for 10min. At the end of this PCR reaction methylated double stranded linear plasmid of mutant *cm*FDH gene was obtained.

Table 3.6.1: Reagents of mutagenesis reaction

Reagents	Volume
10X High Fidelity PCR buffer	5ul
10 mM dNTP	1.5ul
50 mM $MgSO_4$	1ul
Overlapping Primers (10 uM each)	1.5ul (100ug/ul)
Methylated DNA	3ul
Platinum Tag High Fidelity	0.25ul
Autoclaved distilled water	up to 50ul

3.7 Transformation into DH5α-T1

Transformation of mutagenesis mixture was performed by using supplied kit. Transformation was carried out by following steps written below. For each transformation 50ul vial of DH5α™-T1R cells were thawed on ice for approximately 5–7 minutes. 2 ul from each mutagenesis reaction mixtures was directly pipetted into each vial of cells and mixed by tapping gently. Vials were cover completely with ice, and incubated for 7–10 minutes. Vials were transfered to a test tube rack and incubated entire rack at once for exactly 30 seconds in the 42°C water bath. Rack of vials was removed from the 42°C bath and covered with ice for 1 minute. Over each vials 200ul of prewarmed SOC medium (supplied) were added and mixtures were shaked at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. By the end of incubation, 125ul aliquotes from mixtures were gently spreaded on LB agar plate with 100ug/ul amphiciline and incubated at 37°C for 16–20 hours.

At the end of the incubation the several white colonies were selected via micropipet tips and inoculated in 3ml LB with 100ug/ul amphicilin for overnight at 37°C in shaker incubator. The host cell, DH5α-T1 circularizes the linear mutated DNA, and McrBC endonuclease in the host cell digests the methylated template DNA, leaving only unmethylated, mutated product.

After the end of the inoculation period, plasmids isolation from inoculated DH5α-T1 cells were carried out by QIAGEN's QIAprep Miniprep Kit, following written instructions. DNA products were loaded in wells 1% agarose gel and runned. In order to confirm whether isolated have insert or not, pQE-2 plasmids were double

digested with *PstI* and *SacI* at the restriction sides of insert *cm*FDH gene in 1 hour at 37°C. Restricted fragments were visualized in 1% agarose gel by gel electrophoresis.

Purified mutant plasmids were sequenced using the ABI Prism 3100-Avant automated sequencer at the Molecular Biology and Genetics Depertmant for confirmation of mutations. Sequence PCR conditions and reagents in certain amounts were written in Table 3.7.1. Three fluoresent labelled plasmids were used, one for reverse direction, one specific to promoter region and one for forward direction.

Table 3.7.1: Sequence PCR conditions and reagents

Reagents	**Volume**	**Reaction Conditions**		
Big Dye	2ul	Temperature (^{o}C)	Duration	Cycle Number
5X buffer	1ul	95	5min	1
Primers	3,2ul (4pmol)each	95	1min	30
dH_2O	2,8ul	50	30sec	
Template plasmid	1ul	60	4min	
Total	**10ul**	**4**	∞	

Before loading samples to automated sequencer, sequence PCR products were purified by the following procudures: Squence PCR products were first precipitated by adding 1/15th volume at 3M sodium acetate pH5.2 and 2.2 volumes at cold 100% ethanol and incubated -70ºC or on ice for 15 min. Following the precipitation DNA samples were centrifuged at 14,000rpm at 4°C for 15 minutes. Supernatants were discarded and pellets were rinsed once with 200uL 70% (v/v) ethanol/ dH_2O. Samples were centrifuged at 14,000rpm at 4°C for 15 minutes and supernatants were discarded. Ethanol was removed and DNA pellets were dried at 94°C. Over the DNA pellets 20ul formamide was added for redissolving and dissolved DNA samples were denatured at 94°C for 2 min. Samples were immediately transferred at 4°C and kept until loading to sequencer

Follwing the sequence results of samples, pQE-2 expression vector containing His-tagged designed mutant *cm*FDH genes were transformed into *JM*105 host cells by applying procedures, written below, for overexpression of NAD^+ dependent FDH protein.

40ul aliquoted *JM*105 cells were thawed on ice, mixed with 3ul of pQE-2 expression plasmid and kept on ice for 30min. by the end of incubation on ice, cells were introduced heat shock at 42°C for 90 sec. Cells were left on ice for 5min and 200ul LB SOC medium was added and mixed gently. Mixture was incubated for 1 hour at 37°C in shaker incubator. Then, mixture was loaded and spreaded over the LB agar plate with 100ug/ul amphiciline.

3.8 Growth of *JM*105 cells and Expression of Mutant *cm*FDH Gene

A growth and expression procedure was provided by the Qiaexpressionist™ manuscript protocol. By the end of overnight inoculation of transformant *JM*105 cells, cultures were poured into 1000ml LB, including 100mg/ul ampicillin and inoculated at 37 °C approximately for 1,5 h until the absorbance value (A_{600}) was 0,6. Then, 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added to the final concentarion and incubated for 4-6 h at 37 °C in the shaker incubator. After the incubation period, the total 1000ml cultures were harvested by centrifugation at 4000rpm and 4°C for 20 min. The supernants were discarded and cell pellets were stored at - 80°C. Before the centrifugation 1ml sample from each culture was taken to analyze crude protein expression by SDS-PAGE.

3.9 Purification of Mutant *cm*FDH Protein by the Tagzyme™ System

Cells were suspended over the ice in 20 mL of lysis buffer to be purified from centrifuged 1000 mL of culture. Lysozyme was added to 1 mg/mL and the cell suspension was incubated on ice for 30 min before sonication with bursts at 200-300 W with a 10 s cooling period between each burst. 50 mL of sample was collected for determination of total protein expression. The remaining cell suspension was centrifuged at 15,000 g for 15 min at 4°C. 500 mL of the 50% Ni-NTA slurry was added to 20 mL of cleared lysate and mixed gently by shaking (50 rpm on a horizontal shaker) at 4°C for 60 min. The mixture was centrifuged at 5,000 rpm for 1 min, 50 mL of sample was saved to control, and remaining supernatant was discarded. The pelleted resin was washed twice with 3 mL of wash buffer by centrifugation at 5,000 rpm for 1 min and 50 mL of sample was saved to control for each repeat. His-tagged proteins were eluted 6 times with 1 mL of elution buffer by centrifugation at 5,000 rpm for 1 min.

3.10 SDS-PAGE Electrophoresis

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used especially in biochemistry, genetics and molecular biology to separate proteins on the basis of their electrophoretic mobility.

The solution of proteins is introduced with an anionic detergent SDS, which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. By this way, running of different proteins with similar molecular weights due to differences in folding in a different pattern is avoided. Since addition of SDS linearizes the proteins, they are separated by molecular weight, primary structure and or number of amino acids. In this project, purified mutant proteins were seperated by 12% SDS-PAGE.

3.11 Protein Concentration Measurement- Bradford Assay

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

In this assay, 96-well microplate was used and absorbance of proteins were measured with a kind of spectrofotometre, traded as BioRad. In the case of bradford assay, for the accuracy of measurements, standards and eluted proteins were loaded into triplicate well and mean values were calculated. Then the absorbance values of the standards the concentration of eluted protein was calculated.

3.12 Assay of Mutant *cm*FDH Enzyme Activity

Kinetic measurements were performed with a Shimadzu 1700 double beam (10 mm path length) UV-VIS spectrophotometer at 25^{o}C. The reaction mixture contained 20 mM Tris Buffer at pH 8, either 1-40 mM NAD^{+} or 1-40mM $NADP^{+}$, 0–40 mM formate and 0.4 mM enzyme (Mr 40344). Data were analysed using EZ-Fit5 Kinetics Software.

4. RESULTS

In the first step of following experiments, PQE-2 plasmids having wild type *cm*FDH were isolated from host cell *JM*105 cells after overnight inoculation. Three samples were prepared and by supplier kit, 4.8kb PQE-2 plasmids were succeffuly isolated (fig 4.1).

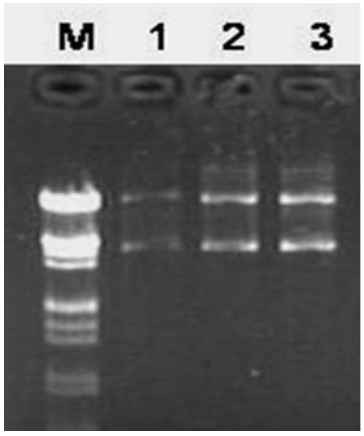

Figure 4.1: Gel electrophoresis picture of double stranded, circular 4.8kb PQE-2 plasmid. M stands for marker, 1,2 and 3 are plasmid samples.

Firstly, circular double stranded PQE-2 plasmids were methylated with 4 IU DNA methylase, then methylated plasmids with overlapping degenerated primers were used to synthesize *cm*FDH gene having single and double mutations. After the mutagenesis reactions mutagenesis products were transformed into DH5α-T1 host cells, in which double stranded linear mutagenesis products were circularized and methylated template DNA was digested by McrBC endonuclease in the host cell, leaving only unmethylated, mutated product.

Before the inoculation steps, whether PQE-2 vectors having *cm*FDH gene insert they were digested with PstI and SacI from insert site (Figure 4.2). DH5α-T1 host cells were inoculated for overnight and plasmids including mutant *cm*FDG gene inserts were isolated for the following sequencing step to confirm mutations.

concentration of eluted protein was calculated as 0,38 mg/ml for D195S and 0,41mg/ml for D195S/Y196R mutant *cm*FDH protein.

Mutant *cm*FDH proteins were overexpressed by *JM*105 cells after the induction of promoter of PQE-2 vector via IPTG. Overexpressed his-tagged mutant proteins were collected from each purification steps by following instructions of Tagzyme^{TM} .

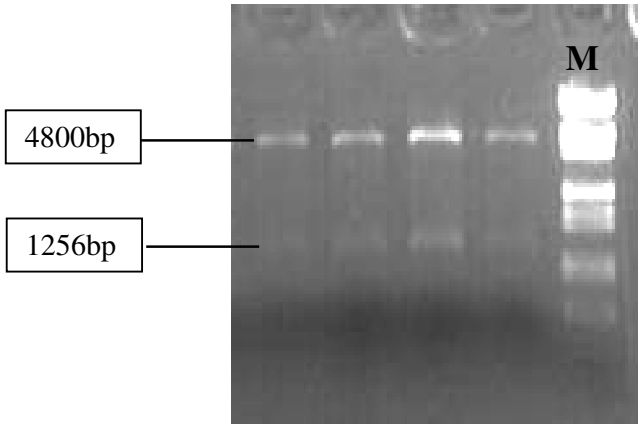

Figure 4.2: Restriction fragments of PQE-2 plasmid, M stands for marker.

System and eluted mutant *cm*FDH proteins were analyzed by SDS polyacrylamide gel electrophoresis (Figure 4.3). Concentration of isolated highly pure mutant proteins were determined by Bradford method, using bovine serum albumin as a standard (table 4.1).

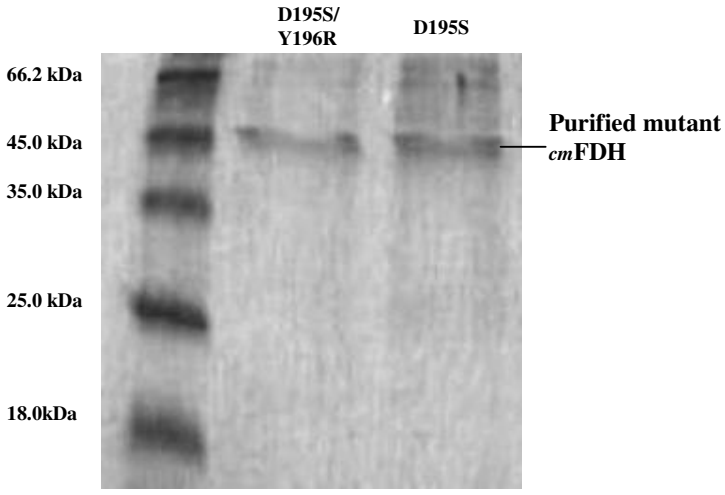

Figure 4.3: SDS gel electrophoresis of double mutant and single mutant his-tagged *cm*FDH proteins after purifications.

According to the measurements, the graphic was drawn and concentration of eluted mutant proteins were calculated. The concentration of eluted protein was calculated as 0,38 mg/ml for D195S and 0,41mg/ml for D195S/Y196R mutant *cm*FDH protein.

Table 4.1: Measurement of Bradford assays

Con. of stndrds	OD of Standards 1	OD of Standards 2	OD of Standards 3	Mean values	Elutions of *cm*FDH	OD values 1	OD values 2	OD values 3	mean value
0	0,4	0,377	0,361	0,379	**D195S**				
0,2	0,514	0,513	0,526	0,517	**E1.1**	0,504	0,493	0,534	0,510
0,4	0,584	0,622	0,567	0,591	**E2.1**	0,508	0,486	0,475	0,489
0,6	0,54	0,525	0,512	0,525	**E3.3**	0,43	0,47	0,48	0,46
0,8	0,551	0,532	0,536	0,539	**D195S/ Y196R**				
1	0,696	0,701	0,661	0,686	**E2.1**	0,625	0,578	0,502	0,563
1,2	0,77	0,821	0,809	0,80	**E2.2**	0,561	0,503	0,496	0.52
1,4	0,76	0,73	0,823	0,771	**E2.3**	0,501	0,487	0,518	0.502

5.DISCUSSIONS

Today, it is obvious that bells are being ringed for environment. An increase in the use of chemical and petroleum-based technologies in the last century resulted in global warming and environmental pollutions, which threat the life in the world. Thus, current agenda of scientist from all disciplines is to develop new green technologies alternative to the out of date technologies. In this aspect, improvement in both chemical catalysis and biocatalysis or enzymes would be the key determinant.

Biocatalysts, namely enzymes offer environmentally friendly processes and enable to perform industrial reactions under the mild conditions, which provide solutions for high energy demands and production of high amount of toxic wastages as well as minimize the problems of isomerization, racemization and rearrangement. Enzymes are chiral molecules with chemoselectivity, diastereoselectivity and enantioselectivity, and can mediate the transformation of prochiral molecules to pharmaceutically important chiral ones.

On the other hand, beside the several examples of direct use of naturally available enzymes, mostly the characteristics of enzymes should be engineered or optimized for industrial processes. Rational design and direct evolution are the two complementary protein engineering techniques. Rational design, which was the scope of this study, is put into practice by site directed mutagenesis system, which necessitates detailed knowledge of protein structure, function and mechanism. As it was applied in this study, in rational design candidate aminoacid/s is replaced with another to alter characteristics of enzyme. The sequence and the structural information requirement or estimated homology modelling of enzyme are the key determinants to define the candidate points. In this study to determine the directed mutation sites homology modelling of *ps*FDH and *cb*FDH were reviewed analytically. In addition to homology, intense decision making had been peformed on the substituted aminoacids' selection, due to the requirement of comprehensive knowledge and various experimental trails. Previous experiment on *cb*FDH, in which the aspartic acid was replaced with the serine aminoacid was the starting point of this study. In our stud, in addition to the same aminoacid substitution in *cb*FDH, a

second, tyrosine at 196th-arginine replacement, was proposed for *cm*FDH. Size, charge andconformation of aminoacid as well as its possible positioning in the 3-D structure of enzyme and interaction with the coenzyme were all taken into consideration during the selection of aminoacid.

One of the fundamental challenge is in protein engineering is to purify individual enzymes, since it needs higher costs, intense labor and time especially. In purification step, so, particularly decreasing process costs and increasing the end-product yields have significant importance.

In the purification of FDH from methyltrophic yeasts, in general various chromotographic procedures, like hydrophobic or ion-exchange chromatography have been applied mostly. Purification methods dependent on affinity chromatography have been described previously.

All these applicable procedures are rather straightforward, yielding less end product and costly as well as time consuming. However, for large-scale production of enzymes in industrial applications, there are no any alternatives to these methods.

Regarding their costly, low efficient and time-consuming process, application of these chromotographic techniques for small-scale enzyme production is not feasible. Instead, new recombinant systems have more advantages in production of target protein comparing the conventional purification systems. These systems could be employed for the production of engineered enzymes, having desired properties, in large scale and lower costs with high efficiency.

One of the mostly used recombinant purification systems is affinity tags and it has been widely used in several areas of studies. As it was used in this project and clearly showed by other similiar studies, the TAGZyme^{TM} system considering its simplicity and providing advance removal of undesired proteins it is the most efficient one within other available systems. This sytem depends on the removal of dipeptides from the N-terminus until the stop codon of sequence encountered by DAPase enzyme. This enzyme is recombinant rat dipeptidyl aminopeptidase and is a part of TAGZyme^{TM} system and has a function in exopeptidase cleavage of his-tag. This approach provides molecular biologist with purification of target protein in short period with high purity and simplicity.

For these reasons, in this project TAGZyme^{TM} system was used to isolate recombinant *cm*FDH. Since its application is very simple and no needs for optimizations in each steps, mutant *cm*FDH proteins were isolated with high purity and enough amount.

Finally, single and douple site directed mutagenesis experiments were successfully carried out and mutant enzymes were expressed and isolated with a high purity. Next step in this ongoing project (Project No: 90188), supported by Turkish State Planning Organisation, is to make kinetics measurement of mutant enzymes to analyze their enzymatic activity or respond to $NADP^+$ and NAD^+.

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