İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INCREASING THE SUBSTRATE SPECIFICITY OF *GEOBACILLUS STEAROTHERMOPHILUS* LDH BY USING ITERATIVE SATURATION MUTAGENESIS

M.Sc. THESIS

Aşkın Sevinç ASLAN

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Programme

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(521131121)

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

TEKRARLAMALI DOYGUNLUK MUTAGENEZ YÖNTEMİ İLE *GEOBACILLUS STEAROTHERMOPHILUS* LDH'IN SUBSTRAT ÖZGÜLLÜĞÜNÜN ARTIRILMASI

YÜKSEK LİSANS TEZİ

Aşkın Sevinç ASLAN
(521131121)

İleri Teknolojiler Anabilim Dalı

Moleküler Biyoloji-Genetik Biyoteknoloji Programı

Tez Danışmanı: Doç. Dr. Nevin GÜL-KARAGÜLER

HAZİRAN 2016

Aşkon Sevinç Aslan, a M.Sc.student of İTU Graduate School of Science Engineering and Technology student ID 521131121, successfully defended the thesis entitled "INCREASING THE SUBSTRATE SPECIFICITY OF *GEOBACILLUS STEAROTHERMOPHILUS* LDH BY USING ITERATIVE SATURATION MUTAGENESIS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below

Thesis Advisor : **Assoc. Prof. Dr. Nevin G. KARAGÜLER** .....

Jury Members : **Prof. Dr. Zeynep Petek ÇAKAR** .....

Assist. Prof. Dr. Nagehan E. TUNALI .....

Date of Submission : 26 April 2016
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To my lovely sister Buket,

FOREWORD

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ABBREVIATIONS

***bs*LDH**	**:** *Geobacillus stearothermophillus* lactate dehydrogenase
AHA	**:** Alpha Hydroxy Acids
BHA	**:** Beta Hydroxy Acids
BSA	**:** Bovine Serum Albumin
CAST	**:** Combinatorial Active-side Saturation Test
DNA	**:** Deoxyribonucleic acid
HA	**:** Hydroxy Acids
IPTG	**:** Isopropyl-Beta-D-Thiogalactopyranoside
ISM	**:** Iterative Saturation Mutagenesis
LB	**:** Luria-Bertani Broth
LDH	**:** Lactate dehydrogenase
NAD^+	**:** Nicotinamide Adenine Dinucleotide
NADH	**:** Nicotinamide Adenine Dinucleotide Hydrogen
OD	**:** Optic Density
PCR	**:** Polymerase Chain Reaction
PHA	**:** Polyhydroxy Acids
PHBA	**:** Polyhydroxy Bionic Acids
SA	**:** Salicylic acid
SDM	**:** Site Directed Mutagenesis
SOC	**:** Super Optimal Broth with Catabolite Repression
1,6-FBP	**:** Fructose - 1, 6 - bisphosphate
3D	**:** Three-dimensional

SYMBOLS

k_{cat}	**:** Turnover number of an enzyme
K_M	**:** Apparent Michaelis constant
min	**:** minute
mM	**:** Normal Power Components
nm	**:** nanometres
^oC	**:** Degrees Celcius
s	**:** seconds
WT	**:** Wild-Type
α	**:** Alpha
β	**:** Beta

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INCREASING THE SUBSTRATE SPECIFICITY OF *GEOBACILLUS STEAROTHERMOPHILUS* LDH BY USING ITERATIVE SATURATION MUTAGENESIS

SUMMARY

Importance of chiral α-hydroxy acids (AHAs) are steadily increasing more in particular for the production of pharmaceutical and cosmetic dermatology. AHAs are indispensable for biotechology due to their importance in the production intermediate component for pharmaceutical industry. They are the main components of cosmetic-personal care product and also they have dermatological constructive effects. Chiral compounds are produced by using enzymes as biocatalyst in industrial biotechnological processes. However, enzymes are not stable under harsh conditions of the industry and recognizing a very small number of chemicals as substrate that has been limited the kemoenzimic production of industrially important chiral AHAs as a single enantiomer. Wild type *Geobacillus stearothermophillus* lactate dehydrogenase (*bs*LDH) can catalyze only the production of lactate from reduced chiral AHA. Therefore, enzyme engineering applications are needed for *bs*LDH which will recognize different chiral AHAs as substrate. In the proposed project, due to the high thermal resistance of *bs*LDH enzymes have been used.

In this study, Iterative Saturation Mutagenesis (ISM) was used in order to expand the substrate range of the enzyme. ISM, only focuses on amino acid position(s) of enzyme defined by experimental or computational studies functional or amino acid position(s) having potential of being functional. Then, ISM forms mutant libraries which is limited with mutations based on candidate amino acid position(s). Due to targeting a few number of selected functional amino acids, formed mutant libraries by ISM are numerically much smaller than obtained libraries by directed mutations techniques. By selecting the best mutant from each mutant candidate library, ISM makes combinations for target mutant enzyme. Thus, ISM was repeated until obtaining the hit target enzyme. So ISM reduces studies in the selection steps in a huge rate and makes much more possible to obtain mutants having the desired properties.

In this project, we aimed to produce *bs*LDH for introducing different industrially important target AHA as a substrate. For this purpose six mutant libraries were designed in an effort to expand the substrate range of *bs*LDH. The eight variants were identified as having enhanced activity toward the selected α-keto acids (oxaloacetate, benzoylformate, phenylpyruvate), belongs to the same library. These new variants now may be useful biocatalysts for chiral synthesis of α-hydroxy acids.

TEKRARLAMALI DOYGUNLUK MUTAGENEZ YÖNTEMİ İLE *GEOBACILLUS STEAROTHERMOPHILUS* LDH'IN SUBSTRAT ÖZGÜLLÜĞÜNÜN ARTIRILMASI

ÖZET

Hidroksi asitler (HA), yapılarında karbonil ve hidroksi gruplarını barındıran organik asitlerdir. Bu organik asitler; α-hidroksi asit (AHA), β-hidroksi asit (BHA), salisilik asit (SA) ve polihidroksi asit (PHA) olmak üzere dört (4) sınıfa ayrılır. Hidroksi asitlerin özellikle de AHA'lerin endüstride kullanımı gün geçtikçe artmaktadır. AHA'lerin dermatolojik olarak yapıcı etkilerinin olması, kozmetik-kişisel bakım ürünlerinin temel bileşenlerinden olması ve ilaç sanayisi için ara ürünlerin üretiminde kullanılmaları nedeniyle endüstriyel biyoteknoloji için vazgeçilmezdirler. AHA'lerin üretimi için organik sentez kullanılan eski yöntemlerden biridir. Çünkü günümüz yeşil teknoloji devridir ve endüstriyel işlemlerde kullanılan kimyasalların yerini enzimler almış durumdadır. Kiral AHA'ler, enzimlerin biyokataliz olarak kullanıldığı endüstriyel biyoteknolojik işlemlerle de elde edilirler. Biyokatalizörler kimyasal katalizörlerle kıyaslandığında; daha az maliyetli koşullarda farklı stereoselektif ve rejioselektif transformasyonları katalizleyebilmesi gibi avantajlar sağlamaktadır. Ancak, enzimlerin endüstrinin aşırı (asidik/bazik ve düşük/yüksek sıcaklık) koşullarına dayanıklı olmamaları ve çok az sayıda kimyasalı substrat olarak kabul etmeleri nedeniyle, endüstriyel öneme sahip kiral AHA'lerin tek enantiomer olarak kemoenzimik üretimi çok sınırlı olmuştur. Enzimlerin bu dezavantajları yüzünden modifiye edilerek endüstride kullanıma uygun hale getirilmeleri gerekmektedir. Bu modifikasyonların gerçekleştirilmesi için protein mühendisliği tekniklerine ihtiyaç duyulmaktadır.

Enzimler endüstriyel prosesler için modifiye edilirken; rasyonel tasarım (mantıklı tasarım/akılcı tasarım) metodu ile mutasyonlar tasarlanır. Ancak, ilgilenilen enzimle ilgili çok detaylı yapı bilgisi gerektirmesi, oluşturulacak yararlı mutasyon (lar) un belirlenmesi için uzun süreli çalışmalara ihtiyaç duyan deneysel-hesapsal bilgiler gerektirmesi bu metodun en büyük dezavantajları olarak sıralanmaktadır. Öte yandan, rekombinant deoksiribonükleik asit (DNA) teknolojisinin gelişmesiyle; mevcut rasyonel tasarım metoduna alternatif yeni metotlar geliştirilmiştir. En yaygın kullanılan metot ise ilgilenilen enzimde çeşitli tekniklerle (hata yapabilen (error-prone) polimeraz zincir reaksiyonu (EP-PCR), karma DNA (DNA shuffling) gibi) rastgele mutasyonlar oluşturulması ve elde edilen içeriği [103-106] arasında olan mutant kütüphanesinden, istenilen özelliklere sahip mutant enzimlerin seçilmesi olarak uygulanan iki basamaklı yönlendirilmiş mutasyon teknolojisidir. Bu yöntemde ilgilenilen enzimin bütün amino asitlerinin hedef olarak seçilmesi ve mutasyonun bu amino asitler üzerinden rastgele oluşturulması nedeniyle anlamsız birçok mutantı içeren büyük bir mutant kütüphanesi elde edilmektedir. Bu kütüphane içerisinde, hedef olarak seçilen özelliklere sahip mutant enzimin seçilmesi için etkili, verimli ve kısa sürede sonuç verecek bir seçme tekniğine ihtiyaç duyulmaktadır. İstenilen özellikleri taşıyan mutant koloninin binlerce koloni içerisinden seçilebilmesi için; istenilen özellikleri taşıyan hedef mutant koloniye özgü bir seçme tekniğinin

olmayışı bu metodun en büyük dezavantajıdır. Ayrıca, sayıca çok büyük ve zengin mutant kombinasyonlarını içerecek bir kütüphane oluşturmak için uzun süreler gerektiren protokol iyileştirmelerine ve sonrasında seçme kısmında çok fazla laboratuvar çalışmasına/robotik teknolojilere ihtiyaç duyulmaktadır.

Çalışmada, endüstriyel uygulamalarda AHA'lerin üretimini sağlamak için termal dayanımı yüksek olması nedeniyle *bs*LDH enzimi kullanılmıştır. Yabanıl tip (WT) *bs*LDH, indirgenmiş kiral AHA'lerden sadece laktatın üretilmesini katalizlemektedir. Piruvat ve laktat arasındaki reaksiyonu, NADH/NAD^+ kofaktörlerini kullanarak katalizler. Reaksiyonu yüksek özgüllükte gerçekleştirir. Bu da endüstride kiral madde kullanımına ihtiyaç duyan alanlarda avantaj sağlayabilir. *bs*LDH'ın termal dayanımının yüksek olmasına rağmen substrat aralığının dar olması bu enzimin kullanımı için bir dezavantaj oluşturmaktadır. Bu nedenle, farklı kiral AHA'leri substrat olarak tanıyacak *bs*LDH için enzim mühendisliği uygulamalarına ihtiyaç duyulmaktadır.

Bu çalışmada, enzimin substrat aralığının artırılması için alternatif protein mühendisliği yöntemi olan "Tekrarlamalı Doygunluk Mutagenez" (ISM) metodu kullanılmıştır. ISM, sadece deneysel ve hesapsal çalışmalarla daha önceden fonksiyonel olduğu belirlenmiş ya da fonksiyonel olma potansiyeline sahip az sayıdaki amino asit pozisyon (lar) ına odaklanır. Sonra aday amino asit pozisyon (lar) ı üzerinden sınırlandırılmış mutasyonlar içeren mutant kütüphaneleri oluşturulur. Sadece az sayıda fonksiyonel amino asit pozisyon (lar) ı hedef alması nedeniyle; oluşturulan mutant kütüphanesi, sayısal olarak yönlendirilmiş mutasyon teknikleri ile elde edilen kütüphanelere göre çok daha küçüktür. Her aday kütüphaneden en iyi mutantı seçerek, hedef mutant enzim için kombinasyon yapar. Aynı şekilde ISM en iyi hedef mutant enzim elde edilinceye kadar tekrarlanır. Dolayısıyla, ISM seçme basamağındaki çalışmaları büyük oranda azaltmakta ve istenilen özelliklere sahip mutantın elde edilmesini çok daha fazla mümkün kılmaktadır. Enzimlerin substrat aralığını genişletmek için; ISM metodu, kombine aktif bölge doyurulmuş test (CAST) tekniği ile (ISM/CAST) elde edilen bölgelere uygulanmaktadır. CAST rasyonel tasarım ve rastgele protein tasarım yöntemlerinin avantajlarını kullanarak; enzimin üç boyutlu (3D) yapı bilgisine (X-ışını yada homoloji modelleme yöntemiyle elde edilmiş) ve yapısıyla ilgili yapılmış çalışmalardan elde edilen bilgilere dayanarak; bağlanma bölgesine yakın bir yada daha fazla amino asit pozisyonundan oluşan bölgeleri belirler. İlgilenilen pozisyon ya da bölge, enzimin substrat tanımada etkili olduğu düşünülerek seçilir. Bu nedenle CAST tekniğinde doğru amino asit pozisyonlarını seçmek çok önemlidir.

Tezin amacı, endüstriyel öneme sahip hedef kiral AHA'leri substrat olarak kullanabilen yeni mutant *bs*LDH enzimini üretebilmektir. Bu nedenle, yeni biyokatalizörleri elde etmek için daha küçük ve fonksiyonel kütüphaneler kuran yarı rasyonel tasarım metodunun kullanılmasıyla *bs*LDH enziminin yeniden tasarlanması amaçlanmıştır. Bu çalışmada, altı (6) mutant kütüphanesi, *bs*LDH'ın substrat çeşitliliğinin artırılması için oluşturulmuştur. Kütüphaneler oluşturulurken NRT dejenaratif kodon kullanılmıştır. Bu kodon 8 amino asidi (arjinin, asparajin, aspartik asit, sistein, glisin, histidin, serin, tirozin) kodlar. Kodlanan amino asitler ile oluşturulan kütüphaneler daha küçük ve zengin içeriğe sahiptir. Oluşturulan kütüphanelerin okzalasetat, fenilpiruvat, benzoilformata karşı taramaları yapılmış ve sekiz (8) LDH varyantı tanımlanmıştır. Bu varyantların hedef olarak seçilen α-keto asitlere (okzalasetat, fenilpiruvat, benzoilformat) karşı iyileştirilmiş aktiviteye sahip oldukları görülmüştür. Aktif mutantların kinetik karakterizasyonları, *bs*LDH' in

doğal substratı olan piruvat ve hedef substratlara karşı çalışılmış ve yabanıl tip *bs*LDH ile karşılaştırmaları yapılmıştır. Tüm bu çalışmalar sonunda, hedef substratlara karşı aktif olarak N101R102, D101Y102 ve N101C102 şeklinde isimlendirilen üç mutant elde edilmiştir. Okzalasetata karşı elde edilen N101R102 mutantı, WT *bs*LDH ile karşılaştırıldığında kinetik parametreleri arasında çok fark olmadığı fakat mutantın az da olsa bir iyileştirme gösterdiği görülmüştür. Bir diğer mutant olan D101Y102' nin fenilpiruvata karşı ilgisi (K_M) beş kat azalma gösterirken, turnover sayısında (k_{cat}) beş kat artış görülmüş bunun sonucunda WT ile benzer k_{cat}/K_M oranına sahiptir. Benzoilformata karşı aktivite gösteren N101C102 mutantının ise, WT ile karşılaştırıldığında k_{cat}/K_M oranının 2.5 kat daha aktif olduğu tespit edilmiştir. Literatür çalışmalarında benzoilformatı substrat olarak kullanan bir çalışma olmaması da bu mutantın önemini artırmıştır. Bu proje kapsamında elde edilmiş olan mutantların, kiral AHA' lerin biyoteknolojik olarak üretilmelerinde faydalı biyokatalizörler olabileceklerine inanılmaktadır.

1. INTRODUCTION

1.1 Hydroxyl Acids

Hydroxyl acid (HA) is an organic acid that includes at least one carboxy group. Hydroxy acids can be classified into four groups; alpha hydroxy acids (AHAs), beta hydroxy acids (BHAs), salicylic acid (SA) and poly hydroxy acids (PHAs) [1].

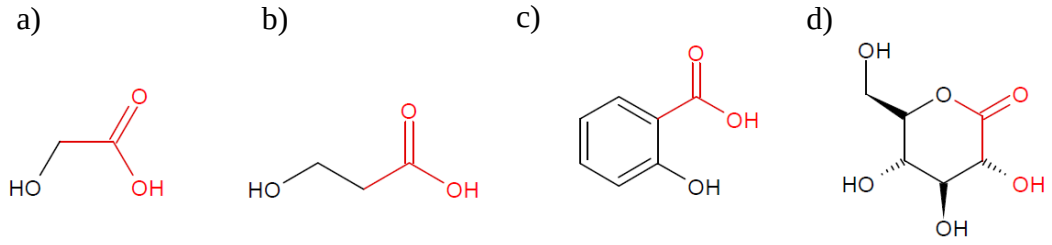


Figure 1.1 : Types of HAs; a) AHA, b) BHA, c) SA (salicylic acid), d) PHA (gluconolactone).

1.1.1 Alpha hydroxy acid

Alpha hydroxy acids (AHAs) represent a class of chemical compounds that are occurring naturally from many biological sources [2]. These carboxylic acids are substituted with a hydroxyl group on the adjacent carbon that can be named as α position [3].

1.1.1.1 Importance of AHAs

Chirality has a significance role in drug discovery, food industry, cosmetic industry, chemistry and biological field. Chiral structures that are not superimposable on their mirror image have two enantiomers and living systems are consisted of chiral compounds [4]. Each enantiomer has a different effect, for example one stereoisomer can be active and provide therapeutic activity but the other can be inactive, toxic or harmful [5].

AHAs are important additives for cosmetic industry. It can remove the top layer of dead skin cells and ensure cellular renewal [6]. One proposed mechanism stated that calcium ions (Ca^{+2}) are very important for skin and AHAs can reduce Ca^{+2} ions in

epidermis and it can induce cell growth and cell differentiation [7]. Use of these compounds may produce critical results depending on the dose. Skin can be more sensitive to ultraviolet light and this can increase the risk for skin cancer [8].

In organic synthesis, AHAs are valuable chiral building blocks. They can be useful for production of diversity of fine chemicals. For example, (R)-2-Chloromandelic acid is a main intermediate for an antidepressant and is an important precursor for synthesis of Clopidogrel [9]. They are also precursors for aldehydes synthesis [10, 11].

Chemical and biotechnological processes have been developed for the synthesis of AHAs [12, 13]. Energy saving and low cost are advantages of biotechnological methods [15, 16]. Therefore, in recent years, studies are moving in biotechnological direction. Lactic acid, glycolic acid, phytic acid, mandelic acid, malic acid, phenyllactic acid can be given as examples of this group (Figure 1.2).

Lactic acid is one of the most common AHA. It is naturally occurring from sour milk, glycolic acid is occurring from sugar cane, malic acid comes from apples [8]. They are generally used as therapeutic agents in many years to regenerate photo-aged skin. Glycolic acid has the smallest molecular size and it has the greatest bioavailability in cosmetic industry. These are helpful to reduce wrinkles, make skin smoother [16]. Phenyllactic acid can be used as an antiseptic agent and as a feed additive [17].

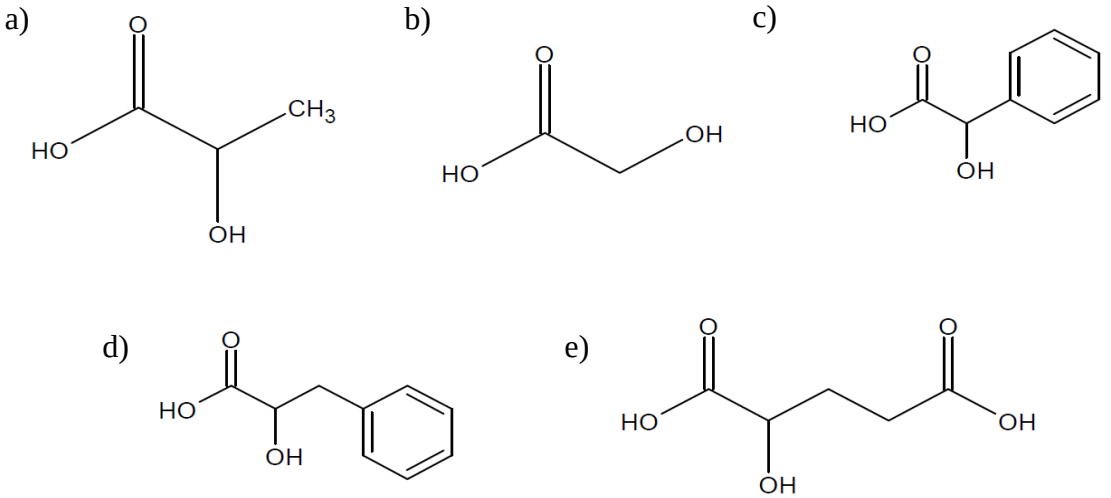


Figure 1.2 : Chemical structures of some AHAs a) Lactic acid, b) Glycolic acid, c) Mandelic acid, d) Phenyllactic acid e) Malic acid.

1.1.2 Beta hydroxy acid

Beta hydroxyl acids (BHAs) are carboxylic acids with one hydroxyl group attached at the beta position of the carboxyl group. They are very similar to AHAs but they are lipid soluble compounds [1, 18].

β-hydroxybutanoic acid is the most common example of this group. It is polyester's precursor, which is a biodegradable plastic [19]. Tropic acid is a lipid soluble BHA that can be used in the synthesis of hyoscyamine and atropine [1, 20]. Malic acid and citric acid have hydroxyl groups that are in a position to one carboxyl group and the other one is in β position to other carboxyl group. Malic acid can be used as a flavor in the food industry and is an ingredient for cosmetic industry. Citric acid is alsofood additive and it can be used as an antioxidant [1, 21].

1.1.3 Salicylic acid

Salicylic acid (SA) can be described as a BHA, but it is not necessarily true. It can also be described as a phenolic acid because hydroxyl group and carboxyl groups are linked to an aromatic benzene ring [22].

SA can be used as a food preservative. It is the main component in skin-care industry and it can be used in order to produce pharmaceuticals like aspirin and sulpiride [23, 24].

1.1.4 Polyhydroxy acid

Poly hydroxyl acids (PHAs) and polyhydroxy bionic acids (PHBAs) are new generation AHAs that include multiple hydroxyl groups bound to carbon atoms or alicyclic chain. PHBAs have an additional sugar molecule compared to PHAs. The effect nearly is the same as AHAs, but cause less irritation responses in skin-care industry [25].

The most known PHAs is gluconolactone that can protect skin against effects of ultraviolet radiation. The other PHA is lactobionic acid. They are also similar to AHAs, but they have larger molecule size when they compared to AHAs. They are also excellent components in dermatologic and cosmetic industry [26, 27].

1.2 Protein Engineering for Synthesis of AHAs

Synthesis of optically pure AHAs is significant for many industries. Racemic mixtures that include both forms of compounds can be a trouble for the production of these compounds. In living systems, reactions can produce enantiomerically pure compounds and various natural products are enantiomerically pure due to use of enzymes as chiral catalysts. So several biocatalytic approaches can be applied for the production of AHAs [4].

Oxidoreductases (lactate dehydrogenase, hydroxy-isocaproate dehydrogenase, leucine dehydrogenase, mannitol dehydrogenase) are a class of enzymes that play a role in redox reactions. They catalyzereactions with high specificities (enantio- or region-) so they can play an important role in many industries which require chirally pure compounds [28, 29, 30].

In industrial processes, enzymes are needed to be modified in order to make them suited for processes. Extreme conditions like high temperature, acidic and basic pH conditions, salinity and limited substrate specificity are obstacles for the usage of enzymes. At this point protein engineering methods can be applied to enzymes in order to overcome these obstacles.

Protein engineering is a multidisciplinary field and has many techniques and knowledge. The goals of protein engineering are to generate of novel molecules, to understand structure function relations and to design enzymes with desired properties (Figure 1.3) [31].

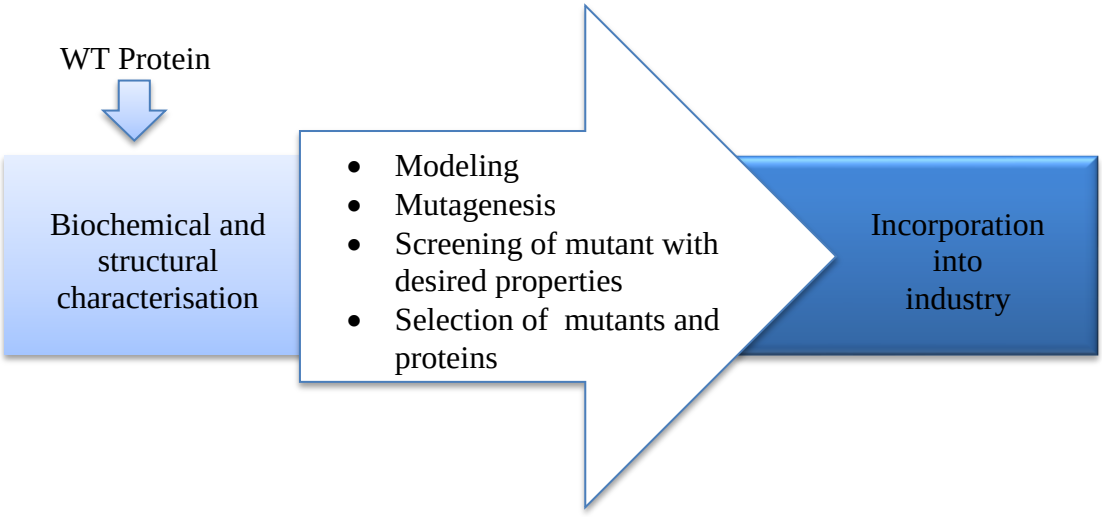


Figure 1.3 : Protein engineering flowchart.

Protein engineering approaches can be classified as “Rational design”, “Non-Rational design” and “Semi Rational Design” [Figure 1.4].

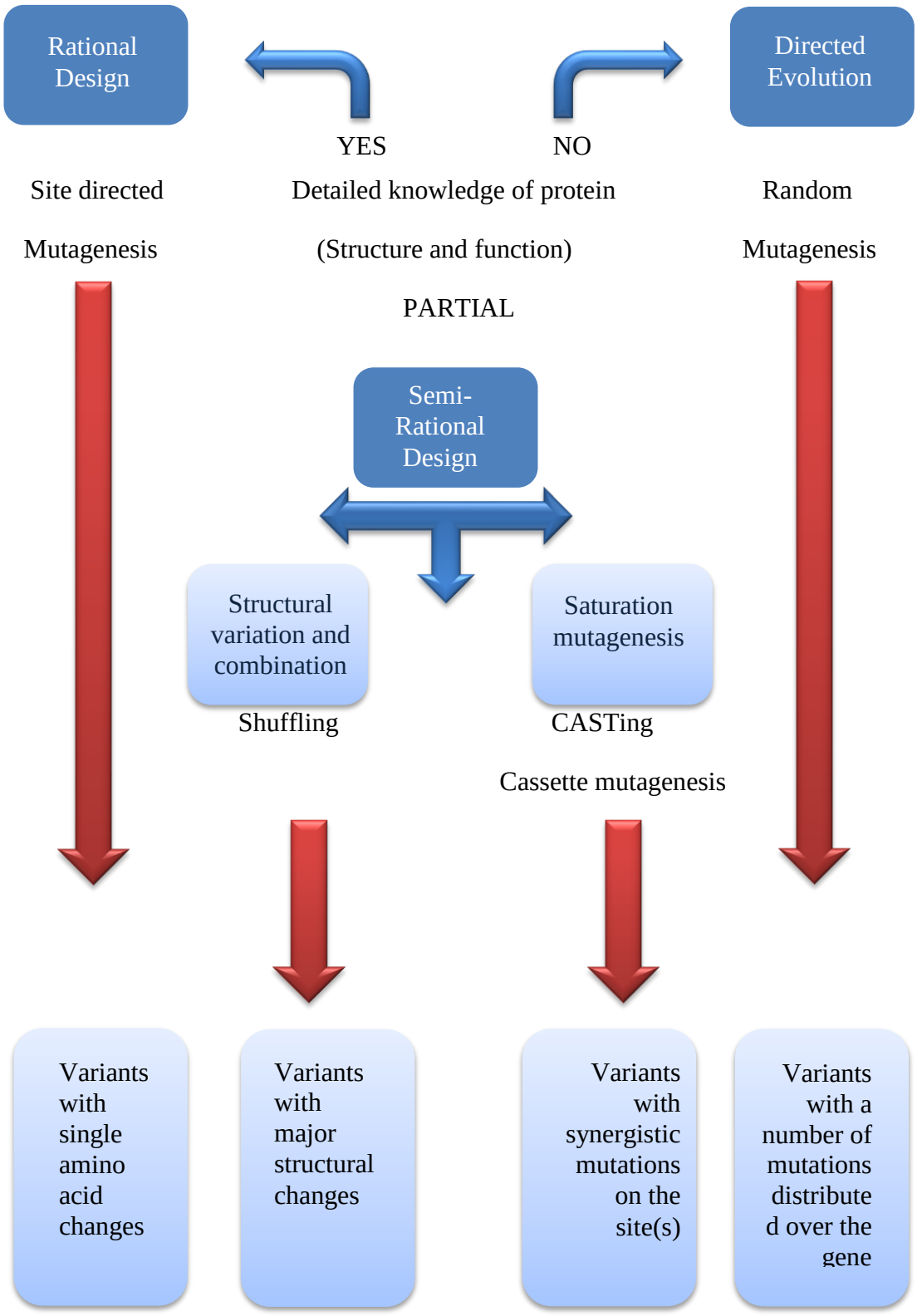


Figure 1.4 : Methods of protein engineering.

1.2.1 Rational design

Rational design requires a detailed knowledge of protein; the amino acid sequence, three-dimensional (3D) structure and the relationship between structure and function in order to create improved and desired protein molecules. Homology modeling can be used to estimate the 3D structure of a protein in the absence of 3D structure. Therefore, suitable modeling studies can be useful to get 3D structure for many of proteins [33].

Site directed mutagenesis (SDM) is a classical and invaluable approach and is widely used in order to re-design a wild type protein with a new functionality. In this technique, mutations are created in one or more amino acids and the effects are evaluated [31, 34, 35]. Structure and function relationship can be investigated by using this technique. Key residues are changed with alanine in order to investigate the roles of active-site mechanism and understand which residues are responsible for substrate binding (it is known as alanine scanning) [36]. Using this technique, properties e.g. substrate specificity, coenzyme, increasing the thermostability of many enzymes can be developed [37, 38, 39, 40].

1.2.2 Directed evolution

Directed evolution, also termed *in vitro* evolution, has been used since 1980s. This technique mimics natural evolution in the laboratory conditions. It does not require 3D structure of the protein [41, 42].

Directed evolution has two main steps; one is to create random libraries and second one is screening and selection of mutants with desired properties. This can be repeated until the desired degree of mutation is obtained [43].

Recombinative and non-recombinative methods can be classes of directed evolution. Non-recombinative method is random mutagenesis of the genes. It is a simple method to create diversity by making substitutions, insertions and deletions. Error Prone PCR can be example of this class. Recombinative method relies on gene fragments recombination which is homologous or non-homologous from different sources. DNA Shuffling and Family DNA Shuffling are given as examples of this class [35].

1.2.3 Semi-rational design

Rational design requires amino acid sequence and 3D structure and directed evolution's screening and selection steps are time consuming. Because of these limitations of both methods, the new strategy namely semi-rational design has been developed. Semi-rational design is a combination of both rational design and directed evolution methods [44, 45].

Site saturation mutagenesis is an efficient method to improve enzyme properties. This method allows getting mutant libraries that include all possible amino acid changes with one or more desired target positions. Hence, the sequence is smaller than the other techniques; it can be screened rapidly [31].

1.2.4 Recent strategies: Iterative saturation mutagenesis and combinatorial active-site saturation test

Iterative saturation mutagenesis (ISM) is a new, invaluable and efficient method in protein engineering. It is based on *in vitro* natural evolution process; library creation, screening and amplification. This strategy reduces screening efforts and it is possible to get high hit rate of mutants (Figure 1.5) [46].

This method is interested infunctional amino acid positions that are determined by experimental or computational studies. ISM only focuses on these positions and mutant libraries that are limited to these positions. Therefore, mutant libraries are much smaller than the other techniques. ISM method's mutations are provided from one initial library. ISM makes combinations and repeats until it obtains the hit target enzyme (Figure 1.6) [47].

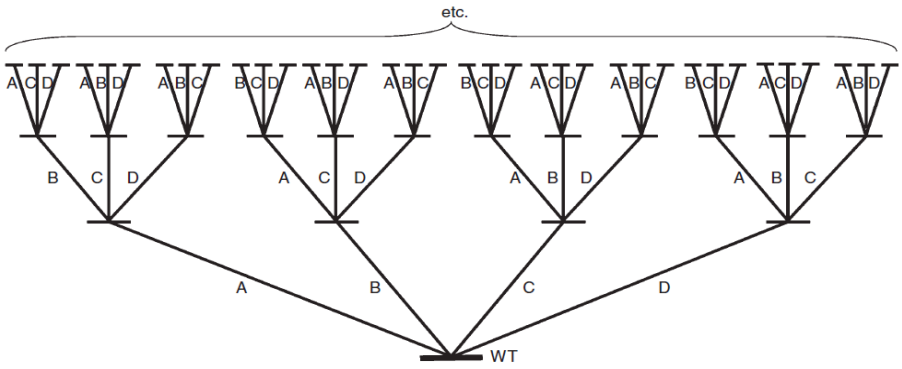


Figure 1.5 : Schematic illustration of ISM [47].

ISM method is used for many practical applications and biotransformation [48, 49, 50]. It can be used for increasing the enantioselectivity and for expanding substrate

specificity of the enzyme. For this purpose, combinatorial active-side saturation test (CASTing) can be applied. It was firstly applied on a lipase to expand substrate specificity [51]. CASTing is used at the active center of the enzyme as a reference and is focused around the binding pocket. Therefore, ISM/CASTing method creates effective and rich mutant libraries and also allows screening these libraries in less time [51].

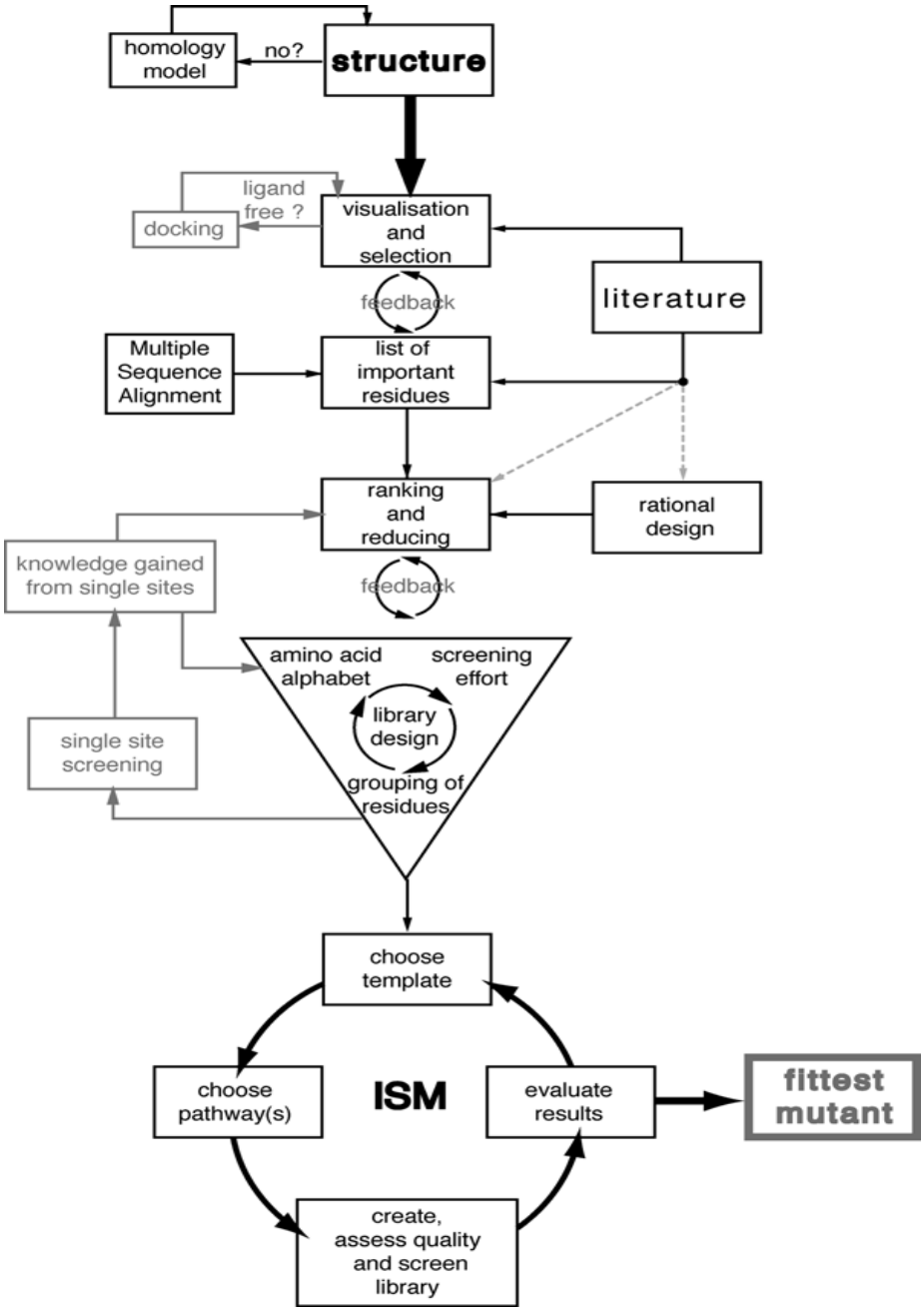


Figure 1.6 : Flow diagram of ISM [46].

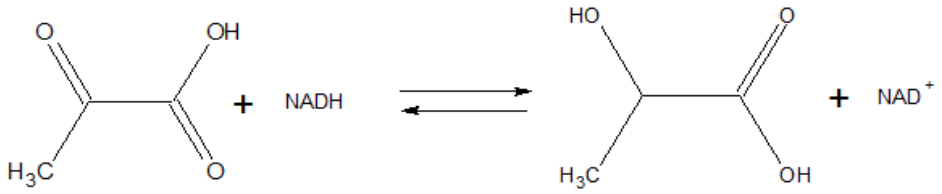
Codon usage is an important issue when applying ISM in order to randomize target amino acid positions (Table 1.1). Degeneracy usages reduce the necessary molecular biological work and screening effort and experimental work [47].

Table 1.1 : Commonly used degenerate codons.

Degenerate Codon	Number of codons	Number of amino acids	Number of stops	Amino acids encoded
NNK	32	20	1	All 20
NDT	12	12	0	RNDCGHILFSYV
DBK	18	12	0	ARCGILMFSTWV
NRT	8	8	0	RNDCGHSY

1.3 *Geobacillus stearothermophilus* L-Lactate Dehydrogenase (*bs*LDH)

Geobacillus stearothermophilus L-Lactate Dehydrogenase (*bs*LDH) (EC 1.1.1.27) is a thermostable 2-hydroxy acid oxidoreductase that can catalyze the reaction between pyruvate (oxo acid) and lactate (hydroxyl acid) by using NADH/NAD^+ pair (Figure 1.7) [52].



Pyruvate Lactic acid

Figure 1.7 : The reaction catalyzed by *bs*LDH.

LDH is the most studied and well-known enzyme. The H form of the enzyme predominates in heart muscle and the L form of the enzyme predominates in skeletal muscle. These forms are combined differently and allow occurring of LDH isoenzymes [53]. LDH is regulated by fructose-1,6-bisphosphate and two molecules of fructose-1,6-bisphosphate bind per LDH [54].

LDH has two forms in nature, L-LDH (EC 1.1.1.27) and D-LDH (EC 1.1.1.28). Because of the different catalytic stereo specificity, it can be used for synthesis of chiral AHAs. LDH is a valuable biocatalyst due to their highest activity for pyruvic acid and α ketonic acids [55]. The substrate specificity of natural LDH can be

improved by altering the enzyme structure. At this point protein engineering can be applied to modify the enzyme [56].

First protein engineering study on *bs*LDH was the replacement of arginine to glutamine at 109th position in 1986 [57]. Because this position is protected in all LDH and it could play an important role in substrate binding and hydride transfer in the reaction. This mutation has no effect on *bs*LDH due to the following alternative hydride transfer mechanism. In 1987, 171st position at the active side of LDH was changed from Arg to Lys and this mutation allowedincrease in k_{cat} and decrease in K_M value [58, 59]. This mutant was applied in different substrates such as pyruvate, α-keto butyrate that have big side chains, K_M value was decreased when the side chains were getting bigger. Therefore this position is important for forming enzyme-substrate complex [59, 60]. Thr246Gly, Asp197Asn and Gln102Arg triple mutation in the enzyme was lead to recognize oxaloacetate as substrate in 1987, but the catalytic activity was insufficient for industrial applications [60]. In 1988, alteration of the substrate specificity of *bs*LDH from pyruvate to malate was achieved by replacement of the glutamine residue by an arginine residue at 102nd [61]. In 1989, Arg171 was changed with Thr and Tyr and different substrates were tried to increase catalytic activity [62]. To obtain succesful mutations, *bs*LDH reaction mechanism was modelled with improved computational techniques in 1991 [63]. In 1992, 109th and 110th positions that are located in mobile region werechanged and *bs*LDH recognized phenyllactate as a substrate [64]. In 1993, it was stated that Thr246 was responsible for hydride transfer due to no difference in k_{cat} and K_M values with the mutations [65]. In 1994 mutations at Gln102 with small amino acids like Ser, Thr lead to recognize different α keto acids as a substrates [66]. In 1996, first directed evolution method was applied on *bs*LDH and with cassette mutagenesis at 101st and 102nd positions, *bs*LDH was screened towards to α-keto acids. Arg101Arg-Gln102Val mutant recognized phenlypyruvate [55]. To get a mutant which shows the same activity without 1,6-FBP, directed evolution method was used for the first time for this purpose. It was achieved by mutations Arg118Cys, Glu203Leu, Asn307Ser but these mutants were not close to active site of the enzyme [67]. In 2001, to decrease substrate inhibition of *bs*LDH, Asp52 was changed with Glu by using site-directed mutagenesis but results were not enough for industrial applications [68]. In 2009, malate specificity was gained by *bs*LDH by using DNA shuffling method [40].

In 2013, LDH utilised L-mandelic acid as a substrate by using site-directed mutagenesis [69]. With the changes Ile240Ala and Thr246Gly, LDH recognized substrate that has aromatic side chain.

1.4 The Aim of the Thesis

*bs*LDH has high industrial potential because of its capability to produce highly enantiopure AHAs from pro-chiral α-ketoacids. As an effective biocatalyst, *bs*LDH can be employed for the synthesis of chiral AHAs, which are important for industrial applications. *bs*LDH has high thermal resistance but it has limited substrate specificity.

Herein, we aimed to expand substrate specificity of *bs*LDH by using semi-rational engineering approach.

2. MATERIALS and METHODS

2.1 General Materials and Methods

2.1.1 Plasmids, Strains and Media

In this study, *bsldh* gene that was previously constructed in His-tagged pQE-2 plasmid was used.

Strains of *Escherichia coli* purchased from New England Biolabs (NEB-5α) and Life Technologies (BL21 Star (DE3)).

Luria Bertani (LB) broth, Luria Borth - Agar Media (LB agar), Yeast Extract Tryptone (2x YT) and Auto Induction Media (AIM), which were used in protein expression experiment, were obtained from Formedium. Super optimal broth with catabolite repression (SOC) medium used for transformation was purchased from Invitrogen.

2.1.2 Chemical Compounds and Laboratory Equipments

Enzymes, chemical compounds and kits and laboratory equipments used in study were shown in Table 2.1, 2.2, 2.3.

Table 2.1 : Enzymes.

Enzyme	Company
Phusion HF DNA Polymerase	BioLabs
DpnI	BioLabs
DNaseI	BioLabs
Lysozyme	Sigma

Table 2.2 : Chemical compounds and kits.

Chemical compounds	Company
Ampicillin	Formedium
IPTG	Formedium
Tris-base	Formedium
Agarose	Fischer Scientific
Imidazole	Sigma-Aldrich
NaCl	Fischer Chemicals
$NaH_2PO_4.2H_2O$	Fischer Chemicals
Glycerol	Fischer Scientific
Sodium pyruvate	Fischer Scientific
Oxaloacetate 96 %	Acros Organic
Phenylpyruvate 98 %	Aldrich
Benzylformate 97%	Acros Organic
Glycolic acid 99%	Aldrich
NADH	Sigma
Fructose 1,6-bisphosphate	Sigma
DNA ladder (1kb)	BioLabs
Protein ladder	PageRuler Prestained
Blue Loading Dye	BioLabs
Protein Stain	Expedeon Instant Blue

Table 2.2 (continued): Chemical compounds and kits.

Chemical compounds	Company
SDS-PAGE gels	NuSep
Protein Assay Kit	Thermo ScientificTM PierceTM BCATM
QIAprep Spin Miniprep Kit	QIAGEN

Table 2.3 : Laboratory equipments.

Equipment	Company
pH Meter	Hanna
Heat Block	VWR
Rocker	Stuart
Biophotometer	Eppendorf
Static Incubator	BINDER KT serie
Orbital Shaker Incubator	Infors HT Multitron Standart
Microplate Reader	Tecan
Microfuge	Thermoscientific
Centrifuge	Eppendorf 5810R, Beckman coulter
Thermocycler	Eppendorf Mastercycler Gradient
Electroporator	BioRad
Nanodrop	Thermoscientific
Sonicator	MSE Soniprep 150
Gel imaging	Syngene

2.1.3 Transformations, starter cultures and glycerol stocks

NEB-5α competent cells were used for mutagenesis, BL21 Star (DE3) competent cells were used for expression. Transformations into *E.coli* strains were performed by heat shock as below.

- 0.2 µl plasmid was added into chemically competent cells.
- Plasmids into chemically competent cells were incubated 30 minutes on ice.
- After 30 minutes, the tubes were placed in a water bath at 42^{o}C for 30 seconds and after that incubated on ice for 1-2 minutes.
- 200 µl pre-heated SOC medium was added into tubes and shaking vertically at 250 rpm, 37^{o}C for 1 hour.
- Plated on LB agar including 100 µg/ml ampicillin.
- Plates were grown at 37^{o}C overnight.

For starter cultures, single colonies were picked from plates and inoculated in20 ml LB medium with ampicillin (100 µg/ml) in 50 ml sterile tubes. These cultures were grown at 37^{o}C overnight with vigorous shaking at 225 rpm.

Glycerol stocks were used for plasmid storage. 400 µl overnight cultures were mixed with 100 µl of 80% glycerol in 2 ml Eppendorf tubes. Tubes were shortly vortexedand stored at -80^{o}C. Inoculating loop was used for scraping the frozen cells and then spread onto LB agar plate with ampicillin (100 µg/ml).

2.1.4 Gel electrophoresis

Agarose gel electrophoresis was carried out with Bio Rad equipment. Agarose gels were prepared with 0.50 mg agarose and 1 µl of SYBR Safe in 50 ml 1X TAE buffer. Gels were run at 90 V for 40 minutes to 1 hour. Bands were observed under UV.

For SDS-PAGE, Tris-Glycine gels (NuSep) were used and was carried with Mini-Protean gel tanks (Bio Rad). First, samples were boiled in Laemmli buffer for 10 minutes at 95^{o}C and were run 120 V for 35-45 minutes. Proteins were stained with InstantBlue Coomassie stain (Novexin).

2.2 Mutation Studies

With ISM/CAST technique, functional or have a functional or potentially functional amino asit positions were determined. These positons were N101, Q102, R109, R171, H195, I240, T246.

2.2.1 Plasmid isolation

For this study, The QIAprep Spin Miniprep Kit was used for plasmid purification.

- Bacterial cells which were grown 16 hours in LB medium with ampicillin (100 µg/ml) at 37^{o}C, 225 rpm. were harvested. The supernatant was discarded.
- Pellets were resuspended in 250 µl of Buffer P1.
- 250 µl of Buffer P2 was added and gently inverted 6 times.
- 350 µl of Buffer N3 was added and inverted immediately 6 times.
- The solution was centrifuged at 13000 rpm for 10 minutes at room temperature.
- The supernatant was transferred to QIAprep column and centrifuged 13000 rpm for 1 minute to bind plasmid to high pure filter column.
- After discarding the flow through, column was washed with 500 µl of Buffer PB and 750 µl of Buffer PE sequentially.
- After washing step, column was placed in a clean 1.5 ml microfuge tube, then 50 µl of binding buffer was added and waited for 10 min.
- Centrifuged at 13000 rpm for 10 minutes and plasmid concentration was determined by using Nanodrop.

2.2.2 PCR primers

Primers were designed for mutagenesis in accordance with Stratagene QuikChange Protocol Primer Design. An NRT degenerative codon (N=A,T,C,G; R=A,G) was choosen torandomize candidate amino acid positions.

Primers were from Eurofins and the sequences were given in the table below. In the Table 2.4, F symbolizes forward and R symbolizes reverse. Red color shows degenerative codon in the related mutation positions.

Table 2.4 : Primer sequences for mutagenesis.

Primer Name	Primer Sequence (5'⟶ 3')
N101Q102 F	GCGCCGGCGCCNRTNRTAAACCGGGCG
N101Q102 R	CGCCCGGTTTAYNAYNGGCGCCGGCGC
R109 F	CCGGGCGAGACGNRTCTTGATCTTGTGG
R109 R	CCACAAGATCAAGAYNCGTCTCGCCCGG
R171 F	GGACGATTTTAGATACGGCGNRTTTCCGCTTTTTGTTGGGC
R171 R	GCCCAACAAAAAGCGGAAAYNCGCCGTATCTAAAATCGTCC
H195 F	GCCTATATTATTGGGGAANRTGGCGACACTGAACTCC
H195 R	GGAGTTCAGTGTCGCCAYNTTCCCCAATAATATAGGC
I240 F	GCCGCCTACCAAATTNRTGAGAAAAAAGGAGC
I240 R	GCTCCTTTTTTCTCAYNAATTTGGTAGGCGGC
T246 F	GAGAAAAAAGGAGCGNRTTACTACGGGATTGC
T246 R	GCAATCCCGTAGTAAYNCGCTCCTTTTTTCTC

2.2.3 PCR profiles

PCR was run using Eppendorf Mastercycler Gradient thermal cyclers. The thermal profiles and PCR reaction components were given in tables below.

Table 2.5 : PCR thermal profiles.

Phase	Step	Temperature(^{o}C)	Time
Initial Denaturation	Denaturation	98^{o}C	30 sec
Amplification 18 cycles	Denaturation	98^{o}C	30 sec
	Annealing	55^{o}C	1 min
	Extension	72^{o}C	2.5 min
Final Extension	Extension	72^{o}C	5 min
	Hold	4^{o}C	∞

Table 2.6 : PCR reaction component.

Reaction Component	Volume (µl)
Sterile dH_2O	37.5
5X Phusion HF Buffer	10
10 mM dNTP mix	1
DNA template (~60 ng)	0.5
10 mM Primer mix	1
Phusion DNA Polymerase	0.5

After PCR, 2 µl DpnI was added to the PCR product and incubated for 3 hours at 37^{o}C to remove the original plasmid that was used as a template DNA. *Dpn*I cuts only methylated DNA so that *Dpn*I does not affect mutated plasmid, which is not methylated.

2.2.4 Transformation

After DpnI digestion, plasmid was ready for transformation. For this purpose, heat shock procedure was used. 1 µl plasmid from DpnI digestion reaction was added to BL21 Star (DE3) competent cells and incubated on ice for 30 minutes. After the heat shock at 42^{o}C for 30 sec., they were incubated on ice for 1-2 min. Then 200 µl SOC medium was added and bacteria were incubated vigorously shaking at 37^{o}C for 1 hour. After 1 hour 100 µl from each transformation plated on LB agar plate with ampicillin (100 µg/ml) and was grown at 37^{o}C overnight.

2.3 Construction of Mutant Libraries

For checking mutations, 1 ml LB was added to agar plate and mixed all colonies for each positions. Then The QIAprep Spin Miniprep Kit was used for plasmid purification. These mutant pool plasmids were sequenced at EuroFins Genomicsin order to check chromatogram results. Mutant pool plasmids were transformed to BL21 Star (DE3) and were grown at 37^{o}C overnight.

Colonies that were obtained from mutagesis were picked and transferred to 96 deep-well plates containing 200 µl LB with ampicillin (100 µg/ml). Wild type *bs*LDH and empty vector were also transferred to wells as positive and negative controls. These deep-well plates were grown overnight at 37^{o}C with shaking, at 250 rpm. These plates were named as "Master Plate" and stored in 20 % glycerol stocks at -80^{o}C.

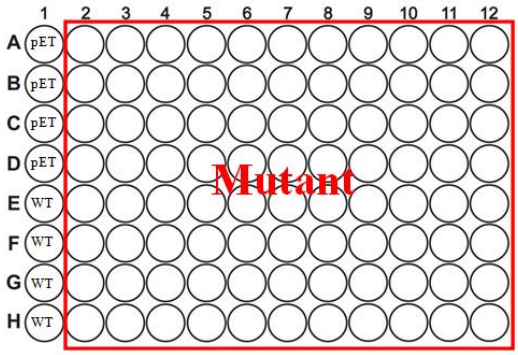


Figure 2.1 : Image of plate for screening.

2.4 Screening of Mutant Libraries

Master plates were copied to 96 deep-well plates in 200 µl LB with ampicillin and were grown overnight at 37^{o}C at 250 rpm for screening. Cells were harvested by

centrifugation at 4000 rpm for 20 min., medium was removed and the pellets were resuspended with lysis buffer that included 1mg/ml lysozyme and DNase in 50 mM Tris-HCl pH 7.4 buffer. After incubation at 30^{o}C for 30 min the plates were centrifugated again at 4000 rpm for 20 min, the supernatants were screened for activity toward the NADH consumption at 340 nm (Figure 2.2).

For screening, 96 well plates were used. 25 µl of supernant was transferred to 96 well plates and then 75 µl reaction mix containing 1.33 mM NADH and 13.3 mM fructose-1,6-bisphosphate (1,6-FBP) in 50 mM Tris-HCl pH 7.4 buffer was added. After adding 20 mM target substrate (oxaloacetate, phenylpyruvate, benzylformate, glycolic acid), absorbance was immediately read and -NADH consumption was observed at 340 nm for 10 minute in a microplate reader. About 150 colonies were screened per library. Positive hits were rescreened again to validate activity.

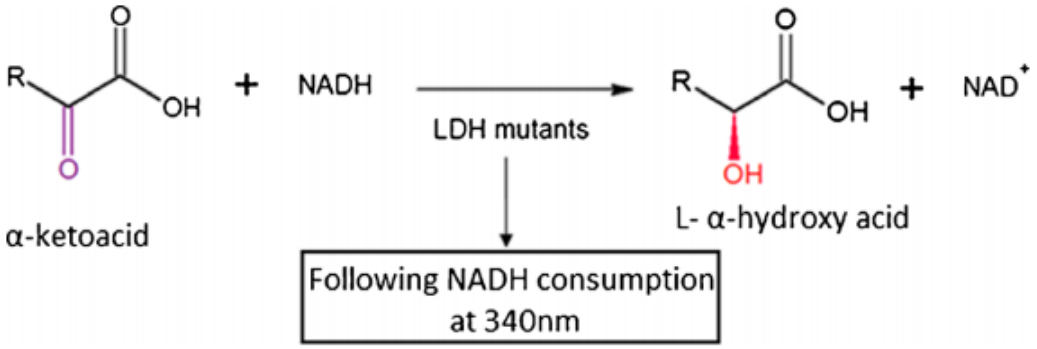


Figure 2.2 : The screening assay for detecting activity of *bs*LDH variants.

2.5 Protein Expression of WT and Mutant *bs*LDHs

WT *bs*LDH single colony was picked from the plate and inoculated into 20 ml LB with 100 µg/ml ampicillin and incubated overnight at 37^{o}C at 250 rpm as a pre-culture. Pre-cultures were grown in different expression conditions in order to understand which condition is the best for protein expression. These conditions had different mediums, different agitation speed and different temperatures. These conditions were given in Table below.

Table 2.7 : Different expression conditions.

Medium	Agitation speed (rpm)	Temperature (^oC)
LB	250	16
LB	250	20
LB	250	25
LB	250	37
LB	200	37
Auto-induction	250	20
Auto-induction	200	25
2X YT	250	37
2X YT	200	37

Pre-cultures were inoculated into 1 liter mediums with ampicillin (100 μg/ml) and incubated at 37^{o}C and the OD_{600} was observed. When OD_{600} reached to 0.6, cultures were induced with 1 M isopropyl β-D-thiogalactoside (IPTG) (except Auto-Induction Media) with different temperatures and agitation speeds. Before and after adding IPTG, 1 ml sample was taken and loaded to SDS-PAGE. According to SDS-PAGE results, best condition was choosen for protein expression (Auto-induction media, 25^{o}C, 200 rpm and 2 days incubation).

Wt *bs*LDH and selected *bs*LDH mutants were grown in auto-induction media at 25^{o}C, 200 rpm for 2 days. Growing cells were harvested by centrifugation at 4500 rpm at 4^{o}C for 20 min. Pellets were resuspended with Buffer A (20 mM sodium-phosphate, 500 mM NaCl, 30 mM imidazole, pH 7.4) with 1 mg/ml lysozyme and cells were placed on ice for 30 min. After incubation, cells were disrupted with sonication (20/20/20) and sonicated cells were collected by centrifugation at 22000 rpm at 4^{o}C for 30 min. Supernatant was used for purification.

2.6 Purification of WT and Mutant *bs*LDHs

The clarified lysates were filtered through a 0.45 µm filter and purified by using Ni-NTA His-Trap Column. First, column was washed with 10 ml water and was equilibrated with 5 ml Buffer A. The filtered samples were loaded onto column twice. Then column was washed with 5 ml Buffer A. After this step, *bs*LDHs were eluted with 3 ml of Buffer A with 100 mM imidazole, 5 ml of Buffer A with 200 mM imidazoleand 3 ml of Buffer A with 400 mM imidazole, respectively and collected as ~ 1 ml fractions. These fractions were loaded to SDS-PAGE in order to control the purity of fractions.

Protein concentration was determined with BCA method. Firstly, set of protein standarts were prepared from Bovine Serum Albumin Standart (BSA). Final BSA concentrations were 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml. 25 µl standarts and samples were replicated into a 96 well-plate. 200 µl Working Reagent (WR) was added, mixed for 30 sec. and incubated at 37^{o}C for 30 min. After incubation, plate was cooled to room temperature and absorbance was measured at 562 nm absorbance on a microplate reader.

2.7 Enzyme Activity Assay

First, the specific activity was determined by monitoring the absorbance changes at 340 nm due to catalyzed redox reactions for wild-type and mutant LDHs. The purified enzyme (10 µl) was transferred to 96 well-plate. After that, 90 µl reaction mix (1.11mM NADH, 11.1 mM 1,6-FBP in 50 mM pH 7.4 Tris-HCl) was mixed with enzyme and the reaction was started by 100 µl 20 mM target substrates (benzoylformate, oxaloacetate, phenylpyruvate, pyruvate). Best mutant *bs*LDHs were determined for their target substrate in accordance with specific activity results.

The steady-state kinetic experiments were done for wild type and best mutant *bs*LDHs. Reaction mixture was contained 1.11mM NADH, 11.1 mM 1,6-FBP, different concentrations of target substrate and enzyme in 50 mM pH 7.4 Tris-HCl. The rates were determined at 340 nm by absorbance change due to NADH consumption and fitted using the GraFit. Experimantal work was summarized in Figure 2.3.

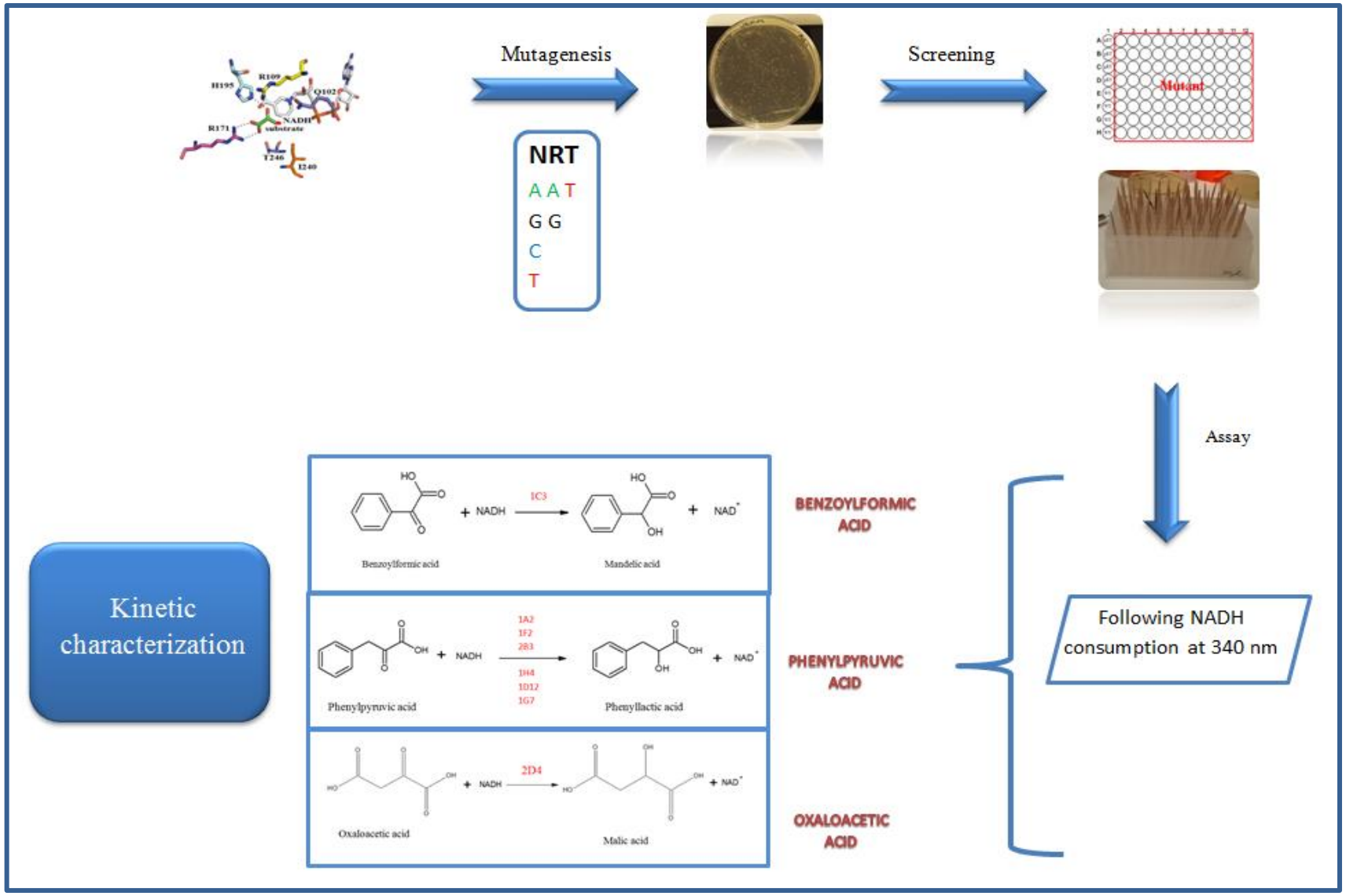


Figure 2.3 : Summary of experimental study.

3. RESULTS AND DISCUSSION

3.1 Mutagenesis Amplification

In order to expand substrate specificity of *bs*LDH, mutant library was constructed by mutagenesis according to Stratagene Quik Change protocol (Quik Change II Site-Directed Mutagenesis Kit) using Phusion polymerase, reaction buffer, dNTPs and designed primers. Six mutant libraries were constructed (N101Q102, R109, R171, H195, I240, T246). The amplified PCR products were run and controlled in agarose gel electrophoresis as shown in Figure 3.1.

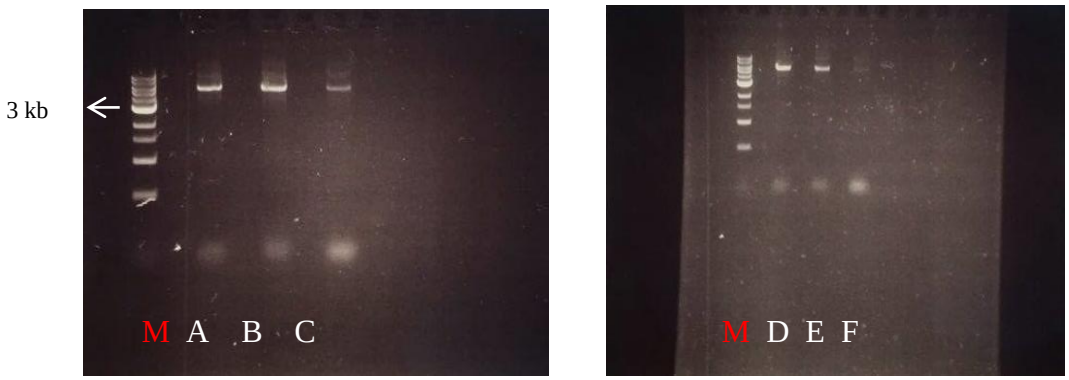


Figure 3.1 : Images of PCR products. M; Marker 1 kb DNA ladder (Biolabs); 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb, 1 kb, 0.5 kb., A(R109), B(R171), C(H195), D(I240), E(T246) and F(N101Q102) are PCR products that are mutated regions.

3.2 Transformation

The amplified PCR products that had amino acid changes at determined sites were transformed into BL21(DE3) competent cells. With transformations, many colonies were obtained for mutated regions at desired mutation sites (Figure 3.2).

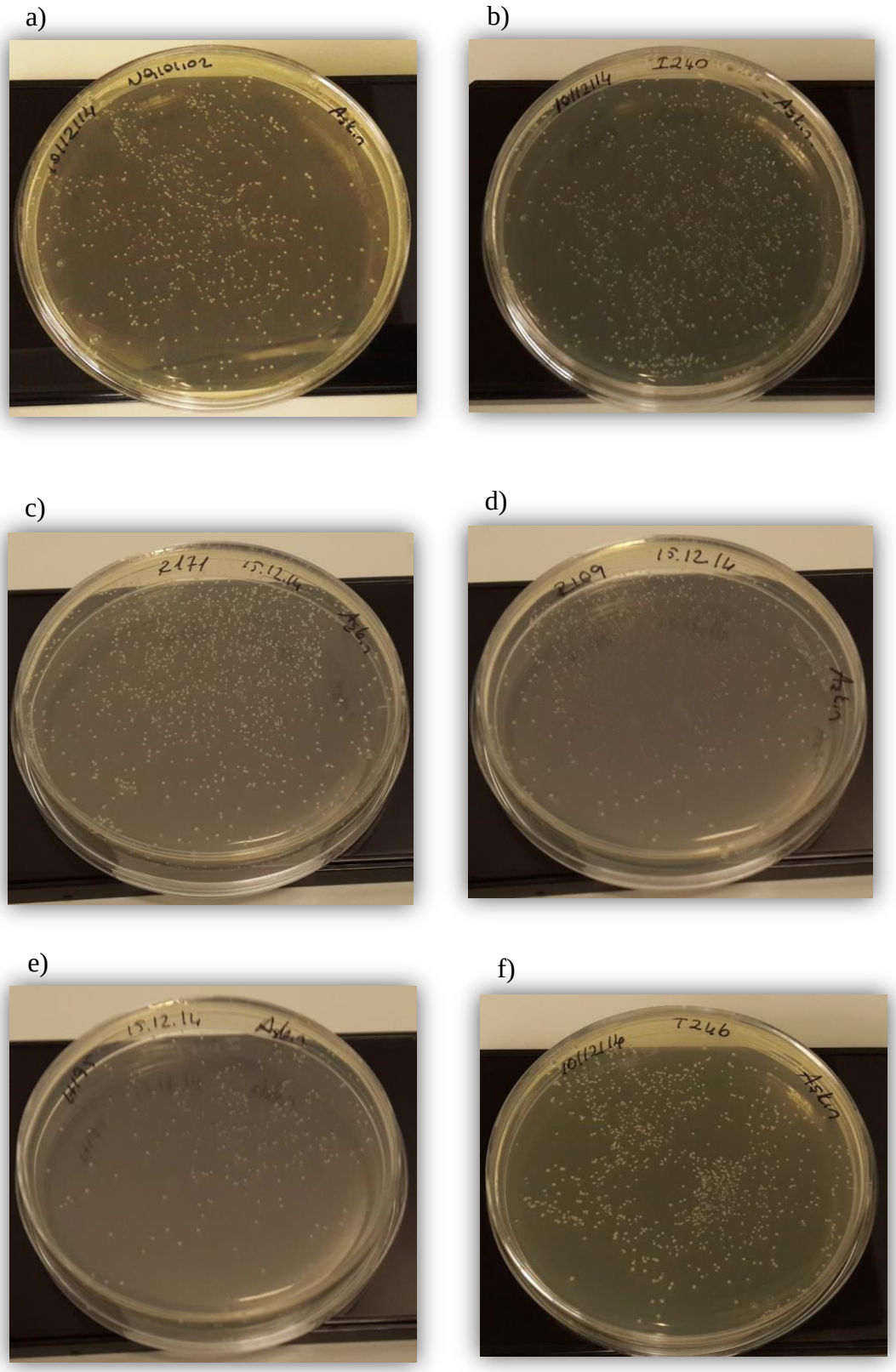
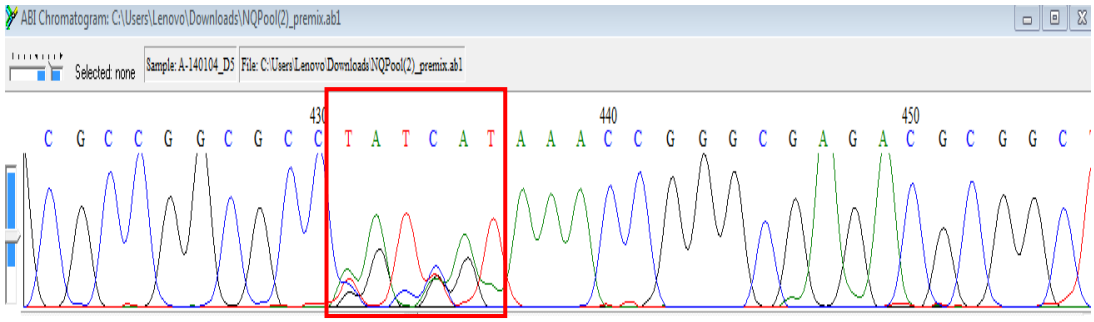


Figure 3.2 : Images of libraries; a) N101Q102, b) I240, c) R171, d) R109, e) H195, f) T246.

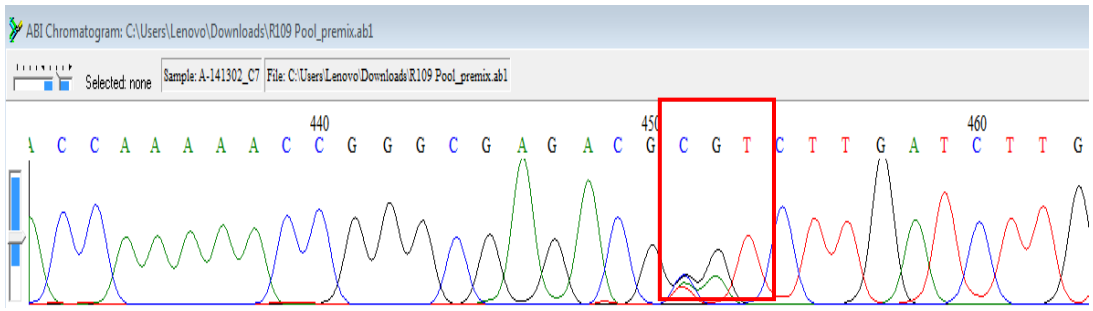
3.3 Mutation Confirmation

In each position, all colonies were mixed and collected into together. Plasmid purification was done and these mutant pool plasmids were sent to sequence against to pQE (5'CCCGAAAAGTGCCACCTG'3). Sequence chromatogram results of mutant pool plasmids showed that desired positions were encoded by NRT degenerative codon. In the Table 3.3 showed that A, G, C, T densities at determined positions.

a)



b)



c)

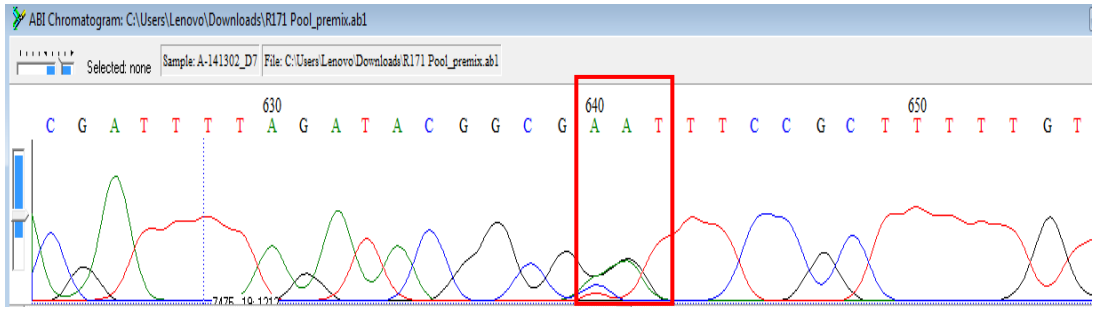
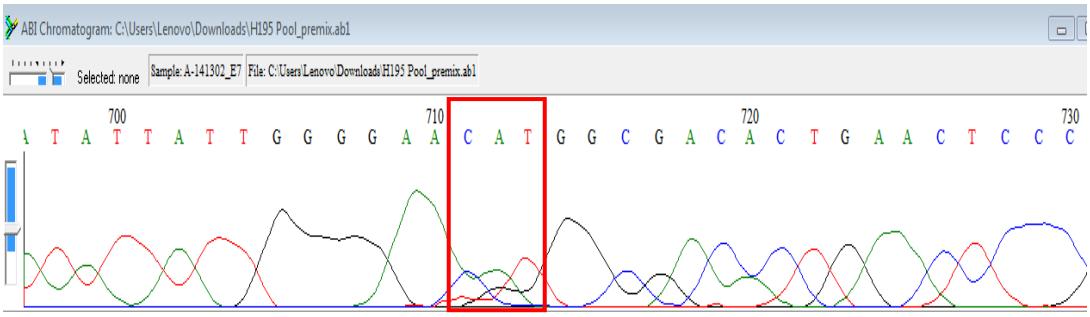
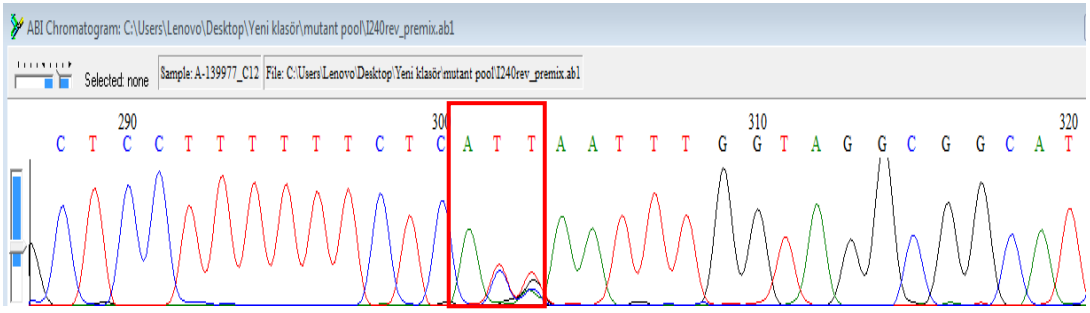


Figure 3.3 : Sequence chromatogram result of libraries; a) N101Q102, b) R109, c) R171, d) H195, e) I240, f) T246.

d)



e)



f)

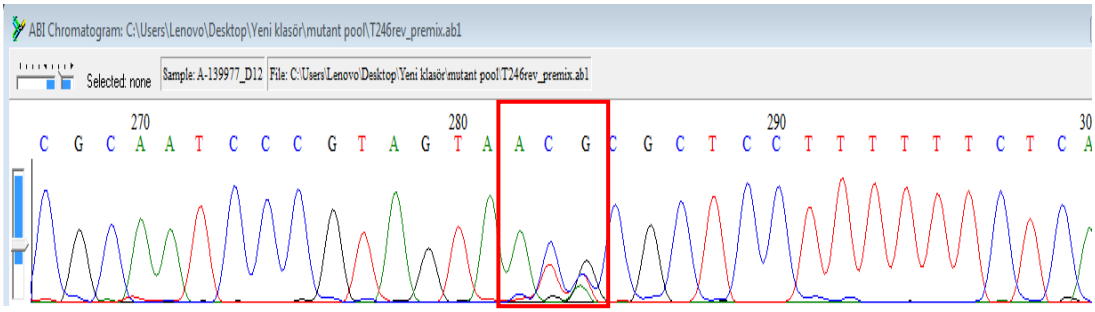


Figure 3.3 (continued) : Sequence chromatogram result of libraries; a) N101Q102, b) R109, c) R171, d) H195, e) I240, f) T246.

3.4 Screening of Mutant Libraries

Replica plates were screened for each position by following NADH consumption at 340 nm. In total, approximately 800 colonies were screened over the six libraries. Eight positive hits showed enhanced activity toward benzoylformate (1C3), oxaloacetate (2D4) and phenylpyruvate (1G7, 1F2, 1A2, 1H4, 2B3 and 1D12) compared to wild-type *bs*LDH. All eight variants came from the same library targeting position (N101Q102). The residue identities for positions 101 and 102 of these eight clones are provided in Table 3.1.

Table 3.1 : The amino acid mutations of *bs*LDH variants.

Substrate	Variants	Position101	Position102
Pyruvate	WT	Asn	Gln
Benzoylformate	WT	Asn	Gln
	1C3	Asn	Cys
Oxaloacetate	WT	Asn	Gln
	2D4	Asn	Arg
Phenylpyruvate	WT	Asn	Gln
	1G7	Asp	Tyr
	1F2	Cys	Asp
	1A2	Asn	Asp
	1H4	Asp	His
	2B3	Gly	Asp
	1D12	Ser	Asp

3.5 Protein Purification

For protein expression, different conditons were tried. Samples were collected before and after IPTG induction. Pellets and supernatants were run on SDS-PAGE (NuSTep gel) for each conditions (Figure 3.4).

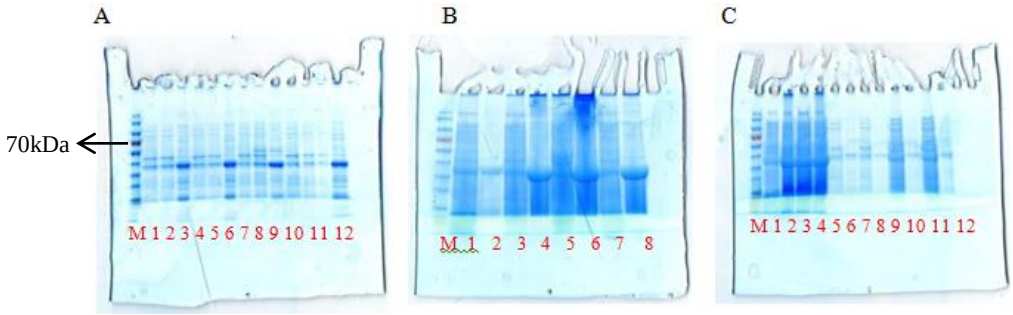


Figure 3.4 : SDS-PAGE images for different expression conditions. M; Marker PageRuler Prestained Protein Ladder (Thermo Scientific); 170 kDa, 130 kDA, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15kDa, 10kDa. A) Using BugBuster: 1-Before IPTG (16^{o}C) 2-After IPTG Supernatant (16^{o}C) 3-After IPTG Pellet (16^{o}C) 4- Before IPTG (20^{o}C) 5- After IPTG Supernatant (20^{o}C) 6- After IPTG Pellet (20^{o}C) 7- Before IPTG (25^{o}C) 8- After IPTG Supernatant (25^{o}C) 9- After IPTG Pellet (25^{o}C) 10- Before IPTG (37^{o}C)11- After IPTG Supernatant (37^{o}C)12- After IPTG Pellet (37^{o}C). B) After sonication: 1- Supernatant (16^{o}C) 2-Pellet (16^{o}C) 3- Supernatant (20^{o}C) 4- Pellet (20^{o}C) 5- Supernatant (25^{o}C) 6- Pellet (25^{o}C) 7- Supernatant (37^{o}C) 8- Pellet(37^{o}C) C) After sonication: 1- Supernatant 20^{o}C 250 rpm auto induction media 2-Pellet 20^{o}C 250 rpm auto induction media 3- Supernatant 25^{o}C 200 rpm auto induction media 4- Pellet 25^{o}C 200 rpm auto induction media 5- Supernatant 37^{o}C 250 rpm LB 6- Pellet 37^{o}C 250 rpm LB 7- Supernatant 37^{o}C 250 rpm 2X YT 8-Pellet 37^{o}C 250 rpm 2X YT 9- Supernatant 37^{o}C 250 rpm non induced LB 10- Pellet 37^{o}C 250 rpm non induced LB 11- Supernatant 37^{o}C non induced 2X YT 12-Pellet 37^{o}C non induced 2X YT.

WT and the eight variants showing improved activity were individually grown in auto-induction media (25^{o}C, 200 rpm, 2 days). Protein purification was done by

using Ni-NTA His-Trap column and the collected fractions were analyzed by SDS-PAGE.

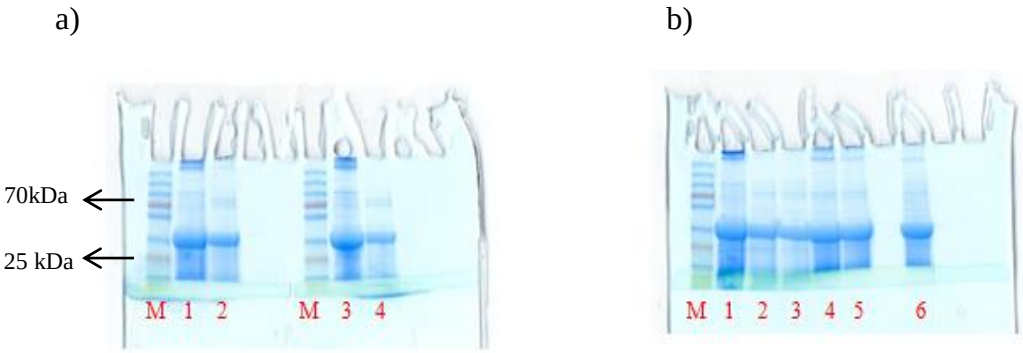


Figure 3.5 : SDS-PAGE images of pure active *bs*LDH variants. M; Marker PageRuler Plus Prestained Protein Ladder (Thermo Scientific); 250 kDa, 130 kDA, 100 kDa, 70 kDa, 55 kDa, 35 kDa, 25 kDa, 15kDa, 10kDa. a) 1-WT 2- N101C102 3- WT 4-N101R102 b) 1-N101D102 2-C101D102 3- S101D102 4- G101D102 5- D101Y102 6- D101H102.

3.6 Kinetic Activity Results

Specific activities of positive hits calculated against 10 mM their target substrates. Specific activities of each variants for the respective new substrate are listed in Table 3.2.

Table 3.2: Specific activity results of positive hits.

Substrate	Residue at Position 101	Residue at Position 102	Specific activity (µmol/min.mg)
Pyruvate	Asn	Gln	48.88 ± 1.87
Benzoylformate	Asn	Gln	7.11 ± 0.98
	Asn	Cys	10.98 ± 1.62
Oxaloacetate	Asn	Gln	23.87 ± 3.52
	Asn	Arg	27.55 ± 1.50
Phenylpyruvate	Asn	Gln	7.39 ± 0.75
	Asp	Tyr	51.26 ± 3.35
	Cys	Asp	30.01 ± 1.24
	Asn	Asp	28.71 ± 2.13
	Asp	His	24.91 ± 2.05
	Gly	Asp	22.60 ± 0.85
	Ser	Asp	21.63 ± 1.95

LDH variants 1C3 (N101C102), 2D4 (N101R102), 1G7 (D101Y102) exhibited the highest activity for benzoylformate, oxaloacetate and phenylpyruvate, respectively, and appeared to be the most improved for these non-natural substrates. These three

LDH variants were also assayed against a range of concentrations of pyruvate and the respective screening substrate to determine steady state kinetic parameters and corresponding activity changes compared to the WT enzyme for the natural and respective non-natural substrate.

The 1C3 variant with a Gln102Cys mutation showed approximately 2.5 fold increase in catalytic efficiency over the wild type enzyme for the novel substrate benzoylformate in addition to a significant loss in pyruvate activity primarily due to a 4.5- fold reduction in k_{cat}. The 2D4 variant did not provide any improvement in oxaloacetate activity, but rather only a reduction in activity on the natural substrate, yielding a 7-fold loss in catalytic efficiency towards pyruvate. The 1G7 variant evolved for activity on phenylpyruvate showed a surprisingly 4.5-fold increase in k_{cat}; however, an equivalent reduction in K_M result in the variant having the same catalytic efficiency as the wild type enzyme. In all cases, the LDH variants showed significant loss in activity for the natural substrate, pyruvate, despite it is not being an aspect of the screening process. A comparison of the substrate specificity of each LDH variant through catalytic efficiencies for the natural and non-natural substrates highlights the large change in the activity caused by these mutations. As shown in Table 3.4, each of the new LDH variants has greater activity for the target substrates (ratio of less than 1), indicating that the enzymes are more active on the non-natural substrates than on pyruvate.

Table 3.3 : The substrate specifity of each variant.

Substrates	*bs*LDH variants	(k_{cat}/K_M) natural / (k_{cat}/K_M) non-natural
Pyruvate / Benzoylformate	WT (N101, Q102)	14.893
	1C3 (N101, C102)	0.558
Pyruvate / Oxaloacetate	WT (N101, Q102)	1.800
	2D4 (N101, R102)	0.206
Pyruvate / Phenylpyruvate	WT (N101, Q102)	8.120
	1G7 (D101, Y102)	0.466

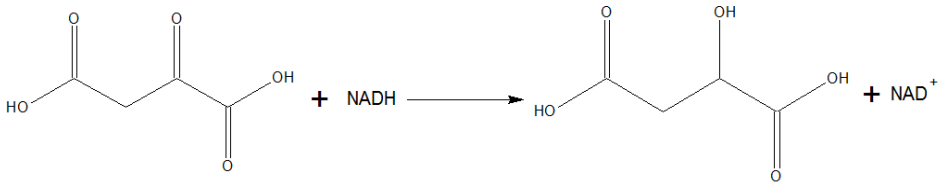
Table 3.4 : Kinetic parameters for *bs*LDH variants

*bs*LDH variants	Substrates					
	Pyruvate			Benzoylformate		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)
WT (N101, Q102)	0.25 ±0.04	25.8 ±0.9	104.55	0.44 ±0.11	3.08 ±0.17	7.02
1C3 (N101, C102)	0.58 ±0.10	5.79 ±0.23	9.90	0.29 ±0.06	5.23 ±0.19	17.74
	Pyruvate			Oxaloacetate		
WT (N101, Q102)	0.25 ±0.04	25.8 ±0.9	104.55	0.25 ±0.04	14.58 ±0.44	58.12
2D4 (N101, R102)	0.35 ±0.07	5.18 ±0.21	14.95	0.21 ±0.04	14.99 ±0.44	72.47
	Pyruvate			Phenylpyruvate		
WT (N101, Q102)	0.25 ±0.04	25.8 ±0.9	104.55	0.44 ±0.10	7.54 ±0.37	17.31
1G7 (D101, Y102)	0.61 ±0.08	3.81 ±0.12	6.27	2.43 ±0.35	35.34 ±1.30	14.49

a)

Benzoylformate Mandelic acid

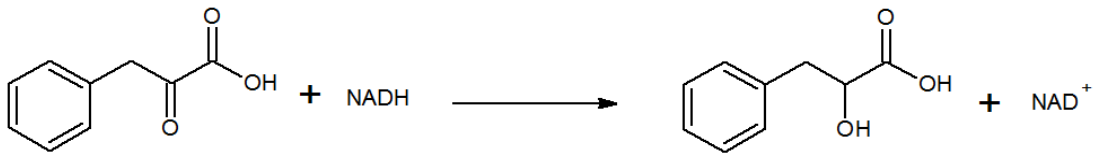
b)



Oxaloacetate Malic acid

Figure 3.6 : The reactions catalyzed by active *bs*LDH variants a) N101C102, b) N101R102, c) D101Y102.

c)



Phenylpyruvate Phenyllactic acid

Figure 3.6 (continued) : The reactions catalyzed by active *bs*LDH variants a) N101C102, b) N101R102, c) D101Y102.

4. CONCLUSIONS

LDH provides reversible conversion between an α-keto acid (pyruvate) and an α-hydroxy acid (lactate), by using NADH/NAD^+ as cofactor. *bs*LDH is extremely enticing to engineer for producing different chiral α-hydroxy acids which can be used in manufacturing pharmaceutical intermediates. Since the experimental determination of the structure of *bs*LDH, several enzyme engineering studies in the last 25 years have targeted different amino acid positions for increasing *bs*LDH substrate specificity. Rational design and directed evolution techniques have so far been tried for this purpose, focusing on residues N101, Q102, R109, R171, H195, I240 and T246 of *bs*LDH to clarify the roles of these residues in substrate binding and catalytic mechanism. Despite this, engineered *bs*LDH variant still remain fairly limited in differing substrate specificity.

In this study, a semi-rational design method was applied to *bs*LDH. The semi-rational design can be a more productive method for constructing a smart and small library of interesting biocatalysts since the potential variation is more selectively chosen as a smaller subset of amino acids. Selection of the right candidate amino acid positions is extremely important to the success of this method. The NRT degenerative codon chosen in this work only encodes eight amino acids, all of which have small or charged side chains. This was intentionally chosen to accommodate larger α-keto acid target substrates within the active site of *bs*LDH without disrupting crucial orientation with the catalytic residues. In addition, NRT considerably reduces the library size needed for screening >95% of all possible combinations of amino acids incorporated at each position, down to 24 variants rather than the 96 required for screening an NNK degenerate codon library.

A screening method was previously developed on solid phase for screening *bs*LDH libraries on α-hydroxy acids. This screen is based on colorimetric detection of redox catalysis via a reaction between dyes and NADH produced by variants able to oxidize target α-hydroxy acids. However, during the application of this method for our LDH construct, colonies were not adequately disrupted and sufficient protein was not available to provide a clear colour change to indicate activity. The new

screening method used in this work was carried out for the generated libraries using the target α-keto acids as substrate and screening in the reduction direction. The lysis buffer and lysozyme applied for cell disruption released sufficient protein to directly screen the cell lysate. Although this method is lower throughput than the solid phase screen, it was still sufficient for screening the focused NRT libraries, and also proved to be more sensitive than the solid phase screen.

Screening identified eight variants that showed higher activity toward the non-natural substrates benzoylformate, oxaloacetate, or phenylpyruvate. Interestingly, all variants with enhanced activity were from one library, targeting positions 101 and 102. The obtained experimental results support the previously characterized functions of R109, R171, H195, I240 and T246 as critical for orienting the substrates (including NADH) and catalyzing the reduction of the α-keto acid substrate. The specific activities of these purified variants were measured and revealed a high level of activity on the non-natural substrates. Three different variants (1C3, 2D4 and 1G7, one for each non-natural substrate) were selected as the best performing enzymes, and steady-state kinetic parameters were determined with the respective screening substrates and pyruvate, revealing that each new variant had altered specificity in favour of the non-natural α-keto acid over pyruvate.

To date, there has not been any reported *bs*LDH activity on benzoylformate. In this study, the selected 1C3 *bs*LDH variant showed 17.74 $mM^{-1}s^{-1}$ as catalytic efficiency, a notable 2.5-fold improvement against benzoylformate from the wild type enzyme, giving a potential biocatalytic route to S-mandelic acid. The Gln102Cys mutation also resulted in an about 10.5-fold decrease in catalytic efficiency for pyruvate. Replacing Gln with Cys in position 102 removes the steric hindrance that likely prevents benzoylformate from binding effectively. Based on molecular modelling studies, the mutation appears to increase the volume of the substrate binding pocket to accommodate the phenyl ring of the new substrate in the active site, while still maintaining the appropriate interactions with catalytic residues.The combination of these significantly improves the kinetic parameters for benzoylformate, creating a *bs*LDH variant that is more efficient for benzoylformate than for pyruvate, but still active on both substrates.

The 2D4 LDH variant with Gln102Arg mutation exhibits essentially the same activity as wild type LDH toward oxaloacetate, but does provide about a 7-fold

decrease in pyruvate catalytic efficiency. These results are consistent with the role already reported for residues at positions 101 and 102 (55), however the more improved Asn101Arg/Gln102Arg variant they identified was not detected in our screen. Although the substitution of Gln102Arg increases the size of the residue, the positively charged Arg side chain presumably provides a more beneficial interaction with the carboxylate of oxaloacetate than the polar Gln side chain in the wild type enzyme.

A similar increase in specific activity toward phenylpyruvate was observed for the six identified variants. The highest improvement of specific activity was identified for variant 1G7 with Asn101Asp and Gln102Tyr mutations. In this variant, k_{cat} was increased approximately 5-fold for against phenylpyruvate. However, due to a 5-fold loss in K_M, the catalytic efficiency of this variant is the same as the wild type enzyme. Similar to the effect of the mutation in variant 1C3, these two mutations are expected to provide the increased active site volume required to bind the large phenylpyruvate substrate. These results of the series of six improved enzymes give more perspective for analysing substrate recognition and its accommodation by the active site pocket of *bs*LDH. In spite of the vastly different amino acids observed in positions 101 and 102, these six variants are all more effective than wild type against phenylpyruvate, which suggests that there is a sizeable range of functional sequence variation permitted at these positions. The acceptance of residues with small, polar, aromatic, positively charged or negatively charged side chains at positions 101 and 102 indicates that *bs*LDH variants can possibly be engineered to accept a diverse array of functionalized α-keto acid substrates that may allow access to a large series of substrates for production of other industrially relevant α-hydroxy acids.

In conclusion, creating pure chiral α-hydroxy acids as building block intermediates is so valuable due to the functional properties they can impart on pharmaceutical chemicals. We have identified three variants of *bs*LDH with significantly enhanced activity towards the selected α-keto acids to produce chiral α-hydroxy acids. The measured K_M and k_{cat} of three novel biocatalysts support that the catalytic efficiency achieved in *bs*LDH is fairly comparable with those resulting from similar efforts on *bs*LDH in the past, however a smaller and more focused library screen was used in the present study.

In further studied, the generated libraries can be screened against different target substrates in order to produce different α-hydroxy acids. In addition to this, different protein engineering approaches can be applied and compared to this study.

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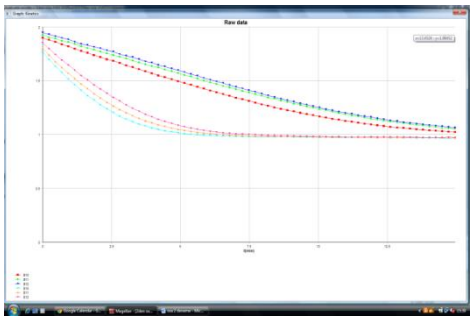
APPENDICES

APPENDIX A: Screening Results
APPENDIX B: Michaelis-Menten graphics of WT and mutant *bs*LDH
APPENDIX C: Amino acids and their 1-Letter and 3-Letter codes.

APPENDIX A : Screening Results

a)



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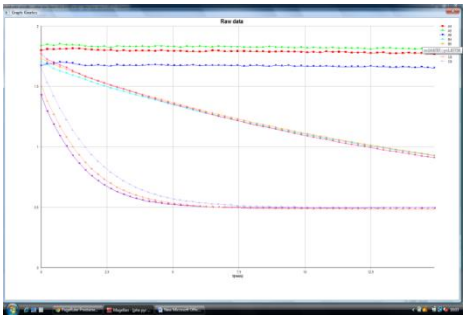

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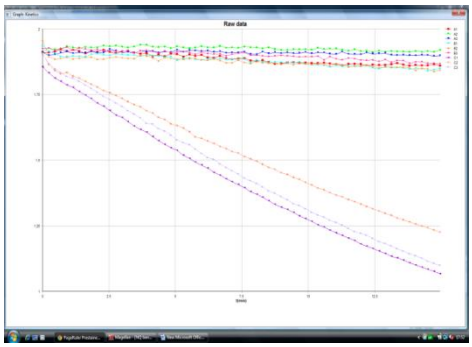

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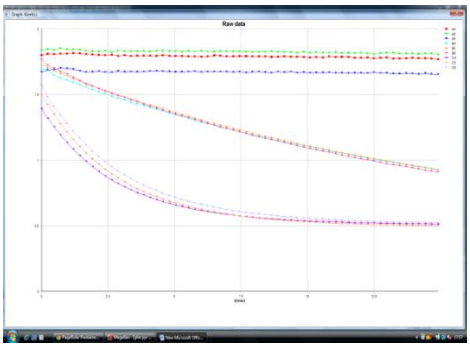

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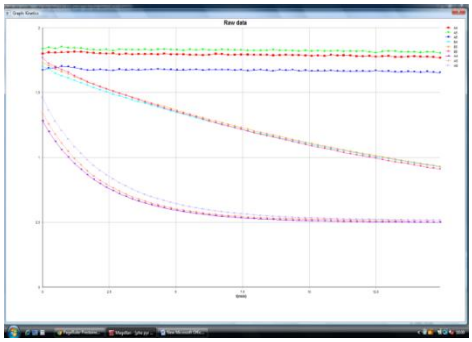

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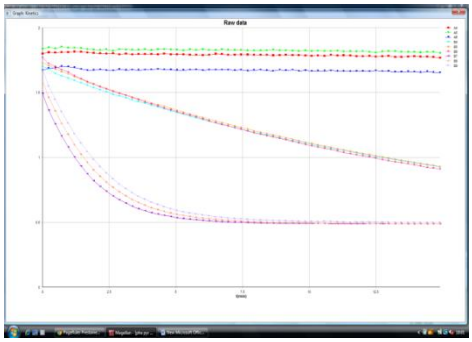
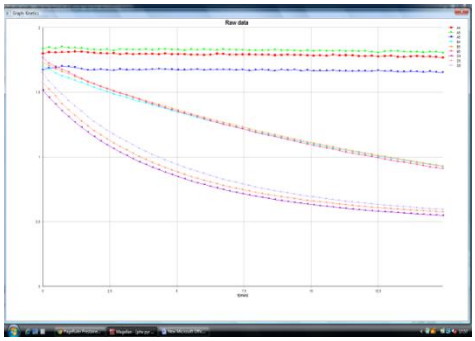


Figure A.1 : Screening results.

g)



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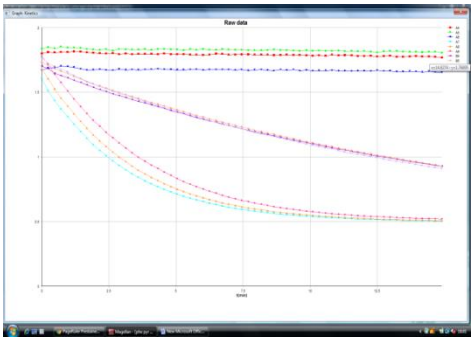

Figure A.1 (continued) : Screening results.

APPENDIX B: Michaelis-Menten graphics of WT and mutant *bs*LDH

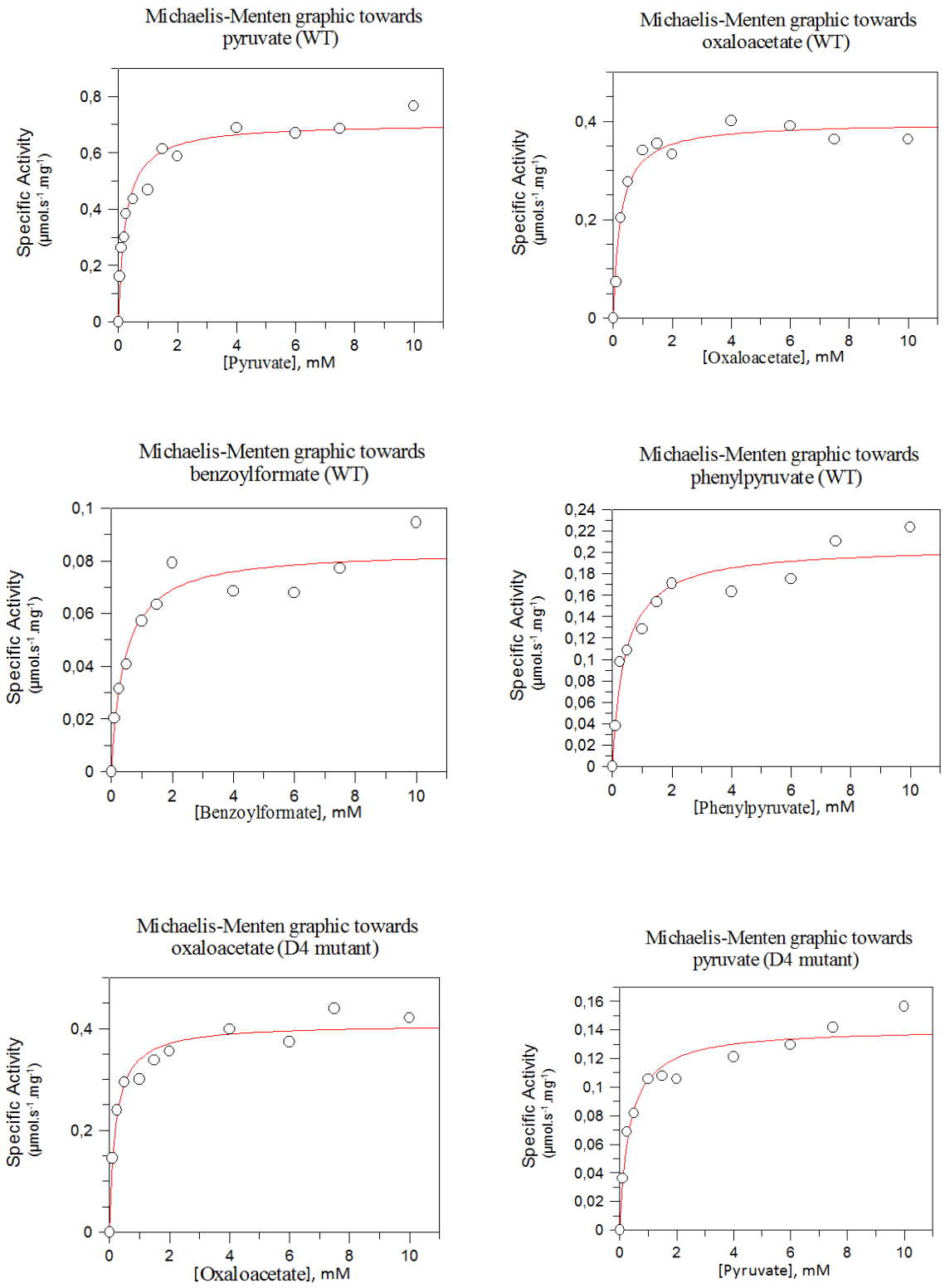

Figure B.1 : Michaelis-Menten graphics.

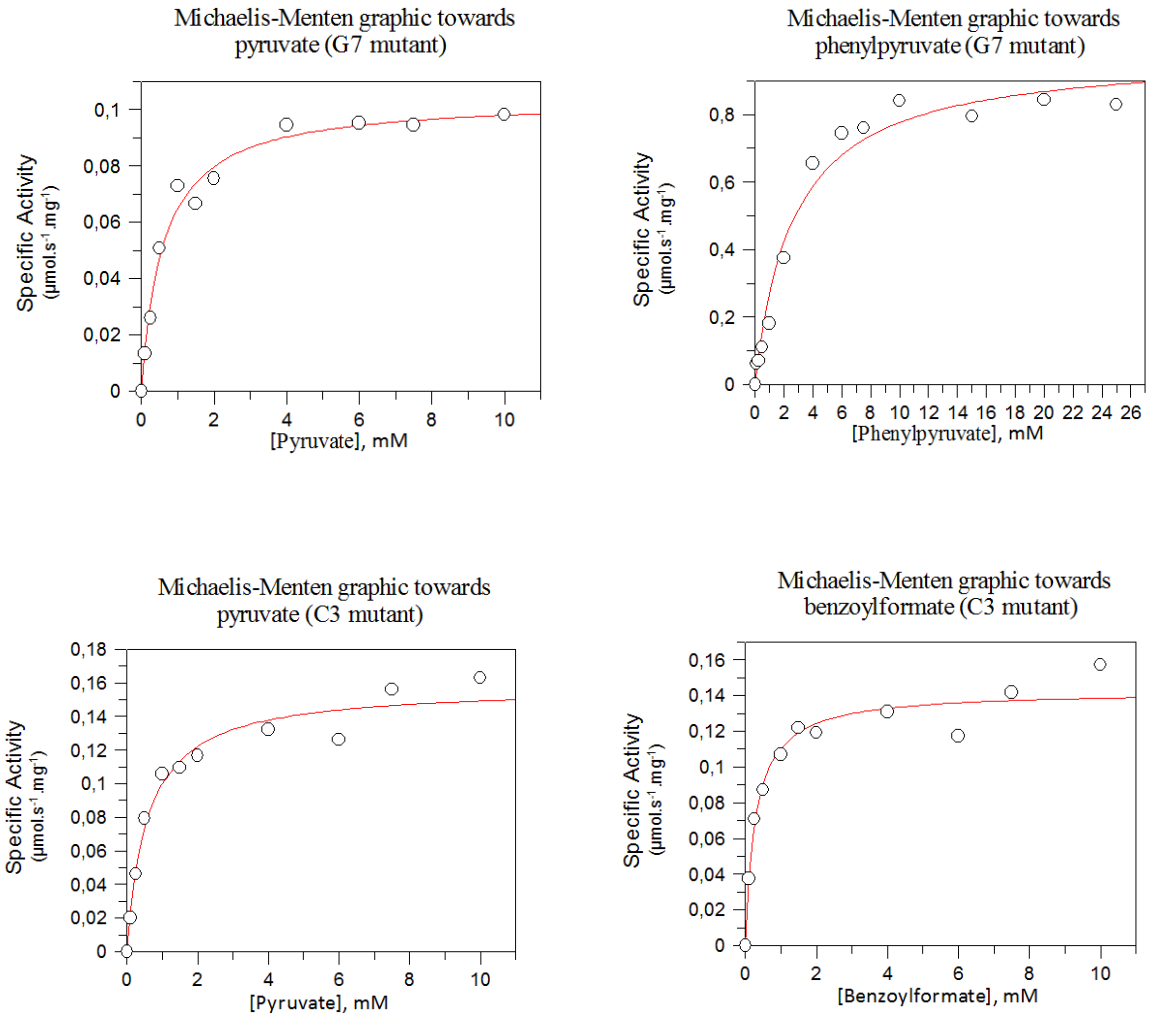

Figure B.1 (continued) : Michaelis-Menten graphics.

APPENDIX C: Amino acids and their 1-Letter and 3 Letter codes.

Table C.1: Amino acids and their 1-Letter and 3-Letter codes.

Amino Acid	1-Letter Code	3-Letter Code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartate	D	Asp
Cysteine	C	Cys
Glutamate	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Trytophan	W	Trp
Tyrosine	Y	Tyr

CURRICULUM VITAE

Name Surname **: Aşkın Sevinç Aslan**

Place and Date of Birth **: İstanbul, 1989**

E-Mail **: s.askin.aslan@gmail.com**

EDUCATION **:**

- **B.Sc.** **:**2013,Yıldız Technical University, Faculty of Science, Chemistry

PUBLICATIONS, PRESENTATIONS ON THE THESIS:

- Aslan, A.S., Birmingham, W.R., Gül-Karagüler, N., Turner, N.J., Binay, B., 2016. Semi-Rational Design of Geobacillus stearothermophilus L-Lactate Dehydrogenase to Access Various Chiral α-Hydroxy Acids, *Appl. Biochem, Biotechnol.*, doi : 10.1007/s12010-016-2007-x.
- Aslan, A.S., Birmingham, W.R., Gül-Karagüler, N., Turner, N.J., Binay, B., Engineering of lactate dehydrogenase from Bacillus stearothermophilus for the synthesis of chiral alpha-hydroxy acids, BioTrans, poster presentation, 2015, Vienna-Austria.