ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DEVELOPMENT OF NOVEL BIOCATALYSTS FOR INDUSTRIAL APPLICATIONS

Ph.D. THESIS

Barış BİNAY

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Programme

NOVEMBER 2012

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DEVELOPMENT OF NOVEL BIOCATALYSTS FOR INDUSTRIAL APPLICATIONS

Ph.D. THESIS

Barış BİNAY
(521052201)

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Assoc. Prof. Dr. Nevin GÜL KARAGÜLER
Co-advisor: Prof. Dr. Nicholas James TURNER

NOVEMBER 2012

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

ENDÜSTRİYEL UYGULAMALAR İÇİN YENİ BİYOKATALİZÖRLERİN GELİŞTİRİLMESİ

DOKTORA TEZİ

Barış BİNAY
(521052201)

İleri Teknolojiler Anabilim Dalı

Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Doç. Dr. Nevin GÜL KARAGÜLER
Eş Danışman: Prof. Dr. Nicholas James TURNER

KASIM 2012

Barış Binay, a Ph.D. student of ITU **Institute of Graduate School of Science and Techmology,** student ID 521052201, successfully defended the **thesis** entitled "**DEVELOPMENT OF NOVEL BIOCATALYSTS FOR INDUSTRIAL APPLICATIONS**", which he 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** İstanbul Technical University	.....
Co-advisor :	**Prof. Dr. Nicholas J. TURNER** University of Manchester	.....
Jury Members :	**Prof. Dr. Dilek KAZAN** Marmara University	.....
	Prof. Dr. Melek TÜTER İstanbul Technical University	.....
	Prof. Dr. Güldem ÜSTÜN İstanbul Technical University	.....
	Assoc. Dr. F. Neşe KÖK İstanbul Technical University	.....
	Assist. Dr. Nagehan E. TUNALI Haliç University	.....

Date of Submission : 11 September 2012
Date of Defense : 21 November 2012

To my endless family,

FOREWORD

I would like to thank my supervisors Assoc. Prof. Dr. Nevin Gül KARAGÜLER and Prof. Dr. Nick TURNER for the opportunity given to study in their laboratories. Special thanks for their guidance, patience, continued advice, supports and discussions in every stage of my thesis. Both of them shared all their sources, materials and the most importantly their valuable time with me. It was a great opportunity for me to work with them and to participate in their research projects.
Special thanks to Assist. Prof. Dr. B. Balta (Istanbul Technical University-ITU), Prof. Dr. A. Russell Clarke (University of Bristol), Dr. Richard B. Sessions (University of Bristol), Dr. I. Rowles (University of Manchester), Dr. S. Willies (University of Manchester), for their continued help and support during my studies. I sincerely thank all of my teachers and friends for sharing their laboratory experiences.
I am greatly indebted to my family; my wife Fatma BİNAY and my daughter Ronya BİNAY my father Ali BİNAY, my mother Hatice BİNAY, my brother Baykal BİNAY, for their deep love, support, endless encouragement and selfless sacrifice all the time. If you have such a perfect family behind you, there is nothing to be afraid of.
The financial support of DPT (State Planning Organization), The Scientific and Technological Research Council of Turkey (TÜBİTAK), Biotechnology and Biological Sciences Research Council in UK (BBSRC), are gratefully acknowledged.

NOVEMBER 2012

BARIŞ BİNAY
(M.Sc.)

TABLE OF CONTENTS

Page

ABBREVATIONS

A	**:**Absorbance
A	**:**Adenine
Amp	**:**Ampicillin
Amp^R	**:**Ampicillin resistance genes
BCA	**:**Bicinchoninic acid
Bp	**:**Base pair
Br-CA	**:**Brom cinnamic acid
Bs	**:***Bacillus stearothermophilus*
BSA	**:**Bovine serum albumin
C	**:**Cytosine
CAST	**:**Combinatorial active-site saturation test
D-AAO	**:**D-Amino Acid Oxidase
dH_2O	**:**Distilled water
DMSO	**:**Dimethyl sulfoxide
DNA	**:**Deoxyribonucleic acid
dNTP	**:**Deoxyribonucleoside triphosphate
dNTPs	**:**Deoxyribonucleotides 5'-triphosphate
dsDNA	**:**Double stranded DNA
dTTP	**:**Deoxytimine triphosphate
E. coli	**:***Escherichia coli*
EDTA	**:**Ethylenediaminetetraacetic acid
epPCR	**:**Error prone PCR
FBP	**:**Fructose - 1, 6 - bisphosphate
FPLC	**:**Fast Protein Liquid Chromatography
G	**:**Guanine
G	**:**Gram
GAO	**:**Galactose Oxidase
HAL	**:**Histidine ammonia-lyase
IPTG	**:**Isopropylthio-β-D- galactoside
K	**:**Kilo
Kb	**:**Kilobase
k_{cat}	**:**Turnover number of an enzyme
kDa	**:**KiloDalton
K_M	**:**Apparent Michaelis constant
L	**:**Liter
L-AAO	**:**L-Amino Acid Oxidase
Lac	**:**Lactate
LB	**:**Luria-Bertani medium
Ldh	**:**Lactate dehydrogenase gene
LDH	**:**Lactate dehydrogenase
M	**:**Molar

MD	**:**Molecular Dynamic
MDH	**:**Malate dehydrogenase
MIO	**:**5-methylene-3,5-dihydroimidazol-4-one
Min	**:**Minute
Mol	**:**Mole
mRNA	**:**Messenger RNA
MWCO	**:**Molecular weight cut off
NAD^+	**:**Nicotinamide adenine dinucleotide
NADH	**:**Nicotinamide adenine dinucleotide hydrogen
Nm	**:**Nanometres
NMR	**:**Nuclear magnetic resonance
^oC	**:**Degrees Celsius
ODX	**:**Optical density at X nm
PAGE	**:**Polyacrylamide gel electrophoresis
PAL	**:**Phenylalanine ammonia-lyase
PCR	**:**Polymerase Chain Reaction
Pfu	**:**Pyrococcus furiosus DNA polymerase
Pyr	**:**Pyruvate
R. glutinis	***:Rhodotorula glutinis***
R. graminis	***:Rhodotorula graminis***
RNA	**:**Ribonucleic acid
Rpm	**:**Revolutions per minute
SDS	**:**Sodium dodecyl sulfate
S	**:**Seconds
ssDNA	**:**Single stranded DNA
T	**:**Thymine
Taq	**:**Thermus aquaticus DNA polymerase
Tris	**:**Tris (hydoxymethyl) aminomethane
U	**:**Units of enzyme activity
UV	**:**Ultraviolet light
v/v	**:**Volume by volume
V_{max}	**:**Maximum rate of catalysed by enzyme
Vol	**:**Volume
w/v	**:**Weight by volume
Wt	**:**Wild type
Å	**:**Angstrom
β – gal	**:**β – galactosidase
3-D	**:**Three-dimensional

LIST OF TABLES

Page

LIST OF FIGURES

Page

DEVELOPMENT OF NOVEL BIOCATALYSTS FOR INDUSTRIAL APPLICATIONS

SUMMARY

Global warming and the resultant problems have made the scientific community and general public realize that there is a need to decrease dependency on oil for energy. It has also shown the need to increase the efficient use of energy in manufacturing and industrial processes. Green processes would be an alternative way of decreasing oil dependency and biotechnology is promoted as a way both to decrease oil dependency and as a source for renewable bio-based products.

Biocatalysis (using enzymes or whole systems) can provide a valuable alternative to traditional chemical processes because it has many advantages such as; efficiency in enhancing the rate of chemical reactions and for their ability to discriminate between potential substrates. Therefore there is the possibility of using biological molecules to catalyse any reaction or modify any product of interest to industry.

Biological catalysts from animal, plant and bacterial sources have evolved to perform most types of organic reactions, producing chirally pure and complex molecules with interesting biological properties. Biocatalysts have thus become important tools in medicine, the chemical industry, food processing and in agriculture. Industrial processes often require extreme conditions such as high pressure, temperature and extreme pH which require a large amount of energy to achieve and may produce unwanted toxic waste. Biological enzymes do not require such conditions and produce chirally pure products often without the disadvantages of unwanted toxic by products.

The use of naturally occurring enzymes is not that common because of limited substrate and coenzyme specificity and kinetic problems with the reactions. Protein engineering is a promising approach which can be used to create enzymes with the desired properties. Enzymes can also be engineered to better understand the molecular basis for their functions so that they will be able to synthesize novel products in non-native environments.

Here, we have aimed to improve the substrate specificities of two industrially important enzymes namely; *Bacillus stearothermophillus* lactate dehydrogenase (*bs*LDH) and *Rhodotorula graminis* phenylalanine amonyum lyase *(rg*PAL).

To improve the properties of these two enzymes, we have applied three routes of protein engineering studies namely; DNA shuffling, site directed mutagenesis and combinatorial active site saturation (CASTing). The substrate specificity of *bs*LDH was successfully altered by using DNA shuffling and site directed mutagenesis approaches. The activity of *rg*PAL for the selected different substrate(s) was also increased by CASTing.

This thesis details various strategies used to produce novel *bs*LDH and *rg*PAL proteins. It is divided into three experimental sections. The first two sections describe

to engineer *bs*LDH and the last section explains the application of CASTing method on *rg*PAL.

*bs*lactate dehydrogenase catalyses the interconversion of an oxo-acid (pyruvate) and hydroxy-acid (lactate) using the NADH/NAD^+ pair as a redox cofactor. The enzyme has a commercial significance as it can be used to produce chiral building blocks for the synthesis of key pharmaceuticals and agrochemicals. Although *bs*LDH allows the synthesis of chiral hydroxy acids from their corresponding oxo-acids with high stereochemical fidelity, this enzyme has the disadvantage of generally not having very broad substrate specificity.

In the first part of the thesis; DNA shuffling was used to alter the substrate specificity of *bs*LDH. The recombinant colonies having shuffled *bs*LDH are blotted and screened for their ability to catalyse the oxidation of malate.

The shuffled mutant LDH has eight amino acid substitutions -Asn4Gln, Ser19Thr, Gln86Arg, Thr91Ser, Ala173Val, Glu183Asp, Gln221Asn and Val267Thr- changing the substrate specificity of *bs*LDH from pyruvate / lactate to malate / oxaloacetate.

The eight amino acid substitutions led to a new malate dehydrogenase that catalysed the oxidation of malate 1009 times faster than the pyruvate does. This result demonstrates that the blind shuffling can achieve a huge shift in specificity which was a known, highly effective single-site mutation designed using structural knowledge.

In the second part of the thesis; we described attempts to change the reactivity of *bs*LDH towards L-mandelic acid. Using the Insight II molecular modelling program and protein engineering techniques, we have successfully introduced substantial mandelate dehydrogenase activity to the enzyme. Energy minimisation modelling studies suggested that two mutations, Thr246Gly and Ile240Ala, would allow the enzyme to utilise L-mandelic acid as a substrate.

Genes encoding for the wild type and mutant enzymes were constructed, and the resulting *bs*LDH proteins were overexpressed in *E. coli* and purified using the TAGZyme system . Enzyme assays showed that insertion of this double mutation to the highly purified *bs*LDH switched the substrate specificity from lactate to L-mandelic acid.

In the last part of the thesis, our aim was to increase the enzymatic activity of the phenylalanine ammonia-lyase (PAL) from *Rhodotorula graminis* for non-natural specific substrates.

PAL catalyses the non-oxidative conversion of L-phenylalanine (L-Phe) to trans-cinnamic acid (t-CA). The reversibility of this reaction has been used to synthesise analogues of L-phenylalanine as pharmacophores in several peptidomimetic drug molecules and are therefore of particular interest to the fine chemical industry.

Five (5) libraries were set up, with at least two residues in each library, around the active site based on CASTing method that is a semi rational design. The degenerate codon NRT was used for all libraries. A total of 2000 colonies were screened using a developed assay 1 and after several rounds of screening, four PAL variants which displayed activity against selected substrate.

Two assays have been developed for facilitating the identification of PAL variants. The first one is applied for screening libraries and measuring the formation of t-CA during the deamination. In the second assay, an oxidase enzyme (as a reporter

enzyme) is coupled for allowing the detection and quantification of phenylalanine production by PAL enzymes.

Following the purification, the mutant *bs*LDH and *rg*PAL enzymes were kinetically characterised. Kinetic studies proved that the substrate specificities of *bs*LDH and rgPAL were successfully altered.

These results imply that protein engineering is a powerful technology for enzyme redesign if an appropriate strategy is applied.

ENDÜSTRİYEL UYGULAMALAR İÇİN YENİ BİYOKATALİZÖRLERİN GELİŞTİRİLMESİ

ÖZET

Küresel ısınma odaklı tartışmalar ve bu konuda oluşan kaygılar son yıllarda bilim insanları ve toplum içinde giderek artmaktadır. Bu durum, petrol kaynaklı üretimler yerine yenilenebilir biyolojik kaynaklara yönelimi teşvik etmektedir. "Yeşil proses" olarak adlandırılabilecek biyokatalizörlerin farklı endüstriyel alanlarda kullanımının önemi yakın zamanda idrak edilmiştir.

Kimyasal katalizörler yerine hayvansal, bitkisel ve bakteriyel kaynaklardan elde edilen enzimlerin kullanımı endüstriyel proseslerde çeşitli avantajlar sağlamaktadır. Kiral merkezli moleküllerin sentezi kimyasal yöntemlerle gerçekleştirildiğinde rasemik karışımlar elde edilmektedir.

Enzimatik reaksiyonlar ise enantiomerik saflıkta ürün üretimine imkan verdiklerinden, biyokatalizörlerin kiral bileşik üretiminde kullanılması çok uygundur. Biyokatalizör kullanımının diğer avantajları enzimlerin biyobozunur olmaları, katalizledikleri reaksiyonda stereo-seçici, bölgesel-seçici ve kimyasal-seçici davranmaları, görece düşük sıcaklıkta, ılıman pH ve basınç koşullarında fonksiyon göstermeleri ve çevre dostu olmaları şeklinde sıralanabilir. Bu avantajlarından dolayı biyokatalizörlerin tıp, kimya, gıda üretimi ve tarım endüstrilerinde kullanım alanları giderek artmaktadır.

Fakat doğal çevreden izole edilen enzimlerin doğrudan endüstriyel proseslerde kullanılabilmeleri herzaman mümkün olmamaktadır. Enzim karakteristik özelliklerinin (aktivite, stabilite, çözünürlük, substrat ve koenzim seçiciliği ve optimum pH aralığı) sıklıkla ilgili endüstriyel üretime göre optimize edilmesi gerekmektedir.

Protein mühendisliği yöntemleri, ilgili proses koşullarına uygun enzimlerin dizayn edilmesine olanak tanımaktadır. Protein mühendisliği, moleküler biyoloji tekniklerinin kullanılması yolu ile enzimlerin yapı ve fonksiyonlarının moleküler düzeyde anlaşılıp, genetik yapılarının değiştirilerek, istenilen yapı ve fonksiyona sahip yeni enzimlerin üretilmesini mümkün kılmaktadır.

Burada, endüstriyel öneme sahip olan *Bacillus stearothermophillus* laktat dehidrogenaz (*bs*LDH) ve *Rhodotorula graminis* fenilalanin liyaz (*rg*PAL) olarak adlandırılan enzimlerin substrat özgüllüklerinin iyileştirilmesini hedefledik.

Bu iki enzimin özelliklerinin iyileştirilmesi için protein mühendisliğinin üç yöntemi uygulanmıştır; DNA shuffling (DNA karıştırma), bölgeye özel mutasyon yaklaşımları ile kombinatoryal saturasyon aktif bölge testi (CASTing).

*bs*LDH'ın substrat spesifikliği, DNA karıştırma ve bölgeye özel mutasyon yaklaşımları ile başarıyla değiştirilmiştir. *rg*PAL'ın aktivitesi ise seçilen farklı substratlara karsı CASTing yöntemi kullanılarak arttırılmıştır.

Bu tez kapsamında yeni *bs*LDH ve *rg*PAL proteinlerinin üretilmesine yönelik farklı stratejiler detaylı bir şekilde incelenmiştir. Üç deneysel bölümden oluşan tezin ilk iki bölümü *bs*LDH'ın düzenlenmesini tanımlarken, son bölüm *rg*PAL için CASTing metodunun uygulanmasını açıklamaktadır.

*bs*LDH, okzo-asitlerin (pirüvat) hidroksi asitlere (laktat) karşılıklı dönüşüm reaksiyonunu NADH/NAD+ çiftini redoks kofaktörü olarak kullanarak katalizler. Enzim, eczacılık ve tarım alanında anahtar role sahip kiral moleküllerin sentezinde kullanılan ara ürünlerin üretiminde kullanıldığından ticari öneme sahiptir. İlgili okzo-asitlerin kiral hidroksi asitlerinin yüksek stereokimyasal seçicilikle üretilmesine imkan vermesine rağmen; *bs*LDH enziminin en önemli dezavantajı son derece sınırlı substrat özgüllüğüne sahip olmasıdır.

Tezin ilk kısmında; *bs*LDH'ın substrat seçiciliğini değiştirmek için DNA karışım yöntemi kullanılmıştır. DNA karışım yöntemi ile elde edilen rekombinant mutant *bs*LDH kolonilerinden, malatın oksidasyonunu katalizleyebilenler uygun tarama ve seçme test yöntemi kullanılarakdiğerlerinden ayırt edilmişlerdir.

Yönlendirilmiş evrim sonucunda sekiz (8) tane amino asit değişimine - Asn4Gln, Ser19Thr, Gln86Arg, Thr91Ser, Ala173Val, Glu183Asp,Gln221Asn ve Val267Thr- sahip olan mutant bsLDH proteininin substrat seçiciliğini pirüvat/laktatdan okzaloasetat/malata değiştirmiştir.

Bahsedilen sekiz mutasyonun *bs*LDH'de malat dehidrogenaz aktivitesi oluşumuna neden olduğu ve malatın oksidasyonunu, pirüvata kıyasla 1009 kat daha hızlı olacak şekilde gerçekleştirdiği görülmüştür. Bu sonuç, yapı bilgisi kullanılarak yüksek verimli tekli mutasyonlarla hedeflenenin gerçekleştirildiği tasarımlara göre, yönlendirilmiş evrimin de özgüllüklerde yüksek değişikliklere yol açabileceğini göstermiştir.

Tezin ikinci kısmında; X-ışını kristal yapısı ve biriken literatür bilgisi kullanılarak *bs*LDH rasyonel olarakmandelik asitle reaksiyona girecek şekilde dizayn edilmiştir. Mandelat dehidrogenaz aktivitesi, InsightII moleküler modelleme programı kullanılarak; *bs*LDH enziminde oluşturulmuştur. Modelleme programı enerji minimizasyonu sonucunda; Thr246Gly ve Ile240Ala mutasyonları ile *bs*LDH enziminin L-mandelik asidi substrat olarak kullanabileceği gösterilmiştir.

Mutanlar oluşturulmuş ve mutant gen *E.coli*'ye aktarılarak sentezi gerçekleştirilmiştir. Ayrıca mutant ve doğal *bs*LDH'i saflaştırmak için TAGZyme saflaştırma sistemi oluşturulmuştur.

Rasyonel olarak tasarlanan *bs*LDH'ın aktivitesi mandelat varlığında ölçülmüştür. Enzim kinetiği sonuçları, bu iki mutasyonun, yüksek saflıkta elde edilen mutant *bs*LDH'ın substrat özgüllüğünü laktatdan L-mandelik asite dönüştürdüğünü göstermiştir.

Tezin son kısmında; *E.coli*'ye ekspresyon vektörüyle klonlanmış *Rhodotorula graminis* kaynaklı fenilalanin amonyum liyaz (*rg*PAL) enziminin doğal olmayan amino asitlere karşı aktivite kazandırılmasını amaçladık.

PAL, oksijensiz ortamda L-fenilalaninin trans-sinamik aside dönüşümünü katalizlemektedir. Bu reaksiyonun geri dönüşüm reaksiyonu, farmakoper olarak birçok peptidomimetrik ilaç molekülünün üretiminde L-fenilalanin analoglarının sentezinde kullanıldığından, enzim kimya endüstrisinde özel bir öneme sahiptir.

Yarı rasyonel dizayn olarak değerlendirilen CASTing metodu çerçevesinde, her bir kütüphane için en az iki tane amino asit içeren beş kütüphane oluşturulmuştur. NRT yıkıcı kodon olarak bütün kütüphaneler için kullanılmıştır. 2000 koloni, geliştirilen protokol 1 ile taranmıştır. Birçok tarama adımından sonra seçilen substrata karşı yüksek aktivite gösteren dört *rg*PAL varyantı belirlenmiştir.

PAL varyantlarının tanımlanmasını kolaylaştırmak için iki tane test yöntemi geliştirilmiştir. Birinci test yöntemi, elde edilen kütüphaneleri taramak ve oluşan trans sinamik asit (t-CA) miktarını ölçmek için uygulanmıştır. İkinci test yönteminde ise, belirleyici enzim olarak kullanılan oksidaz enzimi, PAL enzimleri tarafından üretilen fenilalaninin miktarının kantitatif olarak ölçülmesine olanak tanıyacak şekilde eşleştirilmiştir.

Uygun saflaştırma prosesleri sonrasında, mutant *bs*LDH ve *rg*PAL enzimleri kinetik olarak karakterize edilmişlerdir. Enzim kinetiği çalışmaları, *bs*LDH ve *rg*PAL enzimlerinin substrat özgüllüklerinin başarılı bir şekilde değiştirildiğini göstermiştir.

Elde edilen bu sonuçlar, uygun strateji uygulandığında protein mühendisliğinin, enzimlerin yeniden tasarlanmasında çok güçlü bir teknoloji olduğunu göstermiştir.

1. INTRODUCTION

1.1 Evolution

Charles Darwin started voyage of the Beagle in 1831 as a researcher. After completion of his voyage, precisely twenty-three years later, 1250 copies of his book "On the Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life" was published [1]. In his book, Darwin`s main argument was, by gradual, step by-step transformations from simple beginnings, from primordial entities sufficiently simple to have come into existence by chance. Each successive change in the gradual evolutionary process were arisen by chance was simple enough and relative to its predecessor. Nevertheless, the whole sequence of cumulative steps constitutes anything but a chance process, when you consider the complexity of the final product relative to the original starting point. The cumulative process is directed by non-random survival [1].

Evolution shows that the change in all species living in nature in order to survive under different/hard environmental conditions has been stretched in a long time. In nature, species realize the best possible adaptation form for themselves by accumulated mutation(s) in their own genes. The processes of random mutation, recombination and natural selection act as if an "engineer" adapts a living organism with minimum change to the new conditions, but eliminates those species resisted to adapt to new conditions at the biological molecule level. This "engineer" was the fact of "natural selection" accumulated from Thomas Hobbes to David Hume, from Adam Smith to Thomas Robert Malthus and to Alfred R. Wallace, then finally to Charles Darwin [2].

Each biological molecule has been optimized through evolution to perform its physiological task upon which all forms of life depend. Proteins are the major components of organisms and they are subject to natural selection, which promotes novel adaptive features or removes deleterious mutations.

1.2 Biocatalysts and DNA Technology

One of the most important biological molecules are biocatalysts (referred to as enzymes) which have been naturally evolving over millions of years by accumulating mutations in the DNA of the genes which encode them. Biocatalysts are either proteins (enzymes) or, in a few cases, they may be nucleic acids (ribozymes). They are known as the most remarkable biomolecules because of their extraordinary specificity and catalytic power [3]. The specificity and (enantio- and regio-) selectivity of certain enzymatic transformations make them attractive for the researchers. In the natural environment, Darwinian evolution selects for the altered properties of enzymes. Many enzymes catalyze very different reactions depending on where they are found. However, enzymes have come about by divergent evolution from a common ancestral protein of the same general structure. Therefore, enzymes have been acquired diverse capabilities by the "engineer". Enzymes have all the properties of ideal catalysts for various chemical reactions. In the presence of an appropriate enzyme, a chemical reaction occurs at a much higher rate and the enzymes still continue to survive in reaction environment. The most important feature of the enzymes is to perform very specific transformations that have made them more popular in industries where less specific chemical processes produce unwanted by-products. Purity and predictability are of particular importance in food manufacture, where by-products may be harmful or affect flavor, and because of their specificity, pharmaceutical producers prefer enzymes in the development of new drug. Because of their chiral and position selectivity they are used to produce fine chemicals, which are difficult to make using traditional organic chemistry. The importance of cleaner methods for industry grows as a greater awareness of conservation issue, so enzymes are significant for this area outside of the pharmaceutical and food industries. In the case of enzymes, however, the question whether they can also act as catalysts outside living systems had been of controversy among biochemists in the beginning of the twentieth century. Even there are not any discovered technological tools for transcription of the genetic code into ribonucleic acid (RNA) and translation of the information into protein biosynthesis, together with some methods used in genetic engineering, e.g. the transformation of deoxyribonucleic acid (DNA) into living cells. Although enzymes are formed within cells, they can continue to function in a test-tube and their ability to perform very

specific chemical transformations makes them increasingly useful in industrial processes. The use of industrial enzymes in the industry goes back as far as the 1930s for beverage industry, when Röhm&Haas launched the pectinase "Pectinol K" onto the market for the clarification of apple juice haze and in the 1960s with the use of glucoamylase to produce greater yields, higher purity and easier crystallization of glucose from starch [4-9].

In the late 1980s, a new technology was developed. This technology is based on the artificial manipulation and transfer of genetic material from one organism to another, called recombinant DNA technology. After the development of recombinant DNA technology, many things changed significantly in favor of protein based on biocatalysis. Molecular cloning and heterologous protein expression strategies facilitated the production of large quantities of recombinant biocatalysts much easier and produced cheaper than before.

Enzyme–catalyzed processes have gradually replaced chemical processes in many areas of industry. In less than a century since the start of industrial enzyme production, biocatalyst technology and its products have steadily gained increasing importance. In the industrial production of materials to meet the demands of everyday life, biocatalysts have played an important role. Their application ranges from the production of processed foods such as bread, cheese, juice and beer (Table1.1).

Table 1.1 : The industrial application of enzymes [10].

Industry	**Enzyme**	**Application**
Laundry Detergents	Proteinase	Used in pre-soaks to remove protein - based stains.
	Lipase	Now commonly included to digest oils and fats.
	Amylase	Removes resistant starch residues.
	Cellulase	Digests the cotton "fuzz" which accumulates with excessive washing.

Table 1.1 (contd.) : The industrial application of enzymes [10].

Starch Industry	Amylases, amyloglucosidases and glucoamylases	Converts starch to glucose and other sugar syrups.
	Glucose Isomerase	Converts glucose syrups into fructose syrups.
Dairy Industry	Rennin from the stomachs of young ruminant animals	Manufacture of cheese.
	Lipases	Enhances ripening of blue-mold cheeses.
	Lactases	Break down lactose to glucose and galactose.
Textiles Industry	Amylase	Now widely used to remove starch from woven fabrics. Starch is used as an adhesive (or "size") on the threads of many fabrics to prevent damage during weaving. Traditionally, chemicals were favoured but now bacterial amylases are commonly used.
Brewing Industry	Amylases, glucanases, proteinases	Splits polysaccharides and proteins in the malt
	Proteinases	Reduces clouding of beers
	Amyloglucosidase	Low calorie bee beer
	β-glucanase	Improves filtration characteristics.
Baking Industry	α -amylase	Catalyses the breakdown of starch in flours. Used in the manufacture of bread.
	β-xylanase	Improves the characteristics and rising of bread.
	Proteinases	Reduces the protein in flour. Used in biscuit manufacture.

Table 1.1 (contd.) : The industrial application of enzymes [10].

Leather Industry	Proteinase (trypsin)	The process known as "bating" treats the leather with proteinases to make it more pliable. Trypsin isolated from both slaughterhouses and micro-organisms replaces the old method of using dog and pigeon faeces.
Pulp and Paper Industry	β-xylanases	Emerging technology for enhancing pulp-leaching
	Lipases	Reduces "pitch" which causes paper to stick to rollers and tear.

1.3 Protein Engineering for Ideal Biocatalysts

Meeting the demand for the new products, which increasingly include newly developed and/or sterically pure pharmaceuticals and fine chemicals, has become an important incentive for the further development of biocatalysts technology. However, in many cases, naturally occurring biocatalysts lack features necessary for the application of interest, because they have evolved in nature to serve a purpose rather than the acceleration of industrial processes. There is usually a wide gap between what a biocatalyst found in nature is capable of catalyzing and what is required for a cost effective industrial process; since substrate specificity, substrate or product inhibition, enantioselectivity, rate of catalysis, pH optimum, and stability at elevated temperatures and in organic solvents, will be very specific for any desired reactions. The facilities of recombinant DNA technology and polymerase chain reaction (PCR) supplied the possibilities of the reorder a protein molecule on the nucleotide level by deletion or insertion of fragments, by random changes within defined regions, or by defined changes at specific sites. Recombinant DNA technology paved the way for protein engineering and for synthetic applications of enzymes found in nature. Protein engineering may be achieved by using the transcription, translation, transformation-expression ability of the microorganism, and recombinant DNA techniques and PCR being key technologies.

A while later, in the middle 20^{th} century, to overcome these obstacles due to the properties of enzymes in found nature for application in industry, protein engineering

has emerged as a powerful and versatile means of tailoring biocatalysts in order to adapt their properties to the process requirements. Protein engineering which is described as practical uses ability of "Darwinian evolutionary engineer" based on recombinant DNA technology to construct biomolecules for industry has been developed.

Darwinian evolutionary methods were introduced in protein engineering aiming to improve the properties of biocatalysts with enhanced efficiency, thermal stability, tolerance to organic solvents and other demands by *in vitro* techniques. Clues as to how to engineer better biocatalysts, however, come from studying how nature has done it. Protein evolution shows that biocatalysts are highly adaptable molecules, at least over evolutionary time scales.

The explosion of tools that has come out of protein engineering during the last 25 years has made it possible for us to evolve biocatalysts for features never required in nature. The most effective type of the used method for overcoming these obstacles is the evolutionary methods which mimic the process of Darwinian evolution in the test tube by combining mutagenesis and recombination with selection or screening for improved variants with the desired characteristics [6-7]. The presence of protein engineering has provided biochemists in laboratory bench for the beginning of producing commercial bio-products especially biocatalysts with desired properties for pharmaceutical and agricultural industry. As key discipline catalysis, enabling clean and cost-efficient processes in chemistry for many varieties of different products has a strong economic importance in industry. To date, more than 500 products over a wide spectrum of applications have been manufactured by "ideal" biocatalysts. Well-known examples are ephedrine, aspartame and amoxicillin. A large number of Nobel Prizes in catalysis show high innovation potential of catalysts. The latest prize, in 2010, was awarded jointly to Richard F. Heck, Ei-ichi Negishi, and Akira Suzuki for their supplements in organic synthesis by using catalysts [4-5].

1.3.1 Ideal biocatalysts for industrial applications

The primary objectives are turnover, selectivity and molecular stability for any ideal designed of new biocatalysts. The turnover of an enzyme represents a high degree of substrate conversion to product in the minimum possible time. Although turnover is the key property for the enzymes, enzymes generally used in bioconversions vary

widely; high turnovers are the characteristics of most current commercial biocatalysts. Functional capacity of enzymes is determined by genetically and microenvironment of them which has been formulated and applied. Microenvironment of enzymes having influence over the maximum achievable turnover are temperature, substrate/product solubility, reagent stabilities, exclusion water, ions, solutes and the need to reduce undesirable side reactions [12].

Ideal biocatalysts having broad substrate selectivity are essential, as in the use of different industrial areas. Control and optimization of stereoselectivity in the synthetic biotransformation and chiral resolutions have been seminal focus areas in biocatalysts in recent years. Changing substrate selectivity has progressed to the point where the functionality of biocatalysts can be transformed from one reaction type to another [13].

Substrate selectivity can readily be altered, using molecular or reaction engineering methods, to the extent where compounds of very different steric structure and chemical nature from the natural substrates can be efficiently converted by the designed biocatalysts [14-15].

Molecular stability of biocatalysts is a major issue for all bioprocesses, as it affects process economics at a number of levels. Biocatalyst with poor stability results in longer process operations, increasing frequency of catalyst replacement and reducing product yields. The causes of reduced biocatalyst stability occur in extreme temperature values, ionic strength, pH or presence denaturants like substrates/products, organic solvents etc. [16-18].

In order to change properties of biocatalysts based on these key properties, protein engineering has developed some experimental approaches. These involve four steps: choosing the protein changes (random/rational design), making those changes called mutagenesis, and evaluating the protein variants for improved properties (screening/selection) and lastly characterization of the selected enzyme variants.

1.4 Choosing the Best Protein Engineering Approaches and Methods

Protein engineering is a key method to produce novel biocatalysts for biotechnological industries. Protein engineering has many points used in planning developed approach for engineering protein. By planning correct strategy of protein

engineering, oil recovery is enhanced; cellulosic ethanol is produced [19]. Under favor of selected right protein engineering approaches (Table1.2), proteases are used in detergent with bleach [20]. Polyester is produced by using well-engineered enzymes [21].

Rational design and molecular evolution are main approaches for protein engineering. Rational design introduces mutation(s) to specific protein sites in enzyme for desired properties and requires structure-function relationship and structure information of the enzyme. However, molecular evolution does not need any structure, function of enzymes but randomly generated huge amount mutant enzymes need screening for targeted properties.

Limitations of each approach should be considered to choose the best approach, such as information intensity (detailed structure-function info), methods for mutagenesis in rational design, tools of selection and screening in directed evolution. So, selecting different strategies can lead to different advantages or disadvantages. For example, there is a trade-off between the efforts devoted to rational design versus screening. At one extreme, rational design starting from X-ray crystal structures of a well-understood enzyme can limit the screening effort to a few amino acid substitutions.

At the other extreme, a powerful screening method can eliminate the rational design step completely [9]. Therefore, the choice of the best method is governed by choosing the best locations and how to introduce the substituted amino acids and also screening methods.

While choosing the best location for amino acid substitution strategy in rational design, increasing the thermostability of protein is one common protein-engineering target. A more rigid enzyme can be obtained for high temperatures based on the several approaches including the usage of information from X-ray structure of the enzyme, designing specific stabilizing interactions like salt bridges/disulfide bonds, stabilizing the loop by inserting Pro/removing Gly, focusing mutagenesis where most flexible region of the target enzyme [9].

The enantioselectivity and catalytic activity of the enzymes may be improved by substituting the amino acid residues which are closely located on the catalytic site of the target protein [22].

Error-prone PCR (epPCR), saturation mutagenesis for single amino acid substitution and gene synthesis to add specific changes, shuffling, and simultaneous mutagenesis of many sites for multiple amino acid substitution are widely used strategies for introducing amino acid substitution [23]. A single amino acid substitution leads to increased enantioselectivity of nitrilase and amine oxidase [24-25]. A variant of nitrilase having only Ala190His substitution is the unique reason of increasing enantioselectivity toward an intermediate for the synthesis of atorvastatin [25].

Creating multiple amino acid substitutions in target protein gives extra choices and possibilities but leads to miss any cooperative interactions and creates a big library having many inactive variants.

In order to remove cooperative interaction of multiple amino acid substitutions, F. H. Arnold suggested the stepwise accumulation of single amino acid substitution that each step having a variant is better than the previous one [26]. For finding the paths to the final particular variant of target protein having desired properties, all possible paths should be tested. For amino acid substitutions at five positions, there are 120 possible paths to the final particular variant. Resistance of β-lactamase was improved with 18 paths at each stage [27]. Enhancement of the thermal stability of the enzyme phytase was done. After 9 rounds of optimization, the Tyr277Asp mutation was the only one that resulted from a single base exchange; the other was double-base (three mutations) and three-base exchanges [28].

The previous mutation before the final particular variant can lead to a dead end. Random mutations at several sites avoid this possibility of new mutants and can result in a dead end. Nevertheless, mutation at several sites cost much more screening. Before screening these huge numbers of variant libraries, a selection method can be applied to decrease the number of variants that will be screened [29].

Another way for the reduction of the screening step is to eliminate unfolded or unstable proteins by doing some assumptions [30-31].

With small data sets, it is possible to discern which mutations are beneficial for a given trait, by manual manipulation of the data. However, as more mutations are evaluated in a given library, and each library is screened for a variety of traits, the data set becomes too large analyze by hand. ProSar is a statistical way and has no counterpart in biological evolution for protein structure-activity relationship. ProSar

compares all the data for each variant having the same substitution so it gives an idea about that substitution whether it is functional or not [32-33]. G. W. Fox used ProSar successfully for the evolution of a halohydrin dehalogenase [34].

There are many screening strategies. Most of them are time consuming and need hard work. Sometimes, because of the lack of a suitable screening-selection strategy, final particular variant or the best solution can be missed. However, there are some limited colorimetric screening strategies that have been developed for a limited number of enzymes.

The best protein engineering approach can be defined as one that brings the best solution with the least effort in a short time. Therefore, because of the lack of suitable screening method for all industrial enzymes in directed evolution and difficulty of hitting the best substitution for the desired functions of enzymes in rational design, both of them cannot be unique approaches for protein engineering. A combination of both strategies represented the new route to improve the properties and function of the enzymes and made more rapid progress in protein engineering.

Table 1.2 : Examples of some technologies available for enhancing biocatalyst characteristics [11].

Target improvement	**Technology available**	**Example**	**Activity enhancement**
Increased turnover	Solubilization of enzyme	Solubilization of subtilisin (using isooctane + aerosol OT) for application in organic solvents	Specificity constant k_{cat}/K_M of solubilized subtilisin in octane $=10^3\times$ that of suspended enzyme, and $0.1\times$ that in aqueous medium; stability in octane $10^3\times >$ in aqueous medium.
		Biocatalyst plastics using chymotrypsin and subtilisin	Incorporation of chymotrypsin and susbtilisin in synthetic polymers gave $10^4\times$increased reaction rates with high stability, in polar solvents, facilitating efficient peptide synthesis.
		Solubilization chymotrypsin and subtilisin for use in organic solvents using propanol rinsing treatment	Activity subtilisin Carlsberg 10^3 greater than freeze-dried powder and comparable with, crosslinked enzyme crystal preparation.

Table 1.2 (contd.) : Examples of some technologies available for enhancing biocatalyst characteristics [11].

Altered enantioselectivity	Directed evolution	Improved enantioselectivity in lipases	*Pseudomonas aeruginosa* lipase activity with improved S-enantioselectivity; 2% ee increased to 81% ee after four generations of mutants.
		Reversed hydantoinase enantioselectivity	Conversion of D- to L- selectivity with fivefold increase in total activity, in Art*hrobacter sp.* hydantoinase used for production of L-methionine. No natural L-specific hydantoinase is known.
		Improved enantioselectivity in esterase-catalysed selective resolution	Mutant esterases from *Pseudomonas fluorescens* to hydrolyze bulky giving 25% ee

Table 1.2 (contd.) : Examples of some technologies available for enhancing biocatalyst characteristics [11].

Altered functionality	Rational design and directed evolution	New isomerase activity evolved	Conversion of indole-3-glycerol phosphate synthase into *E.coli* phosphoribosylanthranilate isomerase with 2×lower k_{cat} 15× lower K_M than the native enzyme.
	Rational Design	Desaturase altered to hydroxylase	Change of activity from double-bond insertion to hydroxylase hydroxylation in oleate desaturase, by site-directed mutagenesis at seven sites, selected by comparative analysis of amino acid sequences of oleate desaturases and unrelated hydroxylases.
	Gene shuffling and Screening	Modified β- galactosidase activity	DNA shuffling gave an evolved β- galactosidase with 10-fold increased fucosidase and 40-fold less galactosidase activity due to six amino acid changes.

Table 1.2 (contd.) : Examples of some technologies available for enhancing biocatalyst characteristics [11].

Conversion of non-natural substrates	Directed evolution specificity	Recombination to alter substrate	Genes for biphenyl oxygenase activity from *Pseudomonas pseudoalcaligenes* and *Burkholderia cepacia*, with different substrate specificities, recombined to give dioxygenase activity for efficient PCB conversion.
Increased enzyme stability	Directed evolution	Thermostable esterase developed by directed evolution	Improved thermostability (> 14^{o}C increase in T_m after six cycles of mutation) without loss of activity, showing that the two properties are not mutually exclusive.

Table 1.2 (contd.) : Examples of some technologies available for enhancing biocatalyst characteristics [11].

		Increased thermostability of subtilisin E	Subtilisin was altered by eight amino acid susbstitutions, to give functional thermitase using PCR mutagenesis and staggered extension process recombination.
	Genetic and rational approach	Increased thermostability in a protease	Protease activity with half-life 170 min at 100^{o}C, in the presence of denaturants, and wild-type level of activity, in eightfold mutant of thermolysin-like protease from *Bacillus stearothermophilus.*
Modified reaction milieu	Directed evolution	Increased esterase activity in Aqueous/organic medium	*p*-Nitrobenzyl esterase activity of subtilisin in 30% DMF increased 50× using random mutagnesis.

1.5 Recent Developments in Protein Engineering Approaches

Combinatorial active site saturation test (CAST) is a new structure based method compromise between conventional saturation mutagenesis at single sites and simultaneous randomization at multiple sites in protein engineering was developed by Reetz *et al.* [35]. The CAST has the great advantage over other strategies in that the number of clones that have to be screened is comparatively low. It has already been successfully applied to a number of biotechnologically relevant enzymes (Table 1.2) for substrate acceptance, enantioselectivity, and thermal stability and proved to be a promising alternative to the previous methods.

CAST has been first applied to a lipase from *Psedomanas aeruginosa* to increase the substrate specificity of the enzyme. The mutant protein showed a catalytic activity to the selected new substrates 10^3 more than wild-type lipase. CAST pays a particular attention to complete randomization of pairs of possible synergetic amino acids (targeting more than one amino acid as a set like directed evolution strategies) that are positioned close to each other near or within the active site and point in the direction of the substrate binding site (Fig.1.1). Such an approach requires at least structure of the corresponding model or information of the three-dimensional structure of the respective enzyme like in rational design. Therefore, it is a combination of rational design and directed evolution approach in protein engineering [35].

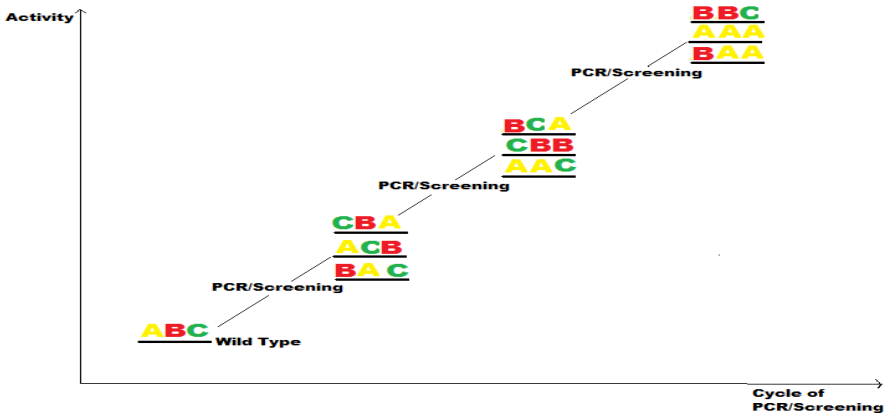


Figure 1.1 : An example of CAST technique having A, B, C library consisting of at least 2 amino acids for a few rounds. Selected candidate amino acids of each library changes in the each cycle of CAST.

CAST has been applied to the epoxide hydrolase from *Aspergillus niger* for improving enantioselectivity [36]. The experiments led to an improvement in the selectivity of factor E from 4.5 for wild-type to 114 for mutant *Aspergillus niger* epoxide hydrolase.

One most important property is the thermal stability of enzymes targeted by CAST in Reetz *et al.* experiments [37]. The final variant of mesophilic lipase from *Bacillus subtilis* shows more than 80 residual activities, however, the wild type does not have any activities over 70^{o}C. Most of the exchanged amino acids are positioned that is not common places for exchange amino acids in thermal stability studies.

CAST needs three-dimensional (3D) information of enzymes for selecting appropriate codon for randomization, so 3D protein structures are of great interest for the CAST of many different types of biological experiments. In the absence of experimental three-dimensional structures of the enzymes of interested homology modeling of enzyme to plan and analyze biological experiments provides important 3D information about them.

1.6 Homology Modeling

Currently, there are about 20000 experimental protein structures collected in the Protein Data Bank (PDB) [38]. However, the number of sequence entries in databases is about 850 000. That means no structural information is available for the vast majority of protein sequences. The gap between the size of the protein sequence data and protein structure data is large and has been increasing.

Even several important developments have been made in the field of experimental structure detection methods by X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) both experimental methods are very costly and time-consuming processes and without guaranteed success.

Therefore, the computational methods for protein structure prediction have gained much more interest in recent years. Homology or comparative modeling is a method of predicting the 3D structure of enzymes and the only method that can reliably generate a 3D model of a protein (target) from its amino acid sequence among all current theoretical approaches. It has been applied in late 1970's using early computer imaging methods by Tom Blundell [39-40]. It covers four steps: searching

databanks for the structural homology, alignment template band target, constructing a model and optimization and the last step is model quality evaluation [39-40].

For successful model building, correct folding (similarity between template and target), model coverage (at least one experimentally solved 3D structure of template), Cα - deviation (root mean square deviation-rmsd- between target and template), alignment accuracy (a significant amino acid sequence similarity between target and template), and placement (optimized placement for template side chains) should care during applied homology protocol.

There are various structural genomics initiatives aiming to speed up the elucidation of new protein structures [41]. Experimental structure elucidation and comparative modeling complement is another one in the exploration of the protein structure space. A key to the efficient coverage will be the careful and optimal selection of the proteins for structural genomics [42]. The growing number of structural templates brings a steadily increasing number of sequences into 'modeling distance' for comparative modeling.

Modeling of protein structures usually requires extensive expertise in structural biology and the use of highly specialized computer programs for each of the individual steps of the modeling process. When a model structure of a template protein molecule is constructed, this candidate model requires minimum potential energy that means stability or acceptable state of the template protein model. The requirement of "polishing and shining" is called as energy minimization. In this step, the potential energy function is minimized by searching for the minimum of this function which corresponds to the optimum geometry of the model. 25–50 steps energy minimization removes atomic overlaps and unnatural strains in the obtained homolog model, stabilizes or reinforces strong hydrogen bonds, breaks weak ones, brings protein to lowest energy in about 1-2 minutes central processing unit (CPU) time [39].

In spite of special care, every model obtained from homology modeling contains errors because of two reasons; one is % sequence identity between reference and model and another is the number of errors in templates. Hence, it is essential to check the correctness of overall fold/structure, errors of localized regions and stereochemical parameters: bond lengths, angles, geometries. Improvements may

include simultaneous optimization techniques in side chain modeling and loop modeling or developing better optimizers and potential function, which can lead the model structure away from template towards the correct structure.

1.7 Molecular Dynamic Modeling

Macroscopic properties are often determined by molecule-level behavior. Quantitative and/or qualitative information about macroscopic behavior of macromolecules can be obtained from simulation of a system at the atomistic level. Molecular dynamics (MD) simulations calculate the motion of the atoms in a molecular assembly using Newtonian dynamics to determine the net force and acceleration experienced by each atom. Each atom i at position r_i, is treated as a point with a mass m_i and a fixed charge q_i.

Steps in molecular dynamics simulations are building realistic atomistic model of the system, simulating the behavior of your system over time using specific conditions (temperature, pressure, volume, etc.) and analyzing the results obtained from MD and relating to macroscopic level properties.

Forcefield, preparing a realistic atomistic model of system and determining specific conditions (temperature, pressure, volume, etc.) will be used in MD is needed for molecular dynamics simulation.

In molecular dynamics, a molecule is described as a series of charged points (atoms) linked by springs (bonds). To describe the time evolution of bond lengths, bond angles and torsions, also the non-bond van der Waals and elecrostatic interactions between atoms, one uses a forcefield. The forcefield is a collection of equations and associated constants designed to reproduce molecular geometry and selected properties of tested structures. One common used forcefield is CHARMm forcefield. Energy terms and function described in the CHARMm forcefield is defined as (1.1) [43];

$$U(\vec{R}) = \overbrace{\sum_{angles} k_i^{angle}(\theta_i - \theta_0)^2}^{U_{angle}} + \overbrace{\sum_{bonds} k_i^{bond}(r_i - r_0)^2}^{U_{bond}} + \overbrace{\sum_{dihedrals} k_i^{dihe}[1 + \cos(n_i\phi_i + \delta_i)]}^{U_{dihedral}} + \overbrace{\sum_i \sum_{j \neq i} 4\epsilon_{ij}\left[\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right] + \sum_i \sum_{j \neq i} \frac{q_i q_j}{\epsilon r_{ij}}}^{U_{nonbond}} \quad \textbf{(1.1)}$$

Ubond = oscillations about the equilibrium bond length

Uangle = oscillations of 3 atoms about an equilibrium angle

Udihedral = torsional rotation of 4 atoms about a central bond

Unonbond = non-bonded energy terms (electrostatics and Lenard-Jones)

Preparing a realistic atomistic model of system includes collecting knowledge related to assigned atom types to identify different elements and different molecular orbital environments, assigned charges to each atom and connectivity between atoms in topology files. Another file is the parameter file having information about force constants is necessary to describe the bond energy, angle energy, torsion energy, nonbonded interactions (van der Waals and electrostatics), and suggested parameters for setting up the energy calculations.

A capable of reading and manipulating this information like X-PLOR, CHARMm, NAMD2, AMBER, GROMOS, EGO, PROTOMOL, etc. are needed.

Minimize the energy of the system in order to reach the most favorable configuration is essential in preparing the system for MD energy minimization. The energy of the system can be calculated using the forcefield. The conformation of the system can be altered to find lower energy conformations through a process called minimization.

The most common minimization algorithms are steepest descent, conjugate gradient, BFGS (quasi-newton variable metric method) and Newton-Raphson algorithm. Steepest descent algorithm slowly converges and is used for highly restrained systems. Conjugate gradients are efficient, using intelligent choices of search direction for large systems. Newton-Raphson algorithm calculates both slope of energy and rate of change.

Biological activity is the result of interactions between molecules and occurs at the interfaces between molecules (protein-protein, protein-DNA, protein-solvent, DNA-

solvent, etc.). Therefore, creating water box or shell to enclose the molecule is critical for MD. A model solvation is needed for many biological processes occurring in aqueous solution, solvation effects playing a crucial role in determining molecular conformation, electronic properties, binding energies, etc. For model solvation, solvent molecules are added to the molecular system or solvent is modeled as a continuum dielectric called explicit treatment and implicit treatment respectively.

MD is defined as changing in conformation over time using a forcefield. MD uses energy function (1.2):

$$U(\vec{r}_1, \vec{r}_2, \cdots \vec{r}_N) = U(\vec{R}) \tag{1.2}$$

For determining the force on each atom, this equation is used (1.3):

$$m_i \frac{d^2 \vec{r}_i}{dt^2} = \vec{F}_i = -\vec{\nabla} U(\vec{R}) \tag{1.3}$$

Newton's equation represents a set of N second order differential equations which are solved numerically at discrete time steps to determine the trajectory of each atom (1.4):

$$\vec{r}_i(t + \Delta t) = 2\vec{r}_i(t) - \vec{r}_i(t - \Delta t) + \frac{\Delta t^2}{m_i} \vec{F}_i(t) \tag{1.4}$$

Based on these equations, several MD ensembles are constructed. Some of them are constant energy-number of particles (NE), constant energy-volume (NVE), constant temperature-volume (NVT), and constant temperature-pressure (NPT) [44].

By using the best fits prepared system, simulation can be performed for a system of a few tenths of angstroms on the length scale and for a few nanoseconds on the time scale.

Initial velocities are assigned at a low temperature for the system. Periodically, new velocities are assigned at a slightly higher temperature and the simulation is allowed to continue. This is repeated until the desired temperature is reached. After running the simulation, the structure, pressure, temperature and energy become stable with respect to time, which is a point of the equilibration phase.

In analyzing the simulation results, mean energy of structure and root mean square (RMS) difference between two structures are cared and final optimized structure is visualized with molecular graphics programs.

1.8 *Bacillus Stearothermophilus* L-Lactate Dehydrogenase (*bs*LDH)

The reaction in the pyruvate to lactate direction is achieved by reducing a carbonyl group of the oxo-acid substrate with a hydride ion donated by NADH (used as a coenzyme) and a proton from the protein. The disadvantages of LDHs are their inhibition of by excess substrate (pyruvate) and also not having very broad substrate specificity.

LDH catalyses the interconversion of pyruvate (oxo acid) and lactate (hydroxy acid) using the NADH/NAD+ pair as a redox cofactor [45]. The chirally pure α-hydroxy acids, derived from the reduction of the corresponding achiral α-oxo acids, are used to produce various pharmaceuticals such as semi-synthetic penicillin (using S-α-hydroxyisocaproic acid) and antihypertensives (using S-α-hydroxyl-4-phenyl butanoate). They are also used in medicine to diagnose phenyl ketonuria by detecting S-ketoisohexanoic acid. LDH enzymes have been isolated from many species. Among these, the NAD+- dependent lactate dehydrogenase (LDH) enzyme from *Bacillus stearothermophilus* has been the subject of intensive research of its structure and catalytic properties. This has been prompted by its industrial potential in the production of chirally pure hydroxyl compounds. The production of chiral (S) α-hydroxyacids is now at the 10-ton scale in industry and any development facilitating the reduction of pyruvate at concentrations greater than 0.1 M would be profitable. *bs*LDH was cloned and overproduced to analyze in details [46].

After the sequence of *bs*LDH was determined, the first analyzing study in the level of amino acid was done for Arg171 in 1987 [47]. Arg171 residue of *bs*LDH, in the active site of the enzyme, interacts with carboxylate group of an α-keto acid by using its guanidium group, that is of basic importance in orienting the substrate for productive substrate-enzyme complex. Arg171 was changed with Lys by using site-directed mutagenesis. This replacement does not have any effects on coenzyme binding and a small effect on the k_{cat} whereas 2000-fold increase in the K_M of the enzyme. This result is explained with the small increase in the distance of Lys-carboxylate of substrate interaction at this part and the lack of the extra hydrogen bond that occurs in carboxylate of substrate-Arg171 of the wild type enzyme.

Holbrook and his colleagues [48] focused on Arg171 at the substrate-binding site of *bs*LDH and changed it with Lys. They observed that this change resulted loosing of a

strong carboxylate-guanidium interaction in the mutant and this mutation decreased the binding energy of substrate (pyruvate, α-ketobutyrate etc.) However, there is a loss of about 1.5 kcal/mol of binding energy for each additional methylene group on the substrate in both mutant and native enzymes. They concluded that the way of the binding productive substrate is the same in both enzymes. While substrate size increases, K_M increases in the wild type but decreases in mutant *bs*LDH. Because of the Arg171, probability of substrates binding in terms of productive substrate enzyme complexes increase in wild type but not in mutant *bs*LDH enzyme.

Another group from Canada changed Arg171 with hydrophobic Tyr and Trp to broad substrate (R=Me, Et, n-Pr, n-Bu, and CH_2OH) specificity of *bs*LDH [49]. Although k_{cat}/K_M of mutant enzyme is lower than wild type enzyme, is more active than the previous mutant (Arg171Lys) which is designed by K.W. Hart. Their one important result is that, losing the Arg171 has no effect on giving optically pure L-lactate by reducing pyruvate catalyzed by Trp171 and the another is secondary polar or hydrogen bonding associations of Arg109 and Thr246 that attributed the retention of L-stereospecificty with the substrate COO-function is major determinant of maintaining substrate orientation.

Introducing oxaloacetate as a substrate to *bs*LDH is the first study about changing the substrate specificity of *bs*LDH. They designed *bs*LDH in order to change preferred substrates from the pyruvate/lactate pair ($R=CH_3$) to the oxaloacetate/malate pair ($R=CH_2$-COO), the volume of the active site was increased with Thr246Gly, an acid was neutralized with Asp197Asn and a base was introduced Gln102Arg. The triple mutant has a catalytic specificity for oxaloacetate over pyruvate of 500, whereas native of *bs*LDH has 1000 [50].

For the same purpose, another attempt was performed by J.J. Holbrook and his colleagues, because previous results (K_M and k_{cat} of mutant *bs*LDH for oxaloacetate) were not satisfactory while compared to native malate dehydrogenase (MDH) from different sources. Therefore, J. J. Holbrook redesigned *bs*LDH framework for a specific, highly active MDH [51]. The double mutant (Thr246Gly, Gln102Arg) was designed but only one mutant (Gln102Arg) showed an effective and highly specific catalyst for oxaloacetate over pyruvate. K_M of mutant enzyme for oxaloacetate is equal to K_M of native *bs*LDH for pyruvate and k_{cat} of the mutant enzyme is double for MDH from the same organisms of the native *bs*LDH.

In 1989, A. R. Clarke and J. J. Holbrook published an article [52]. They described new attempts to design new enzyme recognizing a $–CH_2$-COO- side chain instead of the $–CH_3$ side chain recognized by the naturally evolved enzyme. It was seen that manipulating enzymes for the new substrates is reasonably hopeful, because useful products would be cheaply available from the redesign of natural enzymes. The designs of *bs*LDH were done for large hydrophobic substrate side chains. Although not involved in the catalytic site of enzyme two regions of the *bs*LDH were changed (102-105GlnLysPro-MetValSer and 236-237AlaAla-GlyGly) to increase the tolerance for large hydrophobic substrate side chains(R=CH_3, C_2H_5 etc.). The five changes in *bs*LDH showed broad substrate specificity with a 55-fold improved k_{cat} for α-ketoisocaproate [53].

Designing new enzymes based on the LDH framework has been advanced as far as computational possibilities to be used in the modeling studies of enzymes. Map of the catalytic pathway was drawn for analyzing of the mechanism and structure of LDH [54].

A new phenyllactate deydrogenase was produced by peptide loop exchange on the *bs*LDH framework [55]. The mobile surface loop of polypeptide (residues 98-110) was sufficient for magnitude of substrate specificity. The loops with oligonucleotide overlap extension changed the variable length and sequence loops to produce hydroxyacid dehydrogenase. With one longer loop construction activity against phenylpyruvate was largely unaltered (k_{cat}/K_M=39000 fold).The selectivity of this new construct is enough to monitore phenylpyruvate in the urine of patients.

Importance of the Thr246 at the active site of the *bs*LDH was tested by site-directed mutagenesis to Val, Ala, Leu, and Ser [56]. Effects of these mutants were tested even with different substrates. There is no major change in K_M and k_{cat} but Thr246 can make it easy to transfer hydride from the nicotinamide ring of the NADH cofactor to the carbonyl group of the pyruvate.

Substitutions of the amino acid Gln102 of the *bs*LDH with polar and aromatic residues affect substrate specificity [57]. Gln102 was exchanged with Ser, Thr, Tyr, and Phe to understand the effect of this substitution on substrate recognition. Because of the size of Ser and Thr, Gln102Ser and Gln102Thr mutant enzymes have a broader specificity for α-keto acids. While Gln102Phe, Gln102Tyr have no catalytic efficiency and substrate specificity, all enzymes have a little activity against

branched chain aliphatic substrates. This investigation showed that substrate discrimination depends on size discrimination.

For omega-amino- α-keto acid substrates, having side chain ammonium groups Gln102 of the *bs*LDH was replaced with Glu and Asp acid amino acids [58]. When results are compared to native *bs*LDH, these mutants have 25-fold improvements in k_{cat}/K_M for omega-amino- α-keto acid substrates.

The first broad library based on amino acid residues at position 101 and 102 with site directed mutagenesis for *bs*LDH was done by El Hawrani [59]. They selected some α-keto acids as a substrate (malate, phenyllactate etc.). Asn101Val102 mutant enzyme has a good catalytic activity to phenylpyruvate. At the same time when the position 102 changed with Arg, obtained mutant is as active as many MDHs. Obtained mutants show kinetic rate well for each selected target than designed *bs*LDHs before. Even in the absence fructose-1,6-bis phosphate (FBP) for phenylpyruvate, Asn 101Val102 mutant enzyme has a notable activity.

The first directed evolution of *bs*LDH was done by Stuart J. Allen [60]. The target of the directed evolution was to obtain a mutant working without FBP, because FBP is expensive and representative cofactor is undesirable in industry. After three rounds of directed evolution, obtained mutant (Arg118Cys, Glu203Leu, and Asp307Ser) had 70-fold activation. The big surprise is none of the mutations were near the active site.

The mutation Asp38Glu (previously named Asp52Glu in the LDH from *B. stearothermophilus* reduces the substrate inhibition by approximately two fold. The referred work [61] demonstrates that the designed mutant would weaken the cofactor binding to relieve substrate inhibition.

To introduce mandelic acid as a substrate to LDH was implemented by redesigning *Saccharomyces cerevisiae* falvocytochrome b_2: LDH [62]. Using protein engineering, a specific mandelate dehydrogenase was designed based on LDH framework. Two mutants (Ala198Gly, Leu230Ala) were designed and the mutant enzyme showed a 400-fold improvement on changing the substrate specificity from pyruvate to mandelic acid.

Purification of individual enzymes is a time consuming and costly process. The common purification method of *bs*LDH has many steps, namely lysis, precipitation,

dialysis, chromatography etc. During all these steps wild type and mutant *bs*LDH enzymes may lose their ability to produce product and affinity to bind substrate

Therefore, decreasing the production cost and improving the yield of purified enzymes are important. Production of target proteins using recombinant *E. coli* strains has some advantages compared with the systems based on wild strains. Recombinant systems could be employed for the production of different mutant LDHs with desired properties and large scale production with reduced cost by using protein engineering technology.

TAGZyme purification system is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins making use of coordinative binding of some amino acid residues exposed on the surface. The introduction of immobilized metal ion affinity chromatography, directed toward specific protein side chains has opened a new dimension in protein purification. TAGZyme purification system offers all possibilities for large–scale purification of many industrial enzymes as well as proteins for research in genetics, molecular biology and biochemistry. The introduction of (poly) histidine tags to the N- or C-termini of proteins via genetic manipulation has greatly simplified enzymes purification through affinity chromatography on metal chelate columns [63].

1.9 Phenylalanine Ammonia Lyase from *Rhodotorula graminis*

Phenylalanine ammonia-lyase (PAL) catalyses the non-oxidative conversion of L-phenylalanine (L-Phe) to trans-cinnamic acid (t-CA).

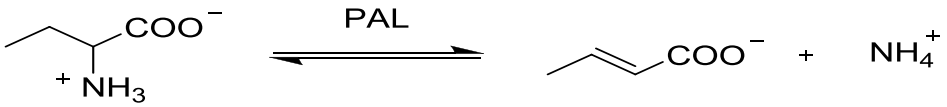


Figure 1.2 : PAL enzyme catalyses the conversion of phenylalanine to trans-cinnamic acid.

Purified PAL from various sources has a molecular mass in the range of 270-330 kDa [64]. The enzyme is tetrameric with identical subunits. Pairs of monomers form a protomer with a single active site. It has been reported that potato tuber PAL is glycosylated and that mannose residues occur on an N-linked oligosaccharide side chain [65].

The first three-dimensional structure of phenylalanine ammonia lyase (PAL) has been determined at 2.1 Å resolutions for PAL from *Rhodosporidium toruloides*. The enzyme PAL has about additional 160 residues extending from the common fold of histidine ammonia lyase. It is believed that catalysis is potentially governed by dipole moments of seven R helices associated with the PAL active site (six positive poles and one negative pole).

Interaction between the amino group of phenylalanine and conserved residues of 5-methylene-3,5-dihydroimidazol-4-one (MIO) :12 methylidene group in N termini of helices was observed in the docking studies for active site of the PAL with phenylalanine. The conserved residues have important roles in activation of MIO, leaving out of product, activation of C3 by polarizing electrons from the phenyl ring of substrate and for recognizing substrate. His is close to C terminus of the helix. This position directs negative pole through the catalytic area causing an increase in the His`s basicity. So highly conserved His residue in N termini of helices behaves as general base. This basicity of the residue functions to abstract the pro-S hydrogen from C3 of substrate [66].

Observed dehydroalanine moiety in the active site of the enzyme gets together with the α-amino group of the substrate during "ordered uni-bi" in the catalytic reaction. In this catalytic reaction, firstly cinnamic acid then ammonium ions are released [67].

Due to having a catalytically important electrophilic group, the presence of dehydroalanine was believed for 30 years but has now been revealed by X-ray crystallography and UV spectroscopy to be a highly electrophilic MIO group.

The electrophilic attack of MIO on the aromatic ring of the substrate that defined as an incomplete Friedel–Crafts type reaction causes to start the reaction observed in experimental studies. This reaction triggers the activation of a β-proton and its stereospecific abstraction. Finally, the elimination of ammonia and reproduction of the MIO group is observed.

In order to detect essential amino acids for PAL, the exchange of Ser202 for Cys by site-directed mutagenesis was done. The effect of this exchange on the catalytic activity of PAL indicates that Ser202 is the precursor of the active site dehydroalanin. Further amino acids essential either for the post-translational modification or for catalysis, Arg174, Glu425, and Lys499 were changed to Ile.

Kinetic analyses for the heterologously expressed mutated genes showed that only the Arg174Ile has a serious decrease for V_{max} [68].

Structure of histidine ammonia-lyase (HAL) that has been solved by X-ray crystallography has a homology with the plant enzyme phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). In order to detect the substrate binding or catalytic residues in PAL; based on analysis of alignment of PAL and HAL, identical or similar Gln348, Ser203, Leu138, Phe400, Tyr110, Gln488, Tyr351, Asn260, and Arg354 residues have been mutated.

Retey and his collogues see that in each case, mutagenesis diminished the activity of the kinetic constants of the over expressed and purified [69]. In the past three decades, PAL has gained considerable significance in several clinical, industrial, and biotechnological applications.

Many successful industrial uses of PAL biocatalyst from *Rhodotorula* strains show that even PAL with its native substrate has a big industrial potential. Therefore, PAL has several commercial applications [70-72]. Despite the widespread distribution of PAL, *Rhodotorula* has been used exclusively for commercial purposes because of its high PAL levels and no fastidious requirements for growth and PAL synthesis [73-74]. *Rhodotorula* PAL is effective in the treatment of certain mouse neoplasms [75], and used to quantitatively analyze serum L-Phe to monitor patients with phenylketonuria [70,63] and to prepare low phenylalanine diets [76]. Whole cells of *Rhodotorula* containing PAL have been used to synthesize L-Phe [74,76] and its methyl ester [70,77] by reversing the physiological reaction.

PAL is specific for L-Phe, and to a lesser extent, L-Tyr. Other common L-amino acids are not deaminated; D-Phe and D-Tyr do not serve as substrates and are competitive inhibitors of PAL [78-79]. Recently, Calabrese *et al.* reported that PAL activities with L-Tyr were either low or undetectable for four PAL isoforms of *Arabidopsis thaliana*, thereby establishing L-Phe as the true physiological substrate [80]. However, the substrate specificity for PAL varies in different species. PAL from different sources has extraordinary properties. PAL from dicotyledonous plants has highly specific for L-Phe. PAL in photosynthetic bacteria has a major specificity for L-Tyr leads to produce p-hydroxycinnamic acid that is a chromophore for the photoactive yellow proteins [81].

In this study, we aimed to increase the activity of PAL from *Rhodotorula graminis* for the selected different substrate(s) by CASTing (Table 1.3) [82].

PAL activity is determined by monitoring and measuring either the amount of L-Phe that has reacted or the t-cinnamic acid that is formed at the end of the enzyme reaction. Conventionally, the activity is calculated spectrophotometrically by measuring the absorbance of product, t-CA, at 290 nm [83].

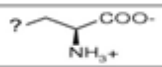
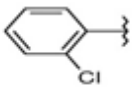
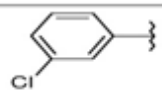
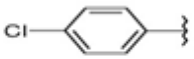
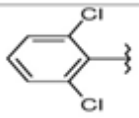
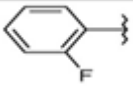
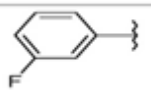
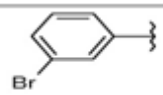
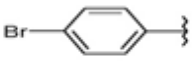
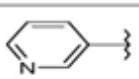
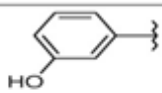
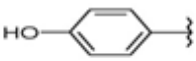
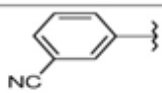
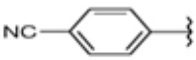
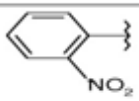
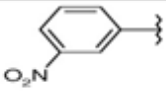
Recently, a liquid phase assay was developed for PAL activity by N.J. Turner group (unpublished). This assay uses purified oxidase enzyme and is conducted in a microtiter plate reader. The advantage of this type of assay is that it can be used to quantify the activity of the oxidase enzyme and allow the measurement of the kinetic parameters of the reaction.

It is important to use a soluble horseradish peroxidase (HRP) substrate for the liquid phase assay, as this assay is performed in a microliter plate reader and precipitation of the colorimetric product will result in a heterogenous mixture and incorrect data being obtained. In the liquid phase assay, several substrates for HRP can be used. These substrates include 4-aminoantipyrine with 3-hydroxy-2,4,6-tribromobenzoic acid, pyrogallol red, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), and scopoletin.

HRP oxidises 4-aminoantipyrine (4-AAP) in the presence of H_2O_2, which subsequently reacts with 3-hydroxy-2,4,6-tribromobenzoic acid (TBHBA) to form quinoneimine, the production of which can be detected at 510 nm in a spectrophotometer or microliter plate reader [84].

The stereoselective reaction of amino acid ammonia lyase that is addition of ammonia to achiral acrylic acid derivatives ends up amino acid. Phenylalanine ammonia lyase from *Rhodotorula* is a valuable industrial biocatalyst because of its native substrate. During catalyzing its own native substrate, PAL has high activity towards its natural substrate, stability under bioprocess conditions and tolerance of high concentrations of substrate and product without displaying significant inhibition.

Table 1.3 : The broad substrate range of PAL from *Rhodotorula graminis*[84].

		Enantiomeric Excess %
	80	>99
	80	>99
	---	---
	24	>99
	80	>99
	80	>99
	80	>99
	80	>99
	---	---
	87	>99
	80	>99
	30	>99
	80	>99
	20	>99
	45	>99
	45	>99

2. MATERIALS AND METHODS

2.1 DNA Shuffling to Construct Shuffled Mutant *bs*LDH

Bacterial strain *E. coli* JM105 {F′ traD36 $proA^+$ $proB^+$ laclq lacZΔM15/Δ (lac-pro) X111 thi rpsL (Strr) endA sbcB supE hsdR9} was used as a host to prepare all double-stranded DNA for mutagenesis and sequencing in the plasmid pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The plasmid pKK223-3 is a transcription expression vector and contains a TAC promoter (Isopropyl β-D-1-thiogalactopyranoside, (IPTG) inducible) which drives expression of recombinant genes and ribosomal termination sequences as well as ampicilin resistance [85]. The same *E. coli* strain was also used as a host for transformation and expression of *bs*LDH proteins in pKK223-3.

The shuffled-*bs*LDH gene was constructed by the method used by Stemmer [86] with some modifications as explained below. When the method was applied to *bs*LDH it first involved isolating the coding DNA (an about 1 kb fragment) with a 5′ region containing an *Eco*RI site and a 3′ region with a *Pst*I site. The product is of the correct size. About 10 μg plasmid containing wild type LDH gene was digested at 37 °C for 1 h with *Eco*RI and *Pst*I in 20 μl 10 × digestion buffers. After purification from 0.8% agarose gel, 2 μg of the plasmid DNA of *bs*LDH was then randomly cleaved with the non-specific endo-DNAse I incubating the reaction mixture including 10 mM TrisHCl (pH 7.4), 10 mM of $MnCl_2$ and 0.0015 U DNase I for 15 min at 20 °C. This should give a smear of randomly sized oligonucleotides smaller than 1 kb.

Fragments with sizes 100–300 bp were purified from the corresponding region of a 2.5 % agarose gel. These fragments were then amplified by PCR (94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s) but without adding primers. This critical step generates sequence variation in the fragments generated in step 1. As expected, the products run on a gel as a characterless smear. The full-length gene is finally isolated by the second PCR (initial denaturation 96 °C for 2 min, 94 °C for 30 s,

55 °C for 30 s, 72 °C for 45 s, for 10 cycles and followed by another 14 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and final extension 72 °C for 12 min) step using the correct primers for the 5′ *Eco*RI (N-terminus) and 3′ *Pst*I (C-terminus) sites and 4 µl of the first PCR product. A high yield of shuffled library about 1 kb *bs*LDH genes was randomly obtained on the gel. All PCRs were carried out in the presence of 0.2 mM of each dNTPs, 1× *Taq* buffer, and 2.5 U *Taq/Pfu* (1:1) enzyme mixtures [87]. The about 1 kb product was digested with *Eco*RI and *Pst*I prior to cloning into similarly cut pKK223-3 DNA and the ligation products were used to transform *E. coli* JM105 competent cells.

2.1.1 Screening for variants with substrate-specificity changes

The screen was initially designed by A.S. El Hawrani for use with a thermophilic protein [59]. Having a thermophilic enzyme expressed in a mesophilic bacterial strain (*E. coli* JM105) allows inactivation of host proteins on the nitrocellulose filters using a heat step. The *bs*LDH library is blotted onto nitrocellulose filters and subjected to a number of steps. Lysis of *E. coli* cells on nitrocellulose filters was carried out and nitrocellulose filters were incubated at 65 °C [51].

The filters were incubated in the dark and any *E. coli* colonies expressing *bs*LDH protein with activity towards the hydroxy acid target substrate were detected by the appearance of a purple coloration around the edges of the bacterial colonies. Double-stranded DNA of shuffled *bs*LDH having activity with oxaloacetate was sequenced to confirm the changes on the gene. Protein over-expression is conveniently established by the appearance of an intense band on an sodium dodecyl sulfate polyacrylamide gel polyacrylamide gel stained with coomassie blue dye.

2.1.2 Construction of Gln102Arg -*bs*LDH

Bacterial strains: Two different bacterial strains are used in this work, namely DH5α™-T1^{R} competent cells ([F^{-}ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A]) which is supplied with Invitrogen™ GeneTailor ™ Site-Directed Mutagenesis System and JM105 as a host for transformation and expression of Gln102Arg mutant of *bs*LDH protein in pKK223-3.

The mutation was introduced into the *bs*LDH gene at required positions by site-directed mutagenesis using Invitrogen Gene Tailor™ Site-Directed Mutagenesis System according to the manufacturer's protocol [88].

The following oligonucleotides;

Forward 5′GGTTGTCATTTGCGCCGGCGCC**CGC**CAAAAACCGG3′

Reverse 5′GGCGCCGGCGCAAATGACAACCAAATCGGCATC3′

were used in order to construct the mutant gene. Base changes are shown in boldface on the mutagenic oligonucleotide primer. According to manufacturer's protocol, only one mutagenic oligonucleotide primer is required to generate a mutation site. The double-stranded DNA extracted from mutated *bs*LDH gene was sequenced in the region of the mutation to check for correct amino acid insertion. Purified DNA samples were sequenced using ABI Prism 3100-Avant automated sequencer at Molecular Biology and Genetics Dept, ITU.

2.1.3 Molecular modeling

Residue replacements and rotamer choices were performed using the molecular graphics and modelling software Accelrys Discovery Studio [89]. Likewise, oxaloacetate was modelled into the active site of shuffled-*bs*LDH by inspection. Energy minimisation of the oxaloacetate ternary complex in explicit water was performed using Discover 2.9.7 and the Cvff forcefield [89].

2.1.4 Cell culture and protein purification

The same protocols were used to purify both wild type enzyme and the mutant *bs*LDHs [90]. Fast protein liquid chromatography was used to purify the enzyme. The protein sample was loaded onto a blue-sepharose column, which had been washed with two column volumes of 100 mM of TrisHCl, pH 7.3. LDH was eluted by using a gradient from 0 M to 1 M NaCl in 100 mM of TrisHCl, pH 7.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and brilliant blue coomassie staining showed the proteins to be 98 % pure after the described purification. Finally, each enzyme was dialyzed against activated charcoal buffer to remove nucleotides.

2.1.5 Steady-state kinetics

All kinetic parameters were measured in the absence and presence of 5 mM FBP, an enzyme activator at 25 °C in a reaction mixture containing 100 mM TrisHCl, pH 7.3, 5 mM NADH, 5 nM enzyme and different concentrations of substrates; pyruvate, oxaloacetate and α-ketomalonate. Kinetic parameters were determined by observing the oxidation of the nicotinamide coenzymes at 340 nm in 10 mm, 1 ml quartz cuvettes mounted in a Perkin Elmer Instruments Lambda 25 UV/VIS spectrometer.

2.2 Mandelic Acid As A Substrate For Highly Purified *Bacillus Stearothermophilus* Lactate Dehydrogenase

2.2.1 General equipment and chemicals

All chemicals were purchased from Sigma-Aldrich. JM105 competent *E. coli* cells, pQE-2 plasmid and 6xHis-tag protein purification system were purchased from QAIGEN. Gene Tailor site- directed mutagenesis system was purchased from Invitrogen and high pure plasmid isolation kit was purchased from Roche. All enzymes, unless otherwise mentioned, were purchased from Fermentas. Microfuge is from Beckman Coulter Microfuge. UV-Visible (Lambda 25) atomic absorption spectrophotometer is from Perkin Elmer Instruments. Microplate reader is Bio-Rad 3550-UV and DNA sequences are analyzed in ABI Prism 3100-Avant automated sequencer from Applied Biosystems.

2.2.2 Improving the purification of *bs*LDH

The N-terminal histidine tag was introduced to clone the wild-type gene of *bs*LDH into the expression vector pQE-2 coding N-terminal fusion 6xHis-tag. Following the cloning strategy that is *PstI*_C-terminal and *SacI*_N-terminal primers were used in order to introduce new restriction enzyme sites, which are suitable for 6xHis-tag pQE-2 expression vector.

Designed mutations were incorporated by an overlapping PCR method using complementary oligonucleotides. Primers were ordered from Eurofins MWG Operon. Primer sequences are listed in Table 2.1. The wild-type *bs*LDH gene in pKK223-3 was amplified according to standard PCR methods (94 °C for 5 min, 95°C

for 1.30 min, 50 °C for 1min, 72 °C for 2.5 min, 72°C for 10 min) in the presence of 0.2 mM of each dNTPs, 0.8 pmol of each primer and 0.6 U *Pfu* DNA polymerase.

Table 2.1 : The used primers for His-tag protocol system and site-directed mutagenesis of rationaly designed *bs*LDH.

Role of Primer	Name	Sequence (5´ to 3´)
Creating restriction sites	*Pst*I_C[a]	AAAACTGCAGTCATCGCGTAAAAGCACG
Creating restriction sites	*Sac*I_N[a]	TACTGAGCTCAAATGAAAAACAACGGTGGAGC
I240A mutation	I240A_fw[b]	GCCTTGACCGTATCAGCCggcCTCGATGGCCTA
I240A mutation	I240A_rv[c]	GGCTGATACGGTCAAGGCAGCGTTTTCGTTA
T246G mutation	T246G_fw[b]	GCATAACGAAAACGCTgccTTGACCGTATCA
T246G mutation	T246G_rv[c]	AGCGTTTTCGTTATGCAAAATGGCGCGAG

Restriction sequences are underlined. [a]*Pst*I and *Sac*I recognition sequences (underlined) were added to the 5´and 3´ ends, respectively, for the His-tag protocol. [b]Nucleotide sequences conferring mutations are shown in lowercase. [c]Reverse primers were designed to be used generally with mutation of same position.

The amplified PCR product was cut with restriction enzymes *Sac*I and *Pst*I and ligated into similarly digested 6xHis-tag pQE-2 expression vector. Ligation product was transformed into *E.coli* JM105 and selected on Luria Broth (LB) Agar containing 100 µg/ml ampicillin. The final construction product double stranded DNA bases were sequenced on an ABI Prism 3100 sequencer according to the supplier's manual. All other cloning procedures and restriction enzyme digests were performed as described [91].

The bacterial cultures harboring the desired plasmid were grown on a rotary shaker in LB supplemented with ampicillin (100 µg/ml). The bacterial cultures harboring the desired plasmid were grown on a rotary shaker in LB supplemented with ampicillin (100 µg/ml). 1mM of isopropyl-ß-D-thiogalactopyranoside (IPTG) was added when the optical density at 600 nm reached 0,6. 50 µl of sample was collected for determination of uninduce total protein expression. After growing overnight, the cells were harvested by centrifugation (15000 g, 2 min). The pelleted cells were stored at -80 ºC.

The native enzyme was purified using nickel nitrilotriacetic acid (Ni-NTA) resin (supplied with kit) having 6xHis-tag protein affinity. The pelleted cells were kept at

37 ºC for 30 min and were suspended in 20 ml of lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH to 8.0 with NaOH. Lysozyme (1 mg/ml) was added and cell supension was incubated on ice for 30 min. 50 µl of sample was collected for determination of total protein expression from each step. Remaining cell suspension was centrifuged at 15000 g for 15 min at 4 ºC.

Batches were prepared by pouring 2 ml of the 50 % Ni-NTA slurry into supernatant from the previous step in 20 ml falcons. The batches were incubated for 60 min on a rocking table at 4°C. 1ml washing buffer containing 50 mM NaH_2PO4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH to 8.0 with NaOH was loaded to the resin four times to remove unbound proteins by centrifugation (13000 rpm, 1 min).

Proteins of *bs*LDH bound to the metal groups of the resin surface were eluted four times with 1ml elution buffer containing 50 mM NaH_2PO4, 300 mM NaCl, 250 mM imidazole, 0.05 % Tween 20, pH to 8.0 with NaOH by centrifugation (13000 rpm, 1 min). The tubes were placed in a flask that contains distilled water with a high concentration charcoal for an overnight dialysis. The amount of obtained fractions from column for both tagged and untagged enzymes were calculated and compared with each other.

2.2.3 Construction of mutated *bs*LDH protein

Residue replacements and rotamer choices were performed using the molecular graphics and modeling software InsightII [89]. Likewise, L-mandelate was modeled into the active site of designed *bs*LDH by inspection. Energy minimization of the L-mandelate binary complex in explicit water was performed using Discover Studio [89]. Site-directed mutagenesis using Gene Tailor Site-Directed Mutagenesis System was applied to construct Thr246Gly and Ile240Ala mutants of *bs*LDH according to the manufacturer's protocol (Invitrogen). I240A_fw, I240A_rv, T246G_fw, T246G_rv primers (Table 2.1) are used for constructing Thr246Gly and Ile240Ala *bs*LDH mutants. Double stranded of plasmid DNA was isolated and methylated with DNA methylase. Methylated DNA was amplified with two overlapping primers to produce linear double stranded DNA. The PCR reaction (for I240A at 94 ^oC for 2 min initial denaturation, 94 ^oC for 5min, 95 ^oC for 1,30 min 50 ^oC for 1min, 72 ^oC for 2,5 min 25 cycles, and at and 72 ^oC for 10 min final extension. For Thr246Gly at 94 ^oC for 2 min initial denaturation, 94 ^oC for 30 sec, 50 ^oC for 30 sec, 60 ^oC for 30 sec,

68 ^{o}C for 5,5 min 20 cycles, and at and 68 ^{o}C for 10 min final extension) was carried out to obtain linear plasmid of *bs*LDH gene with the desired change on it in the presence of 0.3 mM of each dNTPs, 0.3 pmol of each primer and 0,02 U of Platinum*Taq* High Fidelity polymerase. The mutagenesis mixture was transformed into DH5α-T1 cells (supplied by kit). The host cell circularizes the linear mutated DNA, and McrBC endonuclease in the host cell digests the methylated template DNA, leaving only unmethylated, and mutated product. The double-stranded DNA extracted from mutated *bs*LDH gene was sequenced in the region of the mutation to check for desired mutations. pQE-2 expression vector containing His-tag *bs*LDH gene was transformed into JM105 cells for over-expression *bs*LDH.

The mutant enzyme was purified with Tagzyme purification system protocol which was mentioned for the native *bs*LDH before. The collected samples from each step were analyzed by SDS polyacrylamide gel (12 % w/v) electrophoresis. The native and mutant protein concentrations were determined by Bradford's method using bovine serum albumin as the standard [92].

2.2.4 Enzyme assay

The wild type and mutant *bs*LDHs were kinetically analyzed in the presence of 50 mM Tris.HCl pH 7.4 buffers as two sets at 25 °C. Steady-state reaction rates were determined by following change in A340 nm due to the formation and consumption NADH. Rate constant was analyzed using the non-linear least squares fitting program GraFit [93].

2.3 Development of Novel Ammonia-Lyase Biocatalysts for the Production of Unnatural Amino Acids for Industry

2.3.1 Optimization of screening method

Primarily, to find an optimized screening method is so consequential. So we need calibration of the screens that will be used throughout this study.

2.3.1.1 A pH sensing agarose plate assay

pH indicators for compatibility in solid phase screening and selection of PAL activity were tried. Selecting the suitable medium is important in this assay. LB-Agar

medium including substrates, phenol red in 100 mM KH_2PO_4 was prepared. *E.coli* strains having recombinant PAL activity were streaked out. Then plates were kept overnight at 30 ^oC.

2.3.1.2 The solid phase screen

In this assay; minimal medium in the absence of NH_4Cl plus phenylalanine was selected. PAL produces trans-cinnamic acid and ammonium by using phenylalanine. In the absence of NH_4Cl, colonies consume ammonia as nitrogen source. Releasing trans-cinnamic acid makes a decrease on pH. To indicate pH changes phenol red added to medium.

Another solid phase screen based on utilising the release of nitrogen due to enzymatic activity of PAL to support bacterial cell growth. The observed growth is because of nitrogen is being released, due to the activity of the PAL enzyme on the amino acid as a substrate.

2.3.2 Constructing mutant PALs based on generating the CASTing libraries

Through the use CASTing, it should be possible to obtain mutant PAL that accepts 4-bromophenylalanine at greater rate than the wild type PAL enzyme. Select up to 5 CASTing libraries – each library will consist of two residues that will be randomised using site directed mutagenesis. Previously generated 3D structure of PAL with homology modelling is used for selecting the residues that will make up the CASTing libraries [94]. All molecular modelling and *in silico* design of the CASTing libraries was conducted using Discovery Studio in InsightII [89].

2.3.2.1 Generation of saturation mutagenesis libraries with computational study

Tyr369, Asn276, His143, Glu501, Arg372 are five (5) main candidate residues (Fig.2.1). A (His145, Gln146), B (Leu274, Asn278), C (Tyr371, Arg374), D (Glu503, Asn506) are selected amino acids of CAST sites to be randomized simultaneously with the creation of relatively small libraries of mutants in terms of CASTing method. Last site is contact amino acids of PAL mer, E. Site E (Lys475, Ile479). The primers of 5 CAST sites for site-saturation mutagenesis in terms of CASTing method were designed.

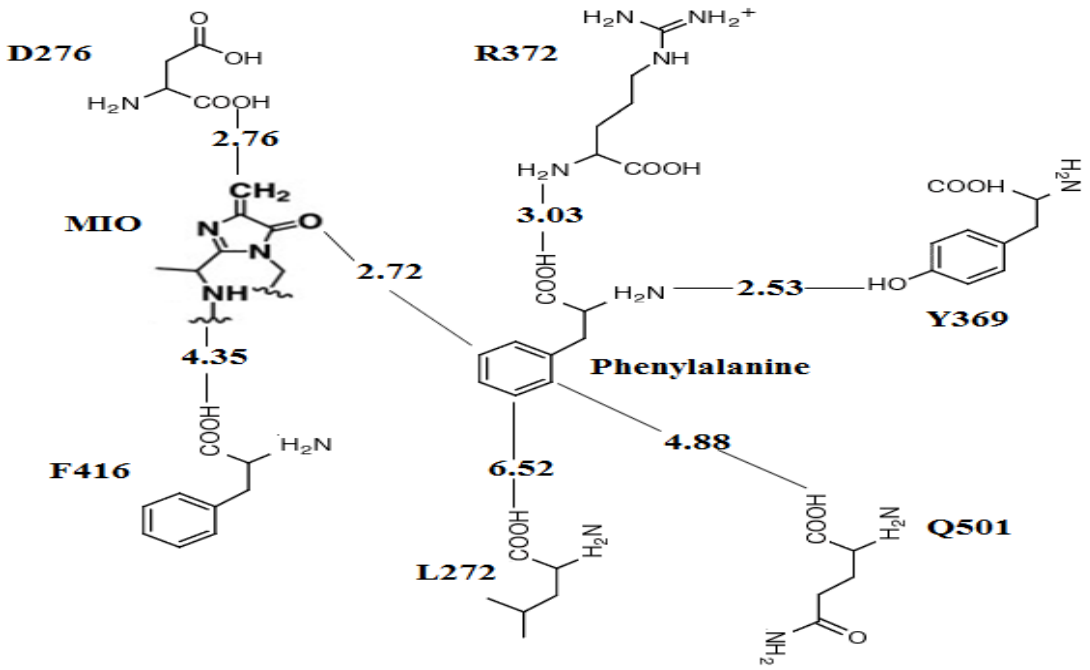


Figure 2.1 : Showing the selected active site amino acids of the whole PAL and substrates with the distance (Å).

2.3.2.1.1 Primers

NRT is selected as degenerative codon due to required low number mutant colony and coding desired amino acids.

Table 2.2 : List of primers used for CASTing sites.

Name	Sequence (5` to 3`)
A site_F	GCTCATCGAGNRTNRTCTCTGCGGCGTGAC
A site_R	CGTCACGCCGCAGAGAYNAYNCTCGATGAG
B site_F	CGAAGGAGGGTNRTGGTCTGGTCNRTGGAACGGCCGTC
B site_R	GACGGCCGTTCCAYNGACCAGACCAYNACCCTCCTTCG
C site_F	CAGGACCGCNYTCCGCTCNYTACGTCGCCTCAGTTCC
C site_R	GGAACTGAGGCGACGTAYNGAGCGGAYNGCGGTCCTG
D site_F	GCTCAACTATCACGGCNRTGGCTTGGACNRTCACATCGCTGCTTACGC
D site_R	GCGTAAGCAGCGATGTGAYNGTCCAAGCCAYNGCCGTGATAGTTGAGC
E site_F	TCCAGCCCGCANRTATGGGTNRTCAGGCCGTCAACTCG
E site_R	CGAGTTGACGGCCTGAYNACCCATAYNTGCGGGCTGGA

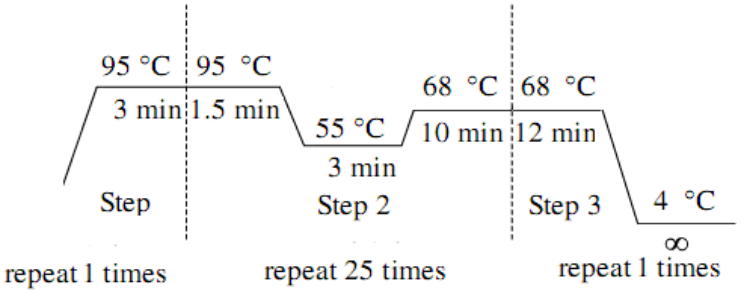
NRT:Degenerative codon.
AYN:Complementary degenerative codon.

In order to decrease the possibility of secondary structure, C base was changed with T base. (ggc:3.0 and ggt:2.8% ratio in *E. coli)*.

First of all, pre-purified pET-16b PAL was transformed to XL-1 Blue chemically competent cells. The plasmid DNA in XL-1 Blue competent cells was purified using Qiagen QIAprep Miniprep Spin Kit, following the protocol provided.

A QuickChange® PCR reaction was carried out with pET-16b PAL as the template [95]. Reaction mixture having 7 µL template (100 ng), 1.5 µL forward primer (10 µM stock), 1.5 µL reverse primer (10 µM stock), 5 µL *Pfu* buffer (10x), 2 µL dNTP mix (10 mM each), 1 µL *Pfu* turbo, 5 µL DMSO and 11 µL H_20. DNA for the saturation mutagenesis reactions using the primers, PCR conditions are detailed below.

2.3.2.1.2 PCR program



After the PCR the parental strand of DNA was digested with 1 µL of DpnI for 3 hours at 37 °C, and then 1 µL of the digested PCR reaction mixture was used to transform XL-1 blue.

For each library, ten colonies were collected for plasmid purification for sequencing. The rest of about 300 colonies for each library were lifted up by using 1 mL of LB Broth. Then plasmid purification protocol was applied to this 1mL LB Broth culture. BL21 (DE3) was used as host cell for transforming 1 µL purified plasmid library.

After application of transformation protocol for BL21 (DE3), each library has about 600 colonies. These colonies were picked and each one used to inoculate 800 µL of LB-amp in a deep-well 96-well plate for growing at 37 °C for overnight. The cells harvested by centrifugation at 15000 rpm for 5 min. The pellet was re-supended in 50 µL of 16 % (w/v) glycerol. After snap freezing of the pellet on liquid nitrogen, it was stored at -80 °C.

2.3.3 Screening of saturation mutagenesis libraries

2.3.3.1 Growth

Each glycerol stock of BL21 pET 16b PAL library A, D, E was inoculated to a 96-well plate having 500 uL aliquots of LB-amp for growing at 37 °C for overnight. Then 20 µL of this each wells were inoculated to 48-well deep well plate having 2 mL of LB-amp for growing at 26 °C for 18 hours. The cells were harvested by centrifugation at 15000 rpm for 5 minutes. 400 µL of 50 mM TrisHCl pH 8.8 was used for re-supending buffer of the cell.

2.3.3.2 Assay

The reaction was started by adding 100 µL of 20 mM L-4-bromophenylalanine to UV compatible 96-well microtiter plate having 90 µL of 50 mM TrisHCl pH 8.8, and 10 µL of the whole cell supension from libraries and wild type PAL and monitor the conversion of L-4-bromophenylalanine to L-4-bromocinnamic acid at 290 nm for 1 hour 30 minutes. The colonies have more activity than wild type PAL have prepared for sequencing.

2.3.3.3 Purification of selected novel mutant PAL enzymes

The native and mutant enzymes were purified using nickel nitrilotriacetic acid (Ni-NTA) resin [63] which the 6xHis affinity tag chelates to and can therefore be selectively removed from a mixture of proteins in solution. After growing overnight, the cells from library A and D were harvested by centrifugation (15000 g, 5 min). The pelleted cells were stored at -80 ºC for overnight. Then the pelleted cells were kept at 37 ºC for 30 min for freeze thaw cell lysis. The thawed cell pellets were supended in 20 mL of lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH to 8.0 with NaOH. Lysozyme (1 mg/mL) was added and cell supension was incubated on ice for 30 min. The remaining cell supension was centrifuged at 15000 g for 15 min at 4ºC.

Batches were prepared by pouring 2 mL of the 50 % Ni-NTA slurry into supernatant from the previous step in 15 mL falcons. A modified method of Qiaexpresionist by Qiagen was applied to mutant enzymes [63]. The batches were incubated for 60 min on a rocking table at 4 °C. 1 mL washing buffer containing 50 mM NaH_2PO_4, 300

mM NaCl, 20 mM imidazole, pH to 8.0 with NaOH was loaded to the resin seven times to remove unbound proteins by centrifugation (5000 rpm, 1 min).

Proteins of PAL bound to the metal groups of the resin surface were eluted three times with 1ml elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH to 8.0 with NaOH by centrifugation (5000 rpm, 1 min). Purified protein was desalted by ultrafiltration and concentrated with 30000 molecular weight cut off (MWCO) of ultrafiltration membranes. The protein concentrations were determined by bicinchoninic acid (BCA) protein assay.

2.3.4 Calculation specific activity of selected mutants of library 1 and 4

5, 10, 20 µL enzyme diluted in variable 50 mM TrisHCl pH 8.8 buffer and incubated at 37 ºC 10 min. Start the reaction by adding then 25 µL 10 mM L-4-Bromophenylalanine monitor the reaction for 1 hour 45 minutes at 290 nm. The specific activity of PAL was calculated using this assay, as 1 U equals 1 nmol of L-phenylalanine produced per minute.

2.3.5 Enantioselectivity of the best mutant PAL

The enantioselectivity of the best mutant PAL reaction was measured by only replacing AAO (amino acid oxidase) in both form D- and L-AAO.

2.3.6 pH profile of the best mutant PAL

The assay is carried out in TrisHCl buffer at pH 8, 8.5, 9, 9.5. The effect of different pH on the kinetic rate of conversion of *trans*-cinnamic acid and $(NH_4)_2SO_4$ to L-phenylalanine was investigated for determining the optimum pH for the best mutant.

2.3.7 Kinetics of the PAL reaction

The standard curve for kinetic rate calculation was produced by adding known quantities of H_2O_2 along with HRP to a fixed concentration of scopoletin and observing the decrease in fluorescence.

In order to grow *E. coli* cells having pET 16b carrying the gene of mutants of PAL, plated from stock to 50 ml LB medium with ampicilin. After 18 hours at 37 °C, the culture transferred to 1 l LB medium with ampicilin for 18 hours at 26 °C. The

protein was purified the by using a modified method of Qiaexpresionist by Qiagen as before [63].

Enzyme assay was carried out in a UV compatible 96-well microtitre plate. Firstly, 50 µL of 50 mM TrisHCl at pH 9, 10 µL of HRP (1 mg/mL), 10 µL of L-AAO (0.02 units of enzyme), 40 µL of scopoletin, native and selected the best 4 PAL mutants were added into the wells. Finally, the reaction was commenced by adding various concentrations of different substrate(s). The solution contains 100 mM of $(NH_4)_2SO_4$ in 100 mM TrisHCl buffer at pH 9. Evolved nmol quantities of H_2O_2 via the activity of the L-AAO upon the amino acid being produced by PAL for 1 hour 30 mins were detected.

2.3.8 Creating molecular models for obtained novel mutant PALs

Modelling the selected amino acid changes into the obtained homology model of PAL was done using Accelrys Discovery Studio 1.6 and 2.0 [89].

3. RESULTS AND DISCUSSION

3.1 DNA Shuffling To Construct Shuffled Mutant *bs*LDH

During the relatively short history of protein engineering two major approaches for altering enzymic activity on known protein scaffolds have emerged, namely rational design and directed evolution. Rational design requires knowledge of the relationship between structure and function in the enzyme scaffold, prediction of appropriate amino acid changes and realization of these changes by site-directed mutagenesis and also protein production before testing for the new activity. Directed evolution approaches require a method of producing many randomly mutated coding genes and a procedure for screening transformed bacteria that overproduce the protein with the new activity. However, if these conditions are met, random mutation and selection have the great advantage of avoiding the difficulties in obtaining incisive structure–activity relationships (SARs) thus successful predictive molecular modeling techniques. Consequently, various methods of directed evolution have been widely adopted by the biotechnology industry and have been proven valuable in ameliorating enzyme properties evolving new functions and in manipulating metabolic pathways [96]. Mutation-selection also has great potential for feeding information back into the rational design approach, if SAR data can be obtained post hoc [97-99].

The *bs*LDH enzyme is extremely stable to thermal denaturation, but has limited substrate specificity. Early studies [51] showed that a complete switching of substrate specificity of *bs*LDH from pyruvate to oxaloacetate by rational design can be achieved by replacement of the Glu residue at position 102 to an Arg residue, found in the naturally occurring MDHs. More recently [59], the substrate specificity of *bs*LDH has been successfully altered by a semi-rational approach in which residues on the substrate-specificity loop were subjected to randomization. The *bs*LDH protein carrying the Gln102Val mutation catalyzes the reduction of phenylpyruvate as efficiently as the wild-type enzyme with pyruvate. Allen and

Holbrook [60] introduced a DNA shuffling approach to produce a novel *bs*LDH that no longer requires the allosteric activator FBP for maximal activity, a clear industrial benefit.

In this study, two strategies of protein engineering have been applied that can potentially create an MDH from a LDH. The problem of altering substrate specificity has been approached from opposite poles. The reason for that a rationally designed MDH-the Gln102Arg variant has been obtained.

3.1.1 DNA shuffling

Applying DNA shuffling to the *bs*LDH gene involved isolating the coding DNA (an about 1 kb fragment) with a 5′ region containing an *Eco*RI site and a 3′ region with a *Pst*I site.

The gene was then randomly cleaved with a non-specific endo-DNAse I followed by PCR without primers to amplify the fragments. The library of mutated full-length genes was generated by another round of PCR in the presence of the primers for the 5′ *Eco*RI (N-terminus) and 3′ *Pst*I (C-terminus) sites. This library was ligated back into pKK223-3 plasmid, transformed into *E. coli* (JM105) cells and plated out. In the second cycle of PCR, the product would be obtained in different sizes. This would lower efficiency of transformation due to inclusion of undesired fragments of DNA. Later, the colonies were blotted onto nitrocellulose filters, heat-stepped to denature the mesophilic *E. coli* proteins and treated with assay buffer containing malate, NAD^+, PMS and NBT. Colonies producing mutant *bs*LDH with MDH activity generate nicotinamide adenine dinucleotide (NADH), which are chemically coupled to the colour reaction giving a purple halo. After the first round of mutagenesis, 83 colonies were analysed whether they had purple halo or not. 11/83 colonies having purple halo were picked up and checked out if they have shuffled-*bs*LDH in their plasmids. Only one of these 11 colonies had shuffled-*bs*LDH insert and showed MDH activity. The colony showing MDH activity was grown up and analysed by SDS-PAGE. Then, the active protein was expressed and purified. This screening result also demonstrated that some of the problems could occur with this assay. It was observed, in our experiment, that ineffective lysis of bacterial colonies and failure to remove unbound materials off the filters could lead to false positive reactions. After sequencing this shuffled gene (shuffled LDH), we identified eight

amino acid changes (Asn18Gln, Ser35Thr, Gln102Arg, Thr108Ser, Ala189Val, h, Gln238Asn and Val285Thr) and two silent mutations (Ala25Ala and Val147Val).

3.1.2 Gln102Arg-*bs*LDH and shuffled-*bs*LDH

As described above, the rational design approach was used nearly twenty years ago to switch the activity of *bs*LDH from pyruvate/lactate to oxaloacetate/malate by a single residue change, Gln102Arg, in the substrate-specificity loop [51]. This residue change provides the binding site for the second carboxylic acid group of oxaloacetate and is characteristic of known malate dehydrogenases. One of the eight mutations in shuffled-*bs*LDH is indeed this 'switch', Gln102Arg. We have re-investigated this single-mutant *bs*LDH to enable a fuller kinetic comparison with shuffled-*bs*LDH. In this way, we can ascertain the effect of the other seven mutations upon the shuffled-*bs*LDH enzyme. It has long been recognized that native *bs*LDH is allosterically activated by FBP [100]. Kinetic experiments were performed both in the presence and in absence of FBP since this was not examined in the original work on Gln102Arg-*bs*LDH. Data was also obtained for the chemically stable substrate α-ketomalonate, since decarboxylation of oxaloacetate to pyruvate can confuse the interpretation of kinetic rates obtained with oxaloacetate and enzymes possessing poor MDH but good *bs*LDH activity. Table 3.1 summarizes the data obtained for native, shuffled and Gln102Arg-*bs*LDH with pyruvate, oxaloacetate and α-ketomalonate, with and without FBP.

3.1.3 A comparison of activities with oxaloacetate in the presence of FBP

*Bs*LDH catalyses the interconvertion of an oxo-acid (pyruvate) and hydroxyl acid (lactate) using the NADH/NAD^+ pair as a redox cofactor. The bidirectional reaction is usually monitored by measuring spectrophotometrically either the increase in NADH at 340 nm produced in the lactate to pyruvate reaction or the decrease in NADH at 340 nm produced in the pyruvate to lactate reaction. Measuring the formation of NADH from NAD in dehydrogenase enzymes is a simple direct way to get kinetic data as it allows real time measurement of OD. Since the kinetic study of *bs*LDH with pyruvate and NADH could be carried out under single turnover conditions, which are significant in the study of many kinetic mechanisms, the reduced nucleotide binds tightly.

The *bs*LDH libraries were screened by using malate as a substrate according to the reverse direction of the reaction. As the product of this reaction, NADH was coupled with phenazine methosulfate (PMS) and nitro blue tetrazolium (NBT) to give a purple halo, which signifies the activity of a *bs*LDH protein with malate used in the screen. However, the kinetic studies of shuffled-*bs*LDH were carried out with oxaloacetate rather than malate for the same reason of wild type LDH as explained above.

As the outcome of the work suggests that the results of kinetic studies are consistent with the results of preliminary screening experiments, the claim of using different substrates for different experiments can be justified.

Native *bs*LDH has a weak K_M (3.42 mM) for oxaloacetate and a low catalytic rate constant; the k_{cat} is 6.3 s^{-1}. In contrast, both the Gln102Arg and shuffled-*bs*LDH have identical low K_M values (60 μM) and high activities (k_{cat} 253 s^{-1} and 377 s^{-1}) respectively. Expressing this as ratios of catalytic efficiencies (k_{cat}/K_M) shows that the Gln102Arg mutant is about 2200 times more efficient as an MDH than the native *bs*LDH, while shuffled-*bs*LDH is about 3400 times more efficient.

3.1.4 A comparison of activities with α-ketomalonate in the presence of FBP

It has been recently reported that certain lactate dehydrogenases can catalyse the decarboxylation of oxaloacetate under standard activity assay conditions [100-101]. This can result in apparent MDH activity as the LDH turns over the pyruvate produced by decarboxylation. The compound α-ketomalonate is an alternative MDH substrate that binds into the active site in a position analagous to oxaloacetate [102] and is not sensitive to decarboxylation.

Although the K_M of α-ketomalonate is some 20-fold higher than oxaloacetate for native *bs*LDH, the k_{cat} is only some 3-fold slower. The activities against α-ketomalonate with Gln102Arg-*bs*LDH and shuffled-*bs*LDH enzymes are kinetically indistinguishable, with k_{cat} around 250 s^{-1} (similar to Gln102Arg-*bs*LDH with oxaloacetate) and K_M around 3 mM, some 50-fold higher than with oxaloacetate, reflecting the difference seen with native *bs*LDH.

Table 3.1 : Steady-state kinetic constants for native and mutant *bs*LDH proteins.

Pyruvate

*bs*LDH	+FBP			-FBP		
	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$
Native	140±3	0.062±0.004	2258	240±3	2.7±0.17	89
Q102R	1.1±0.3	2.1±0.01	0.032	0.89±0.06	1.9±0.1	0.077
Shuffled	2.15±0.03	67±2	0.52	1.73±0.01	22.4±1.4	0.47

Oxaloacetate

*bs*LDH	+FBP			-FBP		
	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$
Native	6.3±0.05	3.42±0.02	184	6.1±0.3	4.1±0.2	1.49
Q102R	253±3	0.060±0.003	4217	234±2	0.062±0.003	3774
Shuffled	377±5	0.060±0.003	6283	267±4	0.063±0.003	4238

α-Ketomalonate

*bs*LDH	+FBP			-FBP		
	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$	$k_{cat}(s^{-1})$	$K_M(mM)$	$k_{cat}/K_M(s^{-1}/mM)$
Native	2.13±0.07	70±2	0.03	1.5±0.01	18.8±1.4	0.080
Q102R	244±4	3.2±0.2	76	223±3	3.0±0.2	74
Shuffled	254±4	2.9±0.1	88	247±3	2.52±0.16	98

3.1.5 A comparison of activities with pyruvate in the presence of FBP

Both Gln102Arg-*bs*LDH and shuffled-*b*LDH*s* are about two orders of magnitude less active with pyruvate than native *bs*LDH, although the k_{cat} of shuffled-*bs*LDH is twice that of the single-mutant Gln102Arg-*bs*LDH. However, the K_M for pyruvate is some 30-fold higher for shuffled-*bs*LDH than Gln102Arg-*bs*LDH; this is the most striking kinetic difference between these two proteins. The consequence of the raised K_M for pyruvate with shuffled-*bs*LDH is that the reduction in catalytic efficiency compared with native *bs*LDH is about 70,000-fold, while that of Gln102Arg-*bs*LDH

is reduced by about 4000-fold. The discriminatory power of the two mutant proteins between pyruvate and oxaloacetate is given by the ratio of their catalytic efficiencies for each substrate. Hence, Gln102Arg-*bs*LDH discriminates for oxaloacetate over pyruvate by a factor of 8000 while that of shuffled-*bs*LDH is 200,000.

3.1.6 The influence of FBP upon the reaction kinetics

Crystallography has shown that the major effect of binding the allosteric activator FBP to *bs*LDH is the repositioning of the active site residue R171 to bind the substrate carboxylate group more tightly [103]. This results in a 40-fold reduction in the K_M for pyruvate while reducing k_{cat} less than 2-fold. This effect on the kinetics of native *bs*LDH is almost entirely attenuated in the case of oxaloacetate and somewhat reversed in the case of α-ketomalonate (K_M is raised 3-fold in the presence of FBP).

The kinetic constants of Gln102Arg-*bs*LDH with all three substrates are essentially unaffected by the presence or absence of FBP. There is no reason to suppose that the Gln102Arg mutation will adversely affect the binding of FBP to the enzyme, hence any structural influence of FBP binding is not effectively propagated to the active site when the Gln102Arg switch is made. Likewise, the kinetic constants of shuffled-*bs*LDH with α-ketomalonate are insensitive to FBP. In contrast, minor effects of FBP are observed for shuffled-*bs*LDH with the other two substrates. For pyruvate, the effect is reversed compared with native *bs*LDH, such that the K_M is raised about 3-fold in the presence of FBP. In the case of oxaloacetate, the effect of FBP is expressed in the k_{cat} rather than the K_M value, such that the presence of FBP accelerates the enzyme by a modest 1.4-fold. Although these effects are small, they do indicate that the FBP binding site in shuffled-*bs*LDH is preserved despite the proximal mutation, A189V. Therefore, we can say that the mutants of shuffled-*bs*LDH are not able to be analog of mutants described by Allen and Holbrook [60].

3.1.7 The roles of the eight mutations in shuffled-*bs*LDH

The eight mutations are discussed here in the light of the model of shuffled-*bs*LDH constructed on the native *bs*LDH structure. The general similarities of the kinetic properties of Gln102Arg-*bs*LDH and shuffled-*bs*LDH illustrates that the Gln102Arg mutation is responsible for the majority of the switch from LDH to MDH activity in these proteins.

Fig. 3.1(a) shows how the Gln102Arg mutation allows oxaloacetate to bind in the active site pocket in a pose consistent with the chemical reaction. This supports the original rational design strategy and is also consistent with the mode of binding of α-ketomalonate to porcine MDH as determined by crystallography [104]. The remaining seven mutations are broadly chemically conservative and located at or close to the surface of the protein. The positions of the changes in the context of the monomer are shown in Fig. 3.1(b) and in the context of the tetramer in the (Fig. 3.1(c)). Three residue changes, Asn18Gln, Gln238Asn and Val285Thr are remote from the active site, cofactor binding pocket and any subunit interface hence they are unlikely to affect the catalytic activity. Likewise, the mutation Ser35Thr is unlikely to affect activity since it is located at the "dimer" interface on the *Q*-axis, which is retained in the conversion of tetramer to dimer that accompanies removal of the FBP activator and is remote from the FBP site. The three remaining mutations are all possible candidates for modulating the activity of shuffled-*bs*LDH compared with Gln102Arg-*bs*. The mutation Thr108Ser in the substrate-specificity loop may be partly responsible for the 30-fold higher K_M for pyruvate shown by shuffled-*bs*LDH compared with Gln102Arg-*bs*LDH, since this change will increase flexibility of the loop, which is required to become ordered on substrate binding. The FBP-dependent effects exhibited by shuffled-*bs*LDH, raised K_M for pyruvate and raised k_{cat} for oxaloacetate, are probably caused by perturbations around the FBP site caused by the nearby Ala189Val mutation. Finally, the residue at position 199 is close to the catalytic histidine 195 (6.2 Å, Glu199:CD-His195:CG) in the *bs*LDH crystal structure, hence the change Glu199Asp may modulate activity by structural or electronic perturbations.

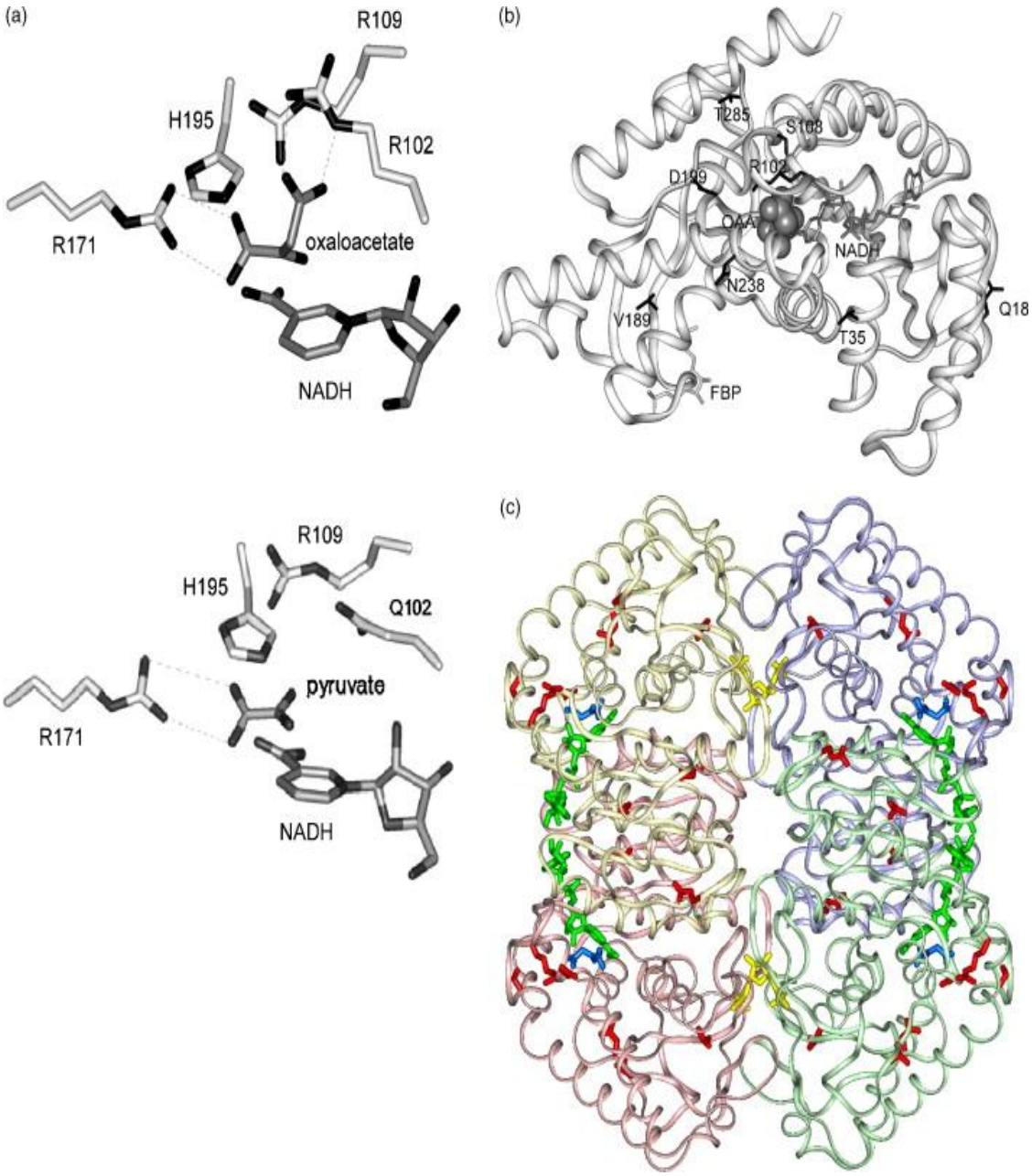


Figure 3.1 : a) Upper image shows oxaloacetate modelled into the activesite of shuffled- *bs*LDH after energy minimisation; lower image shows a similar view of the crystal structure of wild type *bs*LDH with pyruvate and NADH; and shows oxaloacetate modelled into the active site of shuffled-*bs*LDH after energy minimisation; **b)** the positions of the mutations in shuffled-*bs*LDH and **c)** the positions of the mutations in shuffled-*bs*LDH in the context of the tetramer (mutations are red, NADH is green, FBP is yellow and oxaloacetate is blue).

3.2 Mandelic Acid as a Substrate for Highly Purified *Bacillus stearothermophilus* Lactate Dehydrogenase

3.2.1 Expression and purification of mutant and native *bs*LDH proteins

After each PCR reaction, the DNA product isolated after plasmid purification was loaded onto an agarose gel. The results of the agarose gel electrophoresis indicated that the linear plasmid was amplified. The product plasmids were digested and run on an agarose gel prior to sequencing. Site-directed mutagenesis was used to insert mutations at two positions in the *bs*LDH gene, generating the double variant. Presence of the desired mutations was confirmed by DNA sequencing.

*Bs*LDH with 6xHis-tags were purified using the TAGZyme system. Affinity of the 6xHis tag for the chromatography resin facilitates purification of *bs*LDH (Fig.3.2). The lane corresponding to the final protein (lane 4) indicates that *bs*LDH is close to 95 % pure. 8μL for samples of the purified native and mutant *bs*LDH enzymes from each step were loaded onto a (12 % w/v) standard SDS-PAGE gel. As is evident in the final elution samples (lane 4) shown in Fig.3.2, wild type and mutant enzymes were obtained in high yield and had no unexpected bands on the gel, indicating the isolated proteins were free of contamination (Table 3.2).

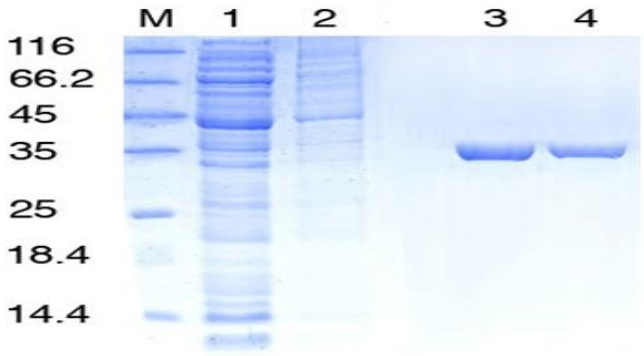


Figure 3.2 : Expression and purification of 6xHis-tagged wild-type and mutant *bs*LDH from *E. coli* protein fractions of SDS-PAGE gel. (M) Standard protein marker (Unstained standard protein weight marker, Fermentas Inc.) (1) Supernatant of the whole cell lysate. (2) Column wash. (3) Fractions of the wild type elution. (4) Fractions of the mutant elution. (Numbers on left show the molecular weight-kDa).

Table 3.2 : Comparison of the purification system for obtained purified *bs*LDH.

Purification Steps	Protein(mg/ml)	Yield(%)	Purification Factor
Crude Extract	1.53	100	1
TAGZyme System	1.31	86	14
Standard Purification System	0.96	62	38

The steady-state kinetic parameters for *bs*LDH were calculated. The average final concentration of *bs*LDHs in elution buffer was 0.53 mg/mL (lane 3-4). Characterisation of the native and mutant *bs*LDH proteins has been facilitated by the utilisation of modified expression and purification protocols. Many recombinant proteins are produced by intracellular expression of heterologous genes in genetically engineered *E.coli* strains. The previous *bs*LDH purification strategy utilised a combination of ammonium sulphate fractionation, ion exchange chromatography, and blue sepharose affinity chromatography. However, these methods are time-consuming and are not suitable for high protein yield and purity. We, therefore, looked for a simple method of purification that does not compromise the purity and yield of active *bs*LDHs. The TAGZyme system, described in the previous section, provides a quick and efficient method for obtaining purified active enzyme. By application of the TAGZyme system, enzyme was isolated with increased purity and decreasing time and complexity of purification. While the TAGZyme system provides the advantages described above, it may affect the kinetic constants of enzymes. After cloning *bs*LDH into a 6xHis-tag expression vector, the protein was obtained in greater than 95 % purity in just three hours from a small scale, with no effect on enzyme. A final dialysis step against active charcoal was added to the purification protocol to remove salts and nucleotides from the product *bs*LDHs.

3.2.2 Enzyme assay of mutant and native *bs*LDH proteins obtained by TAGZyme system

The steady-state catalytic properties of the mutant and native *bs*LDHs obtained by the TAGZyme system were compared to those obtained by the standard chromatographic method. The average yield of *bs*LDHs in the last step of TAGZyme purification was 0.53 mg/mL. The kinetic constants for native *bs*LDH purified by the

former standard method [104] are compared to those of *bs*LDHs purified with the TAGZyme system in Table 3.3 and Table 3.4.

Table 3.3 : Summary of the kinetic parameters k_{cat} and K_M for His-Tagged and wild-type *bs*LDH.

	Pyruvate		
	$k_{cat}(s^{-1})$	K_M(mM)	$k_{cat}/K_M(s^{-1}/mM)$
Wild- type	240±16	2.7±0.08	88.8
His-Tagged Wild-type	116±12	1.02±0.04	113.7

Table 3.4 : Steady-state kinetic properties of native and mutant *bs*LDH proteins with various substrates.

	Pyruvate			Mandelate		
	$k_{cat}(s^{-1})$	K_M(mM)	$k_{cat}/K_M(s^{-1}/mM)$	$k_{cat}(s^{-1})$	K_M(mM)	$k_{cat}/K_M(s^{-1}/mM)$
His-Tagged Wild-type	116±12	1.02±0.04	113.7	n.d.	n.d.	n.d.
I240A/T246G	54±6	18.5±2	2.91	110±5	3.9±0.4	28.2

n.d. : not determined

The Michaelis constant (K_M) of 6xHis-tagged *bs*LDH is reduced relative to that of a stock of wild type *bs*LDH without the 6xHis-tag, indicating that the affinity of 6xHis-tagged *bs*LDH is better than the analogous untagged enzyme. Thus, addition of the 6xHis-tag to the C-terminus of *bs*LDH does not cause any significant change in its conformation.

3.2.3 Molecular modeling of mutated *bs*LDH protein

Residue replacement and rotamer optimisation were conducted using the molecular modeling software Insight II, emphasizing the maintenance of *bs*LDH's ability to facilitate redox chemistry. A representation of the designed mutant active site in the enzyme:NADH:L-mandelic acid complex is shown in Fig.3.3.

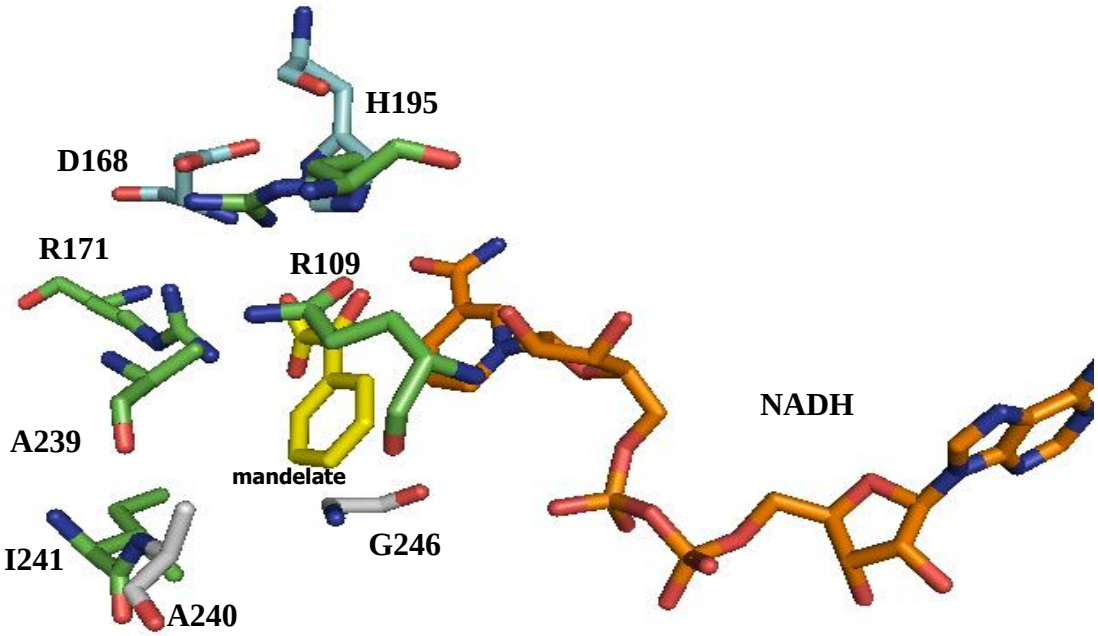


Figure 3.3 : Shows mandelate modeled into the active site of the mutated-*bs*LDH after energy minimization.

A detailed understanding of the structure and mechanism of *bs*LDH is needed to alter the substrate binding site of the enzyme while maintaining the elements essential for catalysing the redox reaction. This is particularly difficult in an enzyme with many amino acid residues and regions important for both catalysis and substrate specificity in the vicinity of the active site. The X-ray crystal structure of *bs*LDH [105] allowed identification of the active site for 2-hydroxy acid dehydrogenation. Previous studies revealed the catalytic importance of His195 as a proton donor/acceptor [25,108] and supports the existence of His195 in the protonated form in the ternary complex [104,48]. Furthermore, Ile250 contributes to the hydrophobic environment for the dihydronicotinamide ring [48], Arg109 stabilises negative charge on the substrate C2-oxygen in the transition state [109], R171 provides a tight, bifurcated binding-interaction with the C1-carboxylate of the substrate [47] and Thr246 constrains the volume of the active site. The role of these amino acids in substrate specificity has been examined in previous studies in which these residues were replaced by smaller variants in an attempt to increase the size of the substrate-binding cavity [51,56,110].

There is also evidence that the specificity of *bs*LDH is determined by at least two regions of the enzyme surrounding the side chain of the substrate. These regions consist of a flexible polypeptide loop that folds over the active site and encloses the substrate in the catalytic pocket, and a helix onto which this loop folds [109]. To accommodate substrates with hydrophobic side chains larger than that of the

pyruvate substrate, mutations were introduced to these two loops. Gln102Met, Lys103Val, Pro104Ser and Ala236Gly, Ala237Gly mutations increase the tolerance for large, hydrophobic branched-chain substituted pyruvate variants. These changes resulted in a 55-fold improvement in k_{cat} with α-ketoisocaproate as a substrate [53].

In the first attempt to change the substrate specificity of *bs*LDH, Atkinson and colleagues designed *bs*LDH to change the preferred substrates from the pyruvate/lactate pair ($R=CH_3$) to the oxaloacetate/malate pair ($R=CH_2$-COO). The volume of the active site was increased with a Thr246Gly mutation, an acid was neutralised with an Asp197Asn mutation and a base was introduced for charge pairing with a Gln102Arg mutation. This triple mutant had a catalytic specificity for oxaloacetate over pyruvate of 500 compared to 1000 for the native of *bs*LDH [50].

A short sequence comparison indicates that residues Thr246 and Ile240 are not well conserved in dehydrogenases for different substrates, supporting the idea that they are important in substrate selection. Examination of the structure native state *bs*LDH enzyme reveals that Ile240 occupies the substrate binding space. Thr246 is close to a tight turn (aa 235-238) between two helices that, together with the mobile coenzyme loop (aa 99-110), shield the bound substrate and the nicotinamide moiety of NADH in the active site from the solvent [108]. The restriction of active site volume imposed by the Thr246 (Fig. 3.3) residue may be responsible for the preference for α-hydroxy acid substrates with small side chains. The hydroxyl group of Thr246, which is part of the active site wall, is favourably positioned to form a hydrogen bond to the carboxylic group of the substrate [45].

Residues Ile240 and Thr246, conserved in all LDHs, are smaller residues in mandelic dehydrogenase (ManDH), presumably to enlarge the volume of the binding pocket and to accommodate the increased size of mandelic acid. Holbrook *et al.* demonstrated that replacement of Thr246 with Gly in the *bs*LDH active site changes the preference for the new substrate over the old, resulting in a 3200-fold reduction in the k_{cat}/K_M for pyruvate and a slight enhancement for oxaloacetate. Thus, Thr246 can affect substrate specificity [51].

Wilks *et al.* targeted 2-oxo and α-hydroxy acids with hydrophobic groups as new substrates for the Thr246Gly mutant of *bs*LDH. They strategised that the Thr246Gly mutation could accommodate the larger side chains of α-hydroxy acids with hydrophobic groups. The kinetic data support this hypothesis only when the side

chain of the substrate is charged, as in the case of oxaloacetate. In this case, the deleterious effect of the Thr246Gly mutation is reversed due to better substrate binding, reflected by the K_M values. As mentioned before, the hydroxyl group of Thr246 is part of active site wall that forms a hydrogen bond with carboxylic group of the substrate. Thus, the Thr246 residue can help dictate preference for small side chain α-hydroxy acids. Replacement of Thr246 with Gly eliminates the possibility of this hydrogen bond and the substrate binding and orientation it facilitates. Additionally, in the specific case of oxaloacetate, water cannot access to the critical active site region in the Gly mutant typically occupied by the side chain of Thr246 [110].

Introduction of mandelic acid as a substrate for LDH was achieved previously by engineering *Saccharomyces cerevisiae* falvocytochrome b2: (LDH). A specific mandelate dehydrogenase was designed from the LDH framework via two mutations (Ala198Gly, Leu230Ala) that increased substrate specificity for mandelic acid 400-fold over the native enzyme [62].

Substrate binding in the wild-type enzyme is achieved by formation of a hydrogen bonding network that excludes water. As the size of substrate side chain is increased, reorientation of the loop may increase the accessibility of water to the active site, thereby decreasing rates of enzyme catalysis. These water molecules may provide alternative H bond partners to various functional groups in the active site. The larger side chain substrates may have a similar effect as water on the active site, such that further increases in size have no additional effects [54].

These previous studies showed that *bs*LDH is sensitive to the charge and size of α-hydroxy acid side chains. Thus, it is likely that *bs*LDH is unable to catalyse the oxidation of L-mandelate because steric interference between the substrate and the side chain of Thr246. The steric interactions force L-mandelate to adopt an unfavourable orientation for proton a*bs*traction at C-2, which may prevent the formation of the transition state. Thr246, a catalytically important residue, restricts the orientation of the mandelate ring. Thus, the redesign of *bs*LDH, while maintaining the important functions of Gln102, His195, Asp68 and Arg171, is necessary to accommodate the large side chain mandelic acid. This was accomplished by enlarging the volume of the active site pocket and orienting substrate/protein contacts. Residues Ile240 and Thr246 were replaced by amino acids

with small side chains to enlarge the volume of the binding pocket and remove possible interactions between the side chain of Thr246 and the aromatic ring of mandelic acid.

Although no additional experimental studies on the role of Ile240 have been conducted, X-ray structure and modelling studies of *bs*LDH indicate that Ile240 is close active site residues and forms a cavity for binding the substrate, as shown in Fig. 3.3. Substituting Ile240 with alanine introduces nucleophilic charge repulsion with the second -COO of new substrate, resulting in a correct orientation for aromatic ring of mandelic acid.

The modelling studies indicated that replacement of Thr246 with Gly most likely leads to a stronger interaction and better binding between the new substrate and Arg171, a hypothesis supported by the K_M value for mandelic acid. Due to the Gly mutation, H bonding with Thr246 is predicted to be eliminated, allowing the aromatic ring of mandelic acid to orientate with Arg171, as seen in Fig.3.3. Furthermore, the aromatic ring of mandelic acid excludes external water from the increased binding pocket formed by the Thr246Gly mutation. Introducing Gly can contribute stabilisation to the mentioned catalytic pocket.

The hydrophobic residues Ala237, Ile240, Ile241 orient the -CH_3 of Thr246 that interacts hydrophobically with the -OH of the substrates. Replacing Thr246 with Gly eliminates the effects of these amino acids on Thr240, thereby decreasing the binding energy for the substrate. However, substituting the methyl side chain of the substrate with an aromatic group generates new interactions between the protein environment and L-mandelate that resulted in increased binding energy. The decreased K_M can be rationalised by these new interactions.

Substituting only Ile240 with any small side chain amino acid may increase the substrate binding pocket to accommodate the aromatic side chain of the substrate, but this single mutation cannot influence the binding because of the its location that is in outside of the α-helix. However, substituting Thr246 with Gly removes a hydrogen bond with the substrate so that the binding energy for substrate will decrease. Furthermore, the CH_3 of the Ala240 mutant makes hydrophobic interactions with aromatic side chain.

These mutations resulted in an enzyme with an increased volume of its binding pocket. His195, Arg171 and Gln102 orient the substrate and transfer H^-. Ultimately, simulations suggested that moving the substrate away from these active residues and towards the Gly246 and Ala240 mutations would lead to a decrease in k_{cat}.

The major rate-determining step for *bs*LDH catalysis is proton abstraction at C-2 of lactate. Thus, the reaction rate for the double mutant *bs*LDH reports on the interaction between mandelate and active site amino acids. The electrostatic interactions of mandelic acid with the crucial Arg171, Gln102, and His195 amino acids suggests a more favourable interaction between the aromatic group of mandelic acid and the Gln102 and Arg171 side chains. An electrostatic interaction may exist between the polar group on the 102 residue and the aromatic ring of the substrate or via a number of mechanisms.

The improved activity of the double mutant compared to the wild type when mandelic acid is the substrate suggests that a combination of better electrostatic interactions and H-bonding between mandelic acid and the active site residues. The combined effects of Gly246 in orienting and accommodating the large side chain of mandelic acid and of Ala240 in supplying more space for mandelic acid are responsible for also improved substrate binding.

The most impressive aspect of this study is being the first time success in introducing the substrate having aromatic side chain. Therefore, these observations led to the suggestion that Thr240 and Ile246 might be crucial residues in accommodating and orienting large side chain substrates for *bs*LDH.

3.3 Development of Novel Ammonia Lyase Biocatalysts for the Production of Unnatural Amino Acids for Industry

3.3.1 Optimisation a screening method for PAL variants

Finding a screening method allowing scanning libraries which have thousands of mutant colonies for the desired properties of engineered enzyme in a short time is a key requirement for any directed evolution experiments. Therefore, primarily the development of both a high-throughput solid phase screen and a microtitre plate based assay to identify and characterize PAL activity was focused on.

3.3.1.1 A pH sensing agarose plate solid assay

Any colour shift after overnight incubation at 30 ^{o}C for pH sensing agarose plate assay was not seen. The similarity pKa of pH indicator to the pH of the reaction mixture ensures that the change of pH leads to a large and linear colour change. It is extremely important to choose an appropriate pH indicator and buffer pair for accurate measurement of PAL activity. The main selection criterion is that the pKa of the buffer and the pKa of the pH indicator should be as close to equal as possible. Desired pH indicator should work most sensitively at pH<8. The colour of the selected pH indicator will gradually change from the colour of used indicator to the coupled of different colour with increasing amount of protons generated by the enzyme. Chosen buffer should have pKa that is very close to that of selected pH indicator dye. The selected reaction mixture should be low enough to maximize sensitivity, while high enough to ensure accurate measurements and small pH changes throughout the assay. The small pH changes are important because kinetic parameters may change with changing pH.

3.3.1.2 The solid phase of growth screen

Another solid phase screen based on utilising the release of nitrogen due to enzymatic activity of PAL to support bacterial cell growth. The observed growth is because of nitrogen released, due to the activity of the PAL enzyme on the amino acid as a substrate. However, the forming of colonies was not observed even after 6 days on Petri plates having M9 media-NH_4Cl, phenylalanine.

A liquid phase assay has been developed by Rowles, which was a construct of the assay by Khan and Valdyananthan [94]. This assay 1 (Fig.3.4) was applied to the screening of small libraries of PAL variants such as those generated during a CASTing experiment.

To aid with the identification of these protein variants two novel assays have been developed for the screening of PAL activity. Assay 1 is a high-throughput whole cell microtitre plate based assay for the deamination reaction of the PAL enzyme. This is applicable for the screening of small focused libraries of PAL variants. Assay 2 is for the determination of the synthetic activity of PAL; this assay uses an enantioselective oxidase enzyme and horse radish peroxidase (HRP) as a reporter system for PAL activity (Fig.3.4).

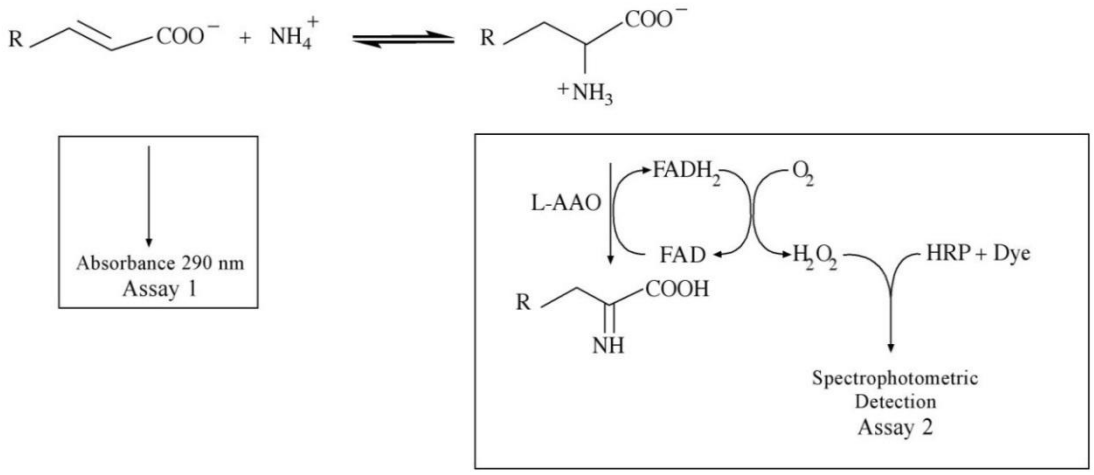


Figure 3.4 : The reaction catalysed by the PAL enzyme and the two developed screenings to detect the activity.

3.3.2 Constructing mutant PALs based on generating the CASTing libraries

An alternative strategy combinatorial active-site saturation test introduced for expanding substrate acceptance of PAL. Based on the obtained three-dimensional structure of the enzyme by homology modeling, sets of five amino acids, whose side chains reside next to the binding pocket, are identified and the respective positions are then randomized simultaneously with the creation of relatively small libraries of mutants. By this method, mutant PALs that have more activity 4-Br-CA than the wild type have been able to obtained.

3.3.2.1 Generation of saturation mutagenesis libraries

On the basis of the homology modelling structure, five CASTing libraries A, B, C, D, E(calling as 1-5 respectively as well) were chosen for saturation mutagenesis using NRT codon degeneracy which encodes Arg, Asn, Asp, Cys, Gly, His, Ser, Tyr. Selected candidate residues composing of five CAST sites to be randomised simultaneously: His145, Gln146, Leu217, Leu274, Val277, Asn278, Tyr371, Asn404, Phe420, Arg474, Lys475, Ile479, Glu503, Asn506. The substrate and 175211 are phenylalanine is MIO respectively in the results of modelling figures (Fig.3.5-11).

The CASTing site of A of the active site of PAL is based on His145 residue.

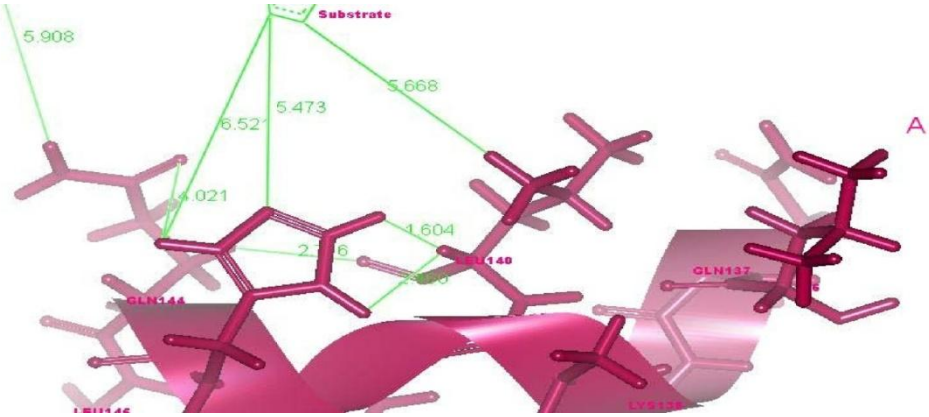


Figure 3.5 : Showing the CASTing sites of A; distance among the selected amino acids and substrates.

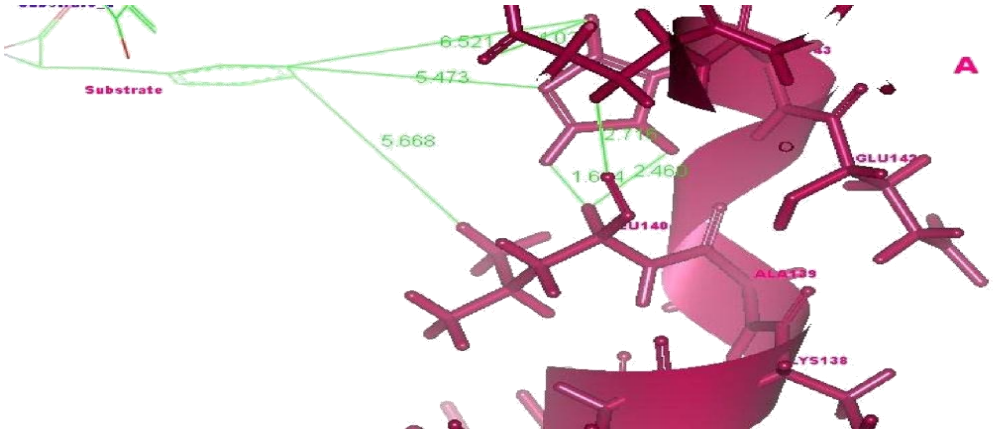


Figure 3.6 : Showing the CASTing sites of A; distance among the selected amino acids and substrates.

The CASTing site of B of the active site of PAL is based on Asn278 residue.

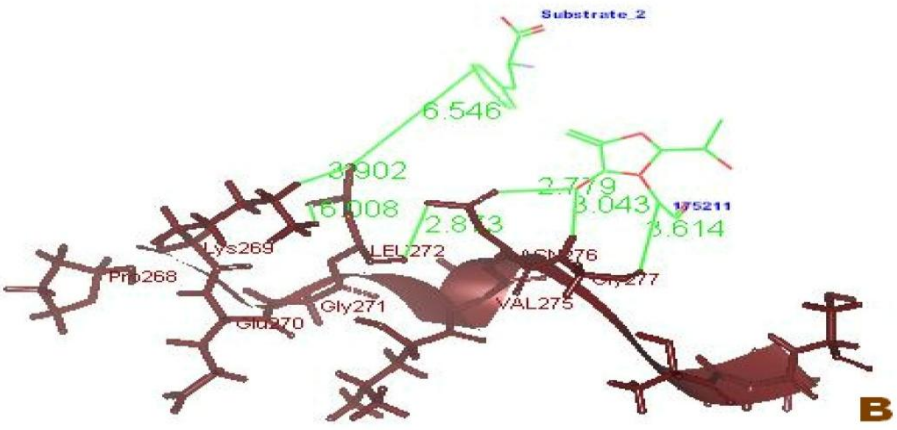


Figure 3.7 : Showing the CASTing sites of B; distance among the selected amino acids and substrates.

The CASTing site of C of the active site of PAL is based on Arg374 residue.

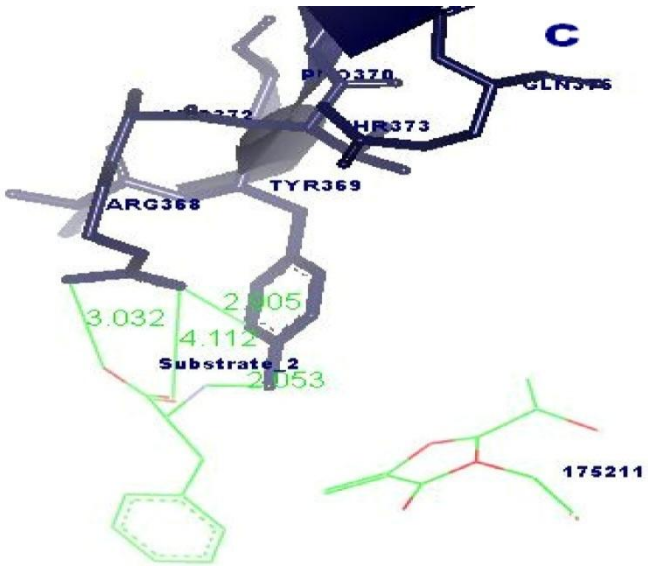


Figure 3.8 : Showing the CASTing sites of C; distance among the selected amino acids and substrates.

The CASTing site of D of the active site of PAL is based on Glu503 residue

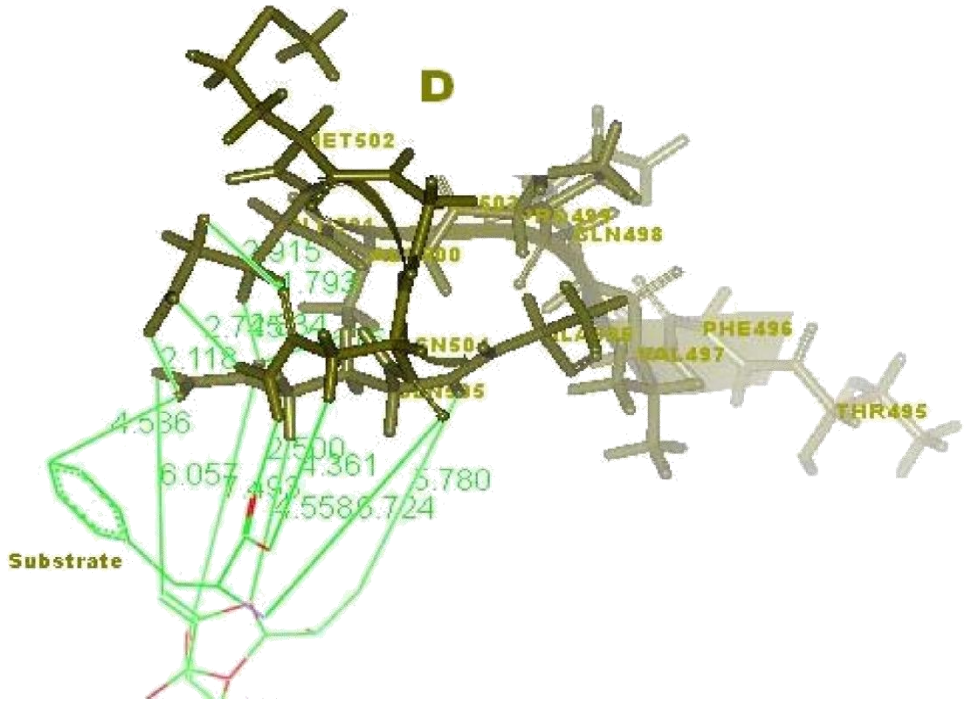


Figure 3.9 : Showing the CASTing sites of D; distance among the selected amino acids and substrates.

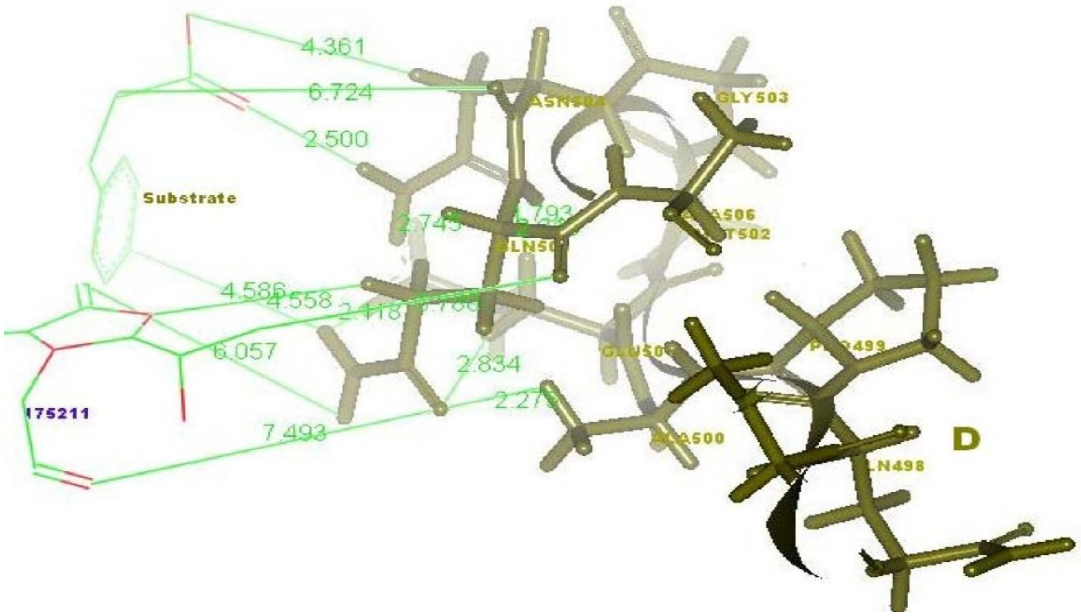


Figure 3.10 : Showing the CASTing sites of D; distance among the selected amino acids and substrates.

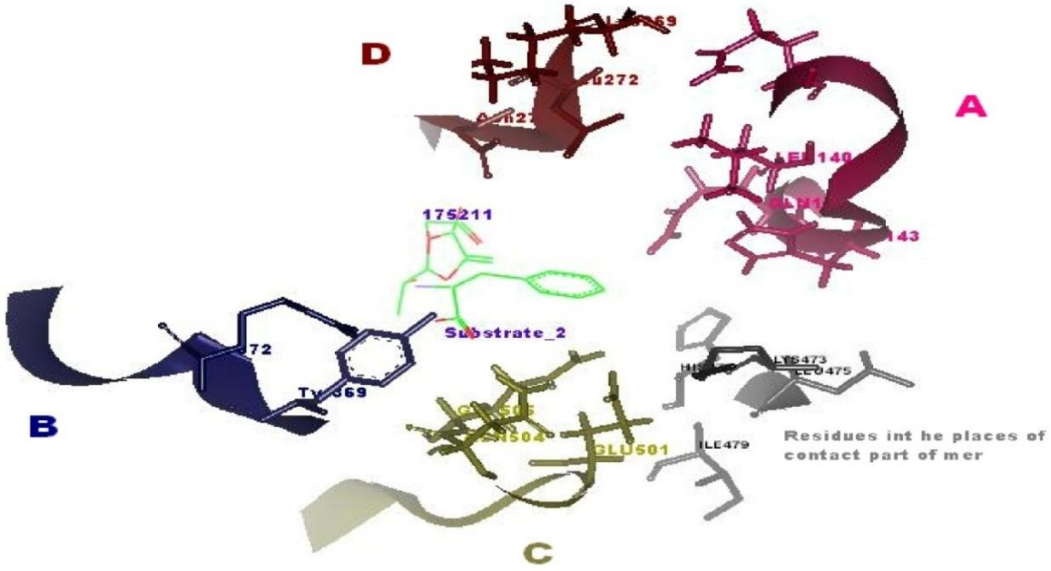


Figure 3.11 : CASTing of PAL leading to the construction of five libraries of mutants (A–D) produced by simultaneous randomization at different number of amino acid sites for each library.

3.3.3 Screening of saturation mutagenesis library and purification of selected novel mutant PAL enzymes

All libraries were screened for lyase activity against 4-bromophenylalanine, which are not accepted by the wild type PAL [83]. This multiple-substrate/step screening required 3000 colonies high throughput screening. Although the simultaneous randomization of a few amino acids yields hundreds unique variants (some being double mutants, others having only a single amino acid exchange plus the original wild type combination), the number of variants to be screened depends on the

randomization technique used in the construction of a library and on the over-sampling required for 95 % coverage of protein-sequence space [110]. Screening of all libraries was accomplished. The purification of the selected novel mutant PAL enzymes was carried out.

3.3.3.1 Sequence results for the selected novel mutant PAL

Library A

421 - GCT TTG ATC GAA **CAT CAA** CTC TGC GGC GTA ACT CCT ACC - 465

141 - A L I E **H Q** L C G V T P T -155

145 **146**

Wild Type PAL: HISTIDINE GLUTAMINE

Mutant diversity of library A after sequenced selected with the screening:

GLYCINE	SERINE
ASPARAGINE	SERINE
GLYCINE	GLYCINE
GLYCINE	CYSTEINE
SERINE	SERINE
GLYCINE	GLYCINE
SERINE	ASPARAGINE
SERINE	GLYCINE
ASPARAGINE	ASPARAGINE
CYSTEINE	ASPARAGINE
GLYCINE	ASPARAGINE
CYSTEINE	SERINE
SERINE	CYSTEINE
ASPARTIC ACID	ARGININE
CYSTEINE	CYSTEINE
PROLINE	ASPARAGINE
CYSTEINE	ASPARTIC ACID
GLYCINE	ASPARTIC ACID
CYSTEINE	GLYCINE
ASPARAGINE	CYSTEINE

Library D

1381- CCT TCT CTC AAT TAT CAC GGT **AAG** GGG CTG GAC **ATC** -1418

468 - S L N Y H G K **L** L D I **I** - 479

475 **479**

Wild Type PAL: LYSINE ISOLEUCINE

Active Mutants of Lib.D after screening

Mutant*: ASPARTIC ACID HISTIDINE

*(inserted primer itself having these mutant but not broken in open reading frame)

Sequence of the mutant:

...ANY_HGRGLDGHIAAY(CTCAATTATCACGGTGATGGTCTGGACCATCAT ATTGCAGCG)ASEL.

Library E

1501 - **GAA** ATG GGT **AAC** CAG GCT GTC AAT TCT CTG GCG CTG -1545

503 - **E** M G **N** Q A V N S L A L - 515

503 **506**

Wild Type PAL: GLUTAMINE ASPARAGINE

Active Mutants of Lib.E after screening

Mutant: GLYCINE ASPARTIC ACID

The mutant PAL enzymes having 6xHis were purified the by using a modified method of Qiaexpresionist by QIAGEN [63]. SDS-PAGE analysis (Fig.3.12) shows that the eluted mutant PAL protein of ~100 kDa and purity of the last eluted fraction is more than 90 % for kinetic studies.

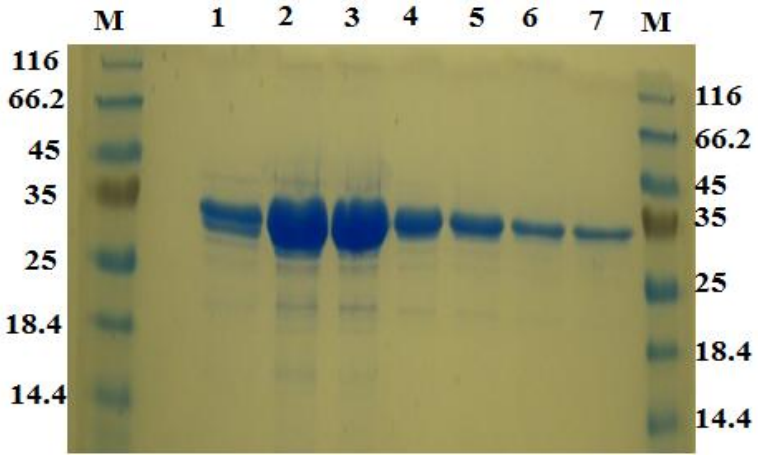


Figure 3.12 : SDS-PAGE shows the purified mutant PALs with 7 step elution. (M): Standard marker (Unstained standard protein weight marker, Fermentas Inc.). (1-7): Eluted fractions. (Numbers on left show the molecular weight-kDa).

3.3.4 Calculation specific activity of the selected mutants of library A and D

Specific activities of the colonies from library A and the single colony from library D which showing more activity in screening were calculated.

Table 3.5 : Specific activity calculations for library A and D (Substrate is 4-bromophenylalanine, Activity-nmole/min/mg protein).

MUTANT NUMBERS	MUTATION OF 145	MUTATION OF 146	SPECIFIC ACTIVITY
1	Cys	Ser	80.50±9.2
2	Gly	Cys	54.90±8.2
3	Pro	Asn	176.20±28.6
4	Ser	Gly	7.90±0.9
5	Cys	Asp	5.70±0.4
6	Ser	Ser	97.70±9.6
7	Gly	Asn	67.99±5.8
8	Cys	Gly	4.90±0.6
9	Asn	Asn	69.24±6.4
10	Asp	Arg	2.92±0.2
11	Asn	Tyr	3.79±0.1
12	Ser	Cys	78.90±14.7
13	Cys	Asn	109.04±8.9
Native PAL	His	Gln	2.30±0.2
15	Cys	Asn	307.28±54.6
16	Gly	Gly	6.16±0.3
17	Cys	Cys	5.56±0.8
18	Gly	Ser	37.25±8.9
19	Gly	Gly	4.81±0.2

Table 3.5 (contd.) : Specific activity calculations for library A and D (Substrate is 4-bromophenylalanine, Activity-nmole/min/mg protein).

20	Asn	Ser	168.34±16.8
21	Ser	Asn	173.32±19.3
22	Asn	His	67.05±4.7
23	From library D		4.23±0.1

Because of CASTing, many variants were identified as hits. Most of the hits originate from library A showing the highest frequency of improved mutants. The most promising enzymes namely 16D, 25E, 29B, 31E variants from library A, were identified as the double mutant His145Asn/Glu146Ser, His145Ser/Glu146Asn, His145Asn/Glu146Cys, His145Cys/Glu146Asn respectively.

3.3.5 Enantioselectivity of the best mutant

4-bromophenylalanine is chiral, so it was therefore of interest to study the enantioselectivity of the best mutant as catalysts in lyase kinetic resolution. The screening system was designed to assess activity, and it is suitable to test the effect of mutations on the enantioselectivtity (Fig.3.13) The enantioselectivity of the reaction of the best PAL variants was determined. This was conveniently achieved by replacing the L-AAO in the assay with D-AAO. A third assay was included as a blank where the oxidase was replaced with a buffer solution to determine the background decrease in fluorescence. When the blank bar compared with the D-AAO bar, it can conclude that there is no increase in activity when a D-amino acid oxidase is instead incorporated in the assay, thus demonstrating the enantioselectivity of the PAL enzyme variants.

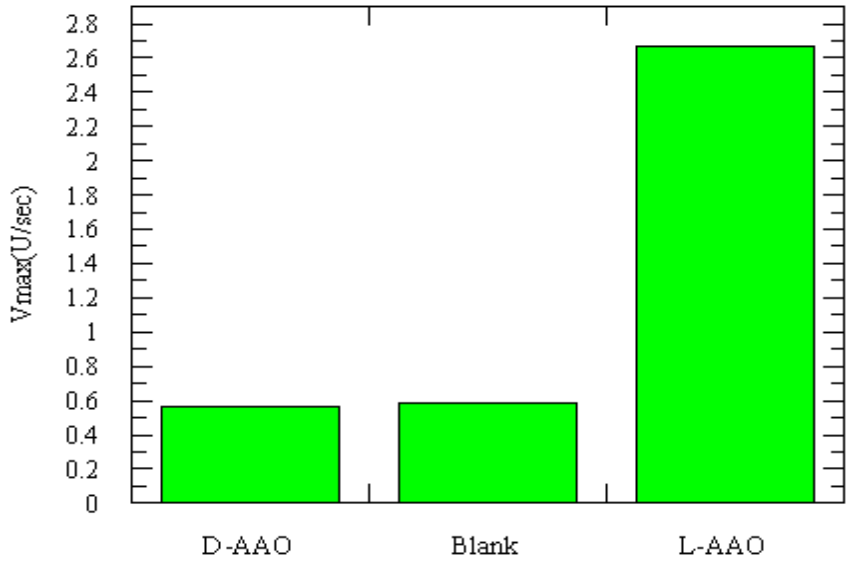


Figure 3.13 : The enantioselectivity of the best mutant PAL.

3.3.6 The best mutant PAL pH profile

The results show that PAL from *Rhodotorula graminis* has an optimum pH of 10 and is performed at 30 °C, the temperature optimum for *R. graminis* growth for the amination reaction, with a tolerance of 0.5 pH units over which the enzyme retains >90 % of its activity (Fig.3.14).

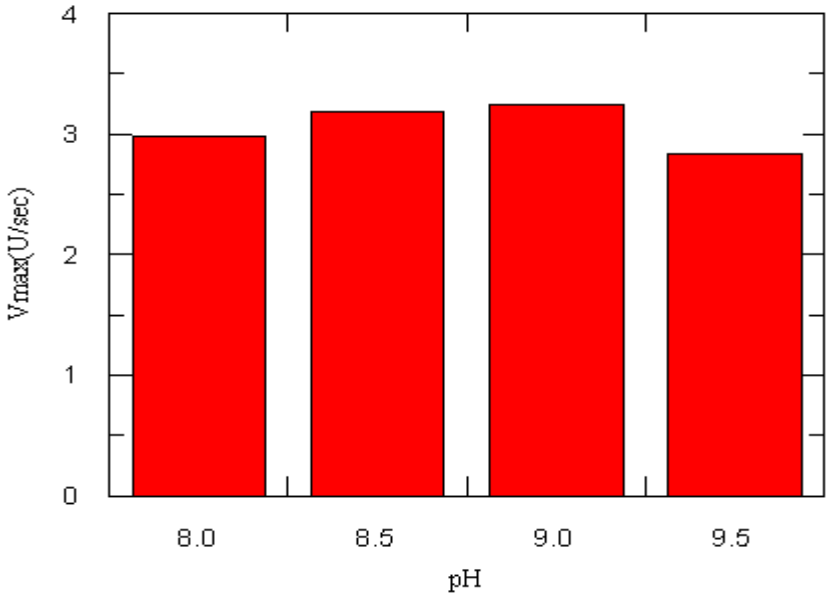


Figure 3.14 : The reaction catalysed by the best mutant PAL at the pH 8, 8.5, 9, 9.5.

3.3.7 Kinetics of the PAL variants

The liquid phase assay was used to determine the kinetics of the PAL synthesis reaction. To this end, a range of substrate concentrations from 12.5 μM to 1000 μM were assayed and the activity of the enzyme at each concentration measured. The kinetic parameters of the reaction were then determined with the direct Linear method [111].

Fixing the concentration of ammonium sulphate at 100 mM the apparent kinetic constants K_M and k_{cat} were calculated for *trans*-cinnamic acid. The concentration of ammonia was kept at 100 mM so that only the influence of *trans*-cinnamic acid on the kinetics of the reaction is measured (Table 3.5).

In each nmol of H_2O_2 evolved, there is a decrease in the fluorescence by 1414.1 RFU. As can be seen in Fig.3.15, this compound is fluorescent and sensitive in nmol level for activity. These results provide a high sensitive detecting kinetic assay for PAL enzyme. Kinetic parameters were calculated based on assay 2 in Fig.3.4.

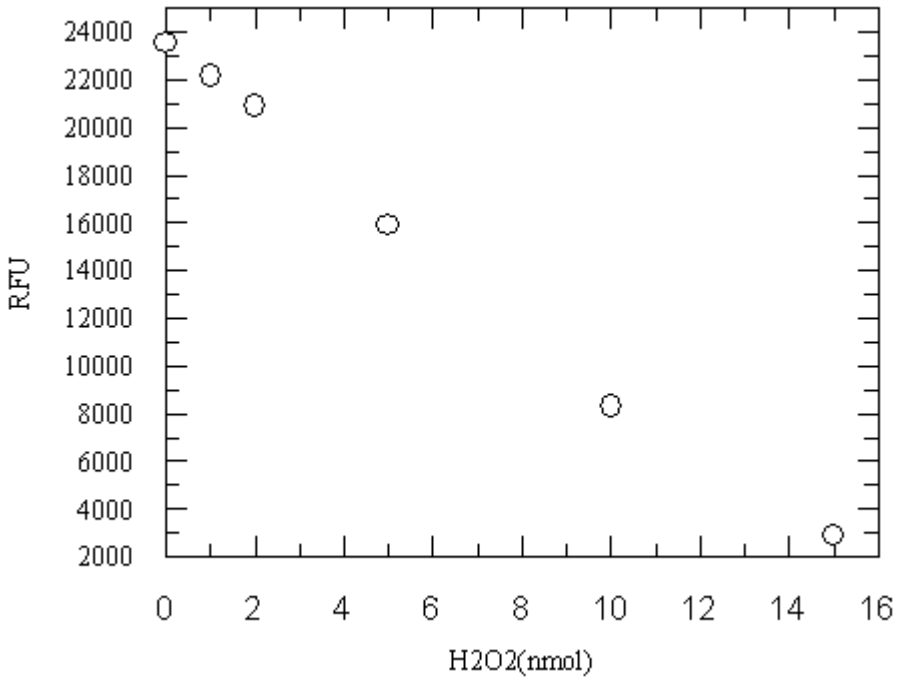


Figure 3.15 : The standard curve of scopoletin fluorescence in the presence of hydrogen peroxide.

Table 3.6 : Summary of the steady state kinetic constants of the PAL variants against 4- Br-CA.

	4-Br-CA		
PAL Variant	**k_{cat} (min^{-1})**	**K_M (μM)**	**k_{cat}/K_M ($min^{-1}\mu M^{-1}$)**
wt	4.24 ± 0.57	80.15 ± 16.00	0.0529 ± 0.0127
29B	81.99 ± 2.69	4.53 ± 0.27	18.10 ± 1.85
31E	289.48 ± 16.11	33.66 ± 2.89	8.60 ± 0.62
16D	53.09 ± 0.38	6.24 ± 0.44	8.51 ± 0.58
25E	43.78 ± 0.62	18.85 ± 0.68	2.32 ± 0.09

It is well known that PAL catalyses the reversible non-oxidative deamination of phenylalanine to *trans*-cinnamic acid and ammonia. The reversibility of this reaction has been used to synthesise phenylalanine economically at multi-ton scale [112]. Analogues of L-phenylalanine are incorporated as pharmacophores in several peptidomimetic drug molecules and are therefore of particular interest to the fine chemical industry.

Ile479, Lys475 and His145 are in the contact place of monomers. Asn278 hydrogen bonds with the developing enolate oxygen of MIO increase the electro positivity of the MIO methyldiene group. His143 is responsible for the a*bs*traction of the pro-S hydrogen from C3 of substrate. Development and stabilisation of carbonion are provided by the phenyl ring of the substrate whose electrons are polarized away from carbonion site from interaction Leu223, Leu274 and Val277. The NH_2 adduct is stabilised by sharing an H bond with OD1 of Asn270 and by interaction with phenyl ring of Phe420. Breakdown of NH_3 and MIO are prompted by donation of a proton from the OH group of Tyr371 to the NH_2 group. Tyr371 is stabilised by Arg374. So Tyr371, Asn278, His145, Glu503, Arg374 are main candidate residues.

Using NRT coding Arg, Asn, Asp, Cys, Gly, His, Ser, Tyr as a degenerative codon means that obtaining about 500 mutant colonies after transformation is enough to cover all possibilities of amino acids encoded by the degenerative NRT codon. Therefore, by choosing the NRT codon as the degenerate codon, the number of colonies required to be screened by only incorporating amino acids that provide side chains that are involved in the majority of catalysis decreased.

Without a study based on molecular mechanics/quantum mechanics (MM/QM), explaining the result is difficult. However, molecular modeling studies based on standard molecular dynamics simulations and the results of published papers can help the molecular basis of the recognizing substrate. The results show that a good diversity was obtained for library. In this diversity, for the much more activity, one of the candidate amino acid of library 1 should have small side chain and another should be polar. However, position of both the polar and small side chain amino acid is crucial. For library 4; placing Asp and His does not make any charge differences. In spite of insertion, primer having same mutant for library 4 of the mutant; having correct the open reading frame (ORF) and more activity than native as well.

The mechanism of the reaction of PAL that is identify the dehydroalanine at the active site of the enzyme reacts with the amino group of the substrate, generating a positive charge, thus increasing the leaving ability of the ammonium group of histidine [113]. The prosthetic electrophile was discovered through experiments where the activity of the enzyme was inhibited by strong nucleophiles. The use of radiolabelled inhibitors led to the conclusion that the prosthetic electrophile is dehydroalanine. This mechanism is not able to exactly define the reaction catalysed by PAL because it does not account for the abstraction of the non-acidic β proton of L-phenylalanine by an enzymatic base. Despite this deficiency in the mechanism, it has been widely accepted for PAL for many years.

X-ray crystal structure modeling studies proposed an alternative that ascertain the precursor of the prosthetic dehydroalanine, which led to the postulation of an alternative mechanism for the activity of the ammonia-lyase enzymes PAL [114]. The dehydroalanine was originally thought to arise from the dehydration of a Ser residue, so mutation experiments were performed that is Ser202Ala in PAL from *Petroselinum crispum* by site-directed mutagenesis. This change resulted in a

reduction in enzyme activity by more than a factor of 1000 [115] that supporting the crucial role of Ser residue as precursors of the dehydroalanine residue for catalytic activity.

Then a new mechanism of PAL was proposed that is a Friedel-Crafts type attack of the imidazole ring by the prosthetic electrophile [115]. It has been observed that recombinant PAL from *Petroselinum crispum* reacts faster with m-tyrosine than its natural substrate; this suggested that the mechanism of PAL was a Friedel-Crafts type reaction [116].

Finally, the evidence of X-ray structure for a member of lyase family histidine ammonia lyase (HAL) provided that the prosthetic electrophile was not dehydroalanine but the Michael acceptor MIO [112]. The prosthetic electrophile can be regarded as a modified dehydroalanine residue formed by the post-translational cyclisation of the inner tripeptide Ala142-Ser143-Gly144, during which two water molecules are eliminated [112]. MIO is much more electrophilic than dehydroalanine making the Friedel-Crafts attack at the aromatic ring of phenylalanine feasible [117].

The proposed mechanism of action of PAL is a Friedel-Crafts type attack of the phenyl moiety by the prosthetic electrophile MIO (Fig.3.17-18) [118].

The recent crystal structures of the same family of PAL enzyme have provided further evidence for the elimination type mechanism for the MIO containing enzymes [119-120].

A paper has showed the existence of a glutamic acid in PAL enzyme family leads to the initial binding of the substrate in the active site and indicates that the Friedel-Crafts type attack is favoured [121].

All of our studies have focused on the active site pocket, the investigation regarding the enantioselectivity, pH and the kinetic resolution. In this study, it was demonstrated that due to directed evolution both the preferred enantioselectivity and high kinetic rates for industrial application of mutant enzyme were obtained.

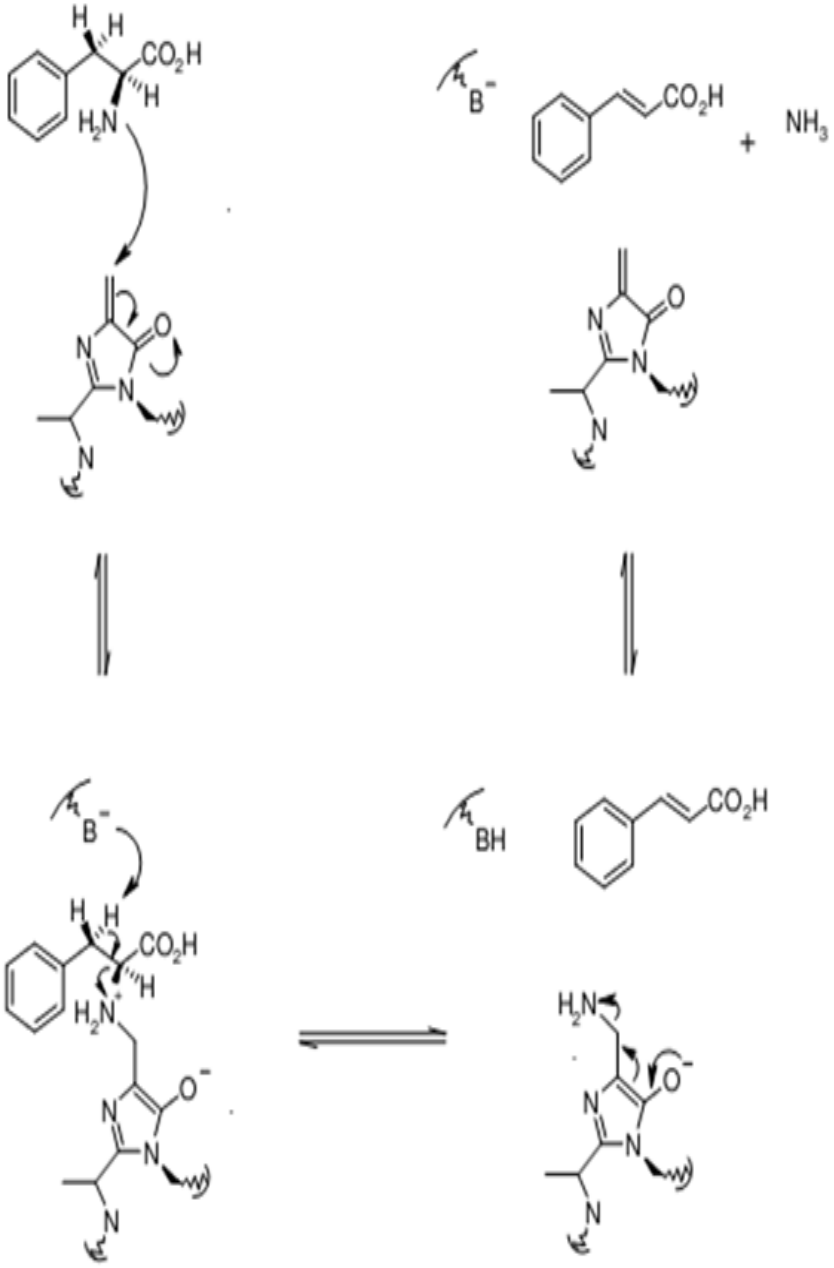


Figure 3.16 : The elimination mechanism of PAL[118].

The theoretical analysis for ammonia lyase family enzymes based on QM/MM studies pinpointed one of the crucial mutations as being His145 (146)/Ser (Cys), which is next to the binding pocket and close to the MIO. It became clear that the mutational change His145(146)/Ser(Cys) causes, among other things, theenlargement of the binding pocket to accommodate the brom at the aromatic ring of 4-Br-CA, that is, to tolerate branching. This result is relevant to the present analysis.

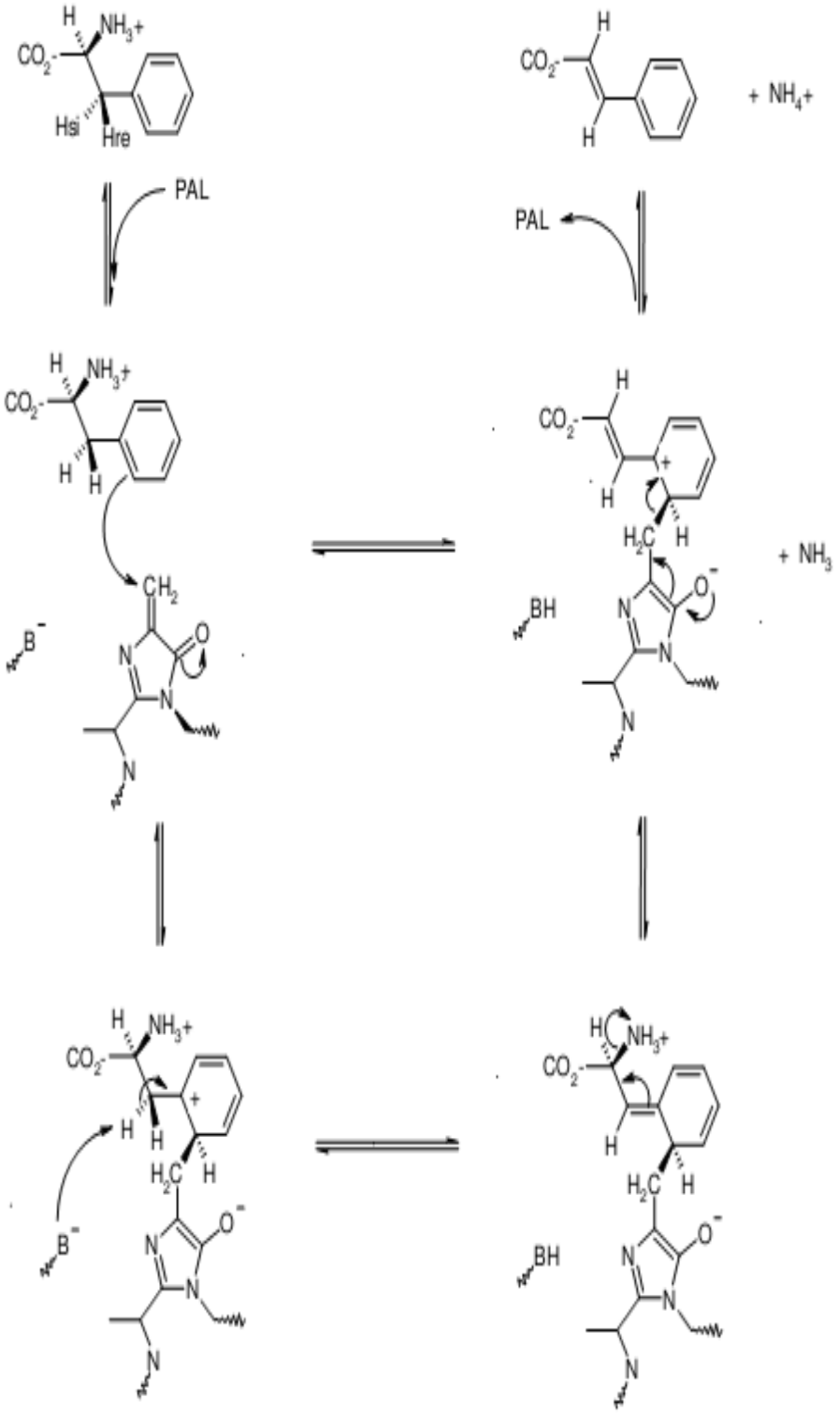


Figure 3.17 : The Friedal-Crafts type mechanism of PAL[118].

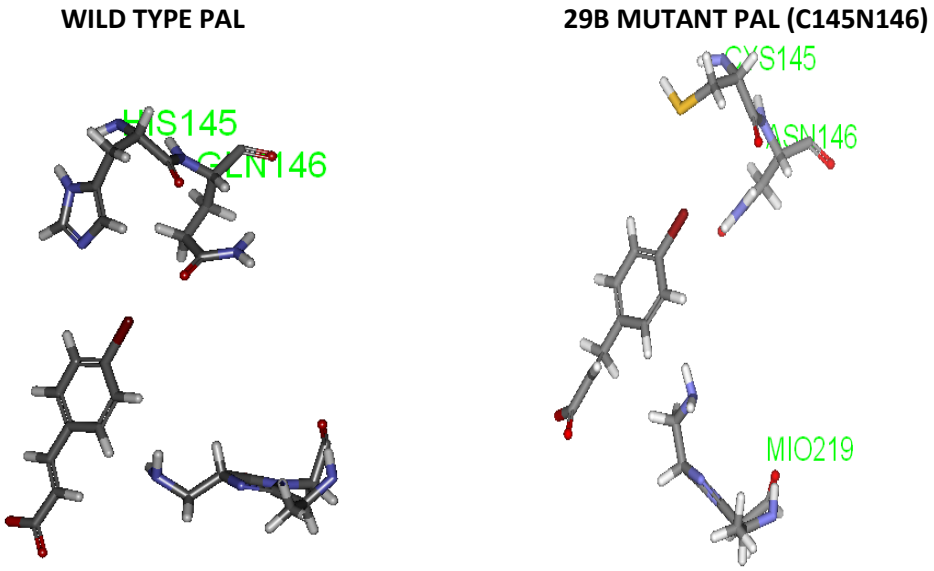


Figure 3.18 : The modeling of the wild type and best mutant PAL with 4-Br-CA.

The mutational changes in 29B, namely His145Asn and Glu146Cys, can be interpreted as follows. The side chain of Cys is sterically smaller than that of the original Gln, thereby providing more space for binding bulky (branched) substrates. Histidine has a positively charged side chain not making easy the Friedel-Crafts type attack. As mentioned above, electorophilic MIO cannot eliminate in the existence of positively charged residue (His145). So replacing the His with Asp or any uncharged amino acids (Ser, Cys) makes fast the elimination of MIO meaning increasing kinetic rate for PAL variants.

REFERENCES

[1] **Moorehead, A**. (1969). *Darwin and the Beagle*, Harper&Row Publisher, New York, USA.

[2] **Ridley, M.** (1996). *The Origin of Virtue*, Penguin Books, London, UK.

[3] **Kaur, J., Sharma, R**. (2006). Directed evolution: an approach to engineer enzymes. *Crit. Rev. Biotechnol.*, ***26***, 165–199.

[4] **Schoemaker, H. E., Mink, D., Wubbolts, M. G.** (2003). Dispelling the myths: biocatalysis in industrial synthesis. *Science*, ***299***, 1694–1697.

[5**] Rubin-Pitel, S. B., Zhao, H**. (2006). Recent advances in biocatalysis by directed enzyme evolution. *Comb. Chem. High Throughput Screen*, ***9***, 247–25.

[6] **Bornscheuer, U. T.** (2005). Trends and challenges in enzyme technology. *Adv. Biochem Engin Biotechnol.*, ***100***, 181–203.

[7] **Dalby, P. A.** (2003). Optimising enzyme function by directed evolution. *Curr. Opin. Struc. Biol.*, ***13***, 500–505.

[8] **Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M., Witholt, B.** (2001). Industrial biocatalysis today and tomorrow. *Nature*, *409*, 258-68.

[9] **Kazlauskas, R. J., Bornscheuer, U. T.** (2009). Finding better protein engineering strategies, *Nature Chemical Biology*, ***5***, 526-529.

[10] **Saul, D. J., Moreland, D. G., Peter L. Bergquist, P. L.** (2009) Biocatalysis:industrial enzymes and the exploitation of microorganisms, XII-Biotech Biocatalysis.

[11] **Burton, S. G., Cowan, D. A., Woddley. J. M.** (2002). The search for the idealbiocatalyst, *Nature Biotechnology*, ***20***, 37-45.

[12] **Adlercreutz, P.** (1991). On the importance of the support material for enzymatic synthesis in organic media-support effects at controlled water activity. *Eur. J. Biochem*., ***199***, 609-614.

[13] **Altamirano, M. M., Blackburn, J. M., Aguayo, C., Fersht, A. R.** (2000). Directed evolution of a new catalytic activity using alpha/beta barrel scaffold. *Nature*, ***403***, 617-622.

[14] **May, O., Nguyen, P. T., Arnold, F. H.** (2000). Inverting enantioselectivity and increasing total activity of a key enzyme in a multi-enzyme synthesis creates a viable process for production of L-methionine. *Nat. Biotechnol.*, ***18***, 317-320.

[15] **Kumamaru, T., Suenga, H., Watanabe, T., Furukawa, K. (1998). Enhanced degradation** of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. *Nat. Biotechnol.****16***, 663-666.

[16] **Hough, D., Danson, M. J.** (1999). Extremozymes. *Curr. Opin. Chem. Biol.*, ***3***, 36-39.

[17] **Daniel, R. M., Cowan, D. A.** (2000). Biomolecular stability and life at high temperatures. *Cell. Mol. Life. Sci.*, ***57***, 250-264.

[18] **Jaenicke, R., Bohm, G.** (1998). The stability of proteins in extreme environments. *Curr. Opin. Struct. Biol.*, ***8***, 738-748.

[19] **Gray, K. A., Zhao, L., Emptage, M.** (2006). Bioethanol. *Curr. Opin. Chem. Biol.*, ***10***, 141-146.

[20] **Estell, D. A., Graycer, T. P., Wells, J. A.** (1985). Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *J. Biol. Chem.*, ***260***, 6518-6521.

[21] **McCarthy, A. A.** (2003). Metaphore Pharmaceuticals: mimicking nature's enzyme. *Chem. Biol.*, ***10***, 893-894.

[22] **Morley, K. L., Kazlauskas, R. J.** (2005). Improving enzyme properties: when are closer mutations better? *Trends Biotechnol.*, ***23***, 231-237.

[23] **Neylon, C.** (2004). Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for directed evolution. *Nucleic Acids Res.*, ***32***, 1448-1459.

[24] **Carr, R., Alexeeva, M., Dawson, M. J., Gotor-Fernández, V., Humphrey, C. E., Turner, N. J.** (2005). Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines. *ChemBioChem*, ***6***, 637—639.

[25] **DeSantis, G., Wong, K., Farwell, B., Chatman, K., Zhu, Z., Tomlinson, G., Huang, H., Tan, X., Bib*bs*, L., Chen, P., Kretz, K., Burk, M. J.** (2003). Creation of a productive, highly enantioselective nitrilase through gene site saturation mutagenesis. *J. Am. Chem. Soc.*, ***125***, 11476-77.

[26] **Bloom, J. D., Labthavikul, S. T., Otey, C. R., Arnold, F. H.** (2006). Protein stability promotes evolvability. *Proc. Natl. Acad. Sci., USA*, ***103***, 5869-5874.

[27] **Weinreich, D. M., Delaney, N. F., DePristo, M. A., Hartl, D. L.** (2006). Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science*, ***312***, 111-114.

[28] **Garrett, J. B., Kretz, K. A., O'Donoghue, E., Kerovuo, J., Kim, W., Barton, N. R., Hazlewood, G. P., Short, J. M., Robertson, D. E., Gray, K. A.,** (2004). Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. *Appl. Environ. Microbiol.*, ***70***, 3041-6.

[29] **Whittle, E., Shanklin, J.** (2001). Engineering delta 9-16:0-acyl carrier protein (ACP) desaturase specificity based on combinatorial saturation mutagenesis and logical redesign of the castor delta 9-18:0-ACP desaturase. *J. Biol. Chem.*, ***276***, 21500-5.

[30] **Bloom, J. D., Romero, P. A., Lu, Z., Arnold, F. H.** (2007). Neutral genetic drift can alter promiscuous protein functions, potentially aiding functional evolution. *Biol. Direct.*, ***2***, 17.

[31] **Gupta, R. D., Tawfik, D. S.** (2008). Directed enzyme evolution via small and effective neutral drift libraries. *Nat. Methods*, ***5***, 939-942.

[32] **Chen, H., Börjesson, U., Engkvist, O., Kogej, T., Svensson, M. A., Blomberg, N., Weigelt, D., Burrows, J. N., Lange, T.** (2009). ProSAR: a new methodology for combinatorial library design. *J. Chem. Inf. Model, 49*, 603-14.

[33**] Fox, R., Roy, A., Govindarajan, S., Minshull, J., Gustafsson, C., Jones, J. T., Emig, R.** (2003). Optimizing the search algorithm for protein engineering by directed evolution. Protein *Eng.*, ***16***, 589-97.

[34] **Fox, R. J., Davis, S. C., Mundorff, E. C., Newman, L. M., Gavrilovic. V., Ma, S. K., Chung, L. M., Ching, C., Tam, S., Muley, S., Grate, J., Gruber, J., Whitman, J. C., Sheldon, R. A., Huisman, G. W.** (2007). Improving catalytic function by ProSAR-driven enzyme evolution. *Nat. Biotechnol.*, ***25***, 338-44.

[35] **Reetz, M. T., Bocola, M., Carballeira, J. D., Zha, D., Vogel, A.** (2005). Expanding the range of substrate acceptance of enzymes: combinatorial active-site saturation test. *Angew Chem Int Ed Engl.*, ***44***, 4192-6.

[36] **Reetz, M. T., Wang, L. W.** (2006). High-throughput selection system for assessing the activity of epoxide hydrolases. *Comb. Chem. High Throughput Screen.*, ***9***, 295-9.

[37] **Reetz, M. T., Carballeira, J. D., Vogel, A.** (2006). Iterative saturation mutagenesis on the basis of B factors as a strategy for increasing protein thermostability. *Angew. Chem. Int. Ed. Engl.*, ***45***, 7745-51.

[38] **Westbrook, J., Feng, Z., Chen, L., Yang, H., Berman, H. M.** (2003). The Protein Data Bank and structural genomics. *Nucleic Acids Res.*, ***31***, 489–491.

[39] **Tramontano, A., Leplae, R., Morea, V.** (2001). Analysis and assessment of comparative modeling predictions in CASP4. *Proteins*, ***45***, 22–38.

[40] **Marti-Renom, M. A., Stuart, A. C., Fiser, A., Sanchez, R., Melo, F., Sali, A.** (2000) Comparative protein structure modeling of genes and genomes. *Annu. Rev. Biophys. Biomol. Struct.*, ***29***, 291–325.

[41] **Brenner, S. E.** (2001). A tour of structural genomics. *Nature Rev. Genet.*, ***2***, 801–809.

[42] **Vitkup, D., Melamud, E., Moult, J., Sander, C.** (2001). Completeness in structural genomics. *Nature Struct. Biol.*, ***8***, 559–566.

[43] **Duan, Y., Wu, C., Chowdhury, S., Lee, M. C., Xiong, G., Zhang, W., Yang, R., Cieplak, P., Luo, R., Lee, T., Caldwell, J., Wang, J., Kollman, P.** (2003). A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *J. Comput. Chem.*, ***24***, 1999-2012.

[44] **Fujitsuka, Y., Takada, S., Luthey-Schulten, Z. A., Wolynes, P. G**. (2004). Optimizing physical energy functions for protein folding. *Proteins*, ***54***, 88-103.

[45] **Holbrook, J. J., Liljas, A., Steindal, S. J., Rossman, M. G.** (1975). *The Enzymes* In P.D. Boyer (Edt.), vol.11, pp. 191-292, Academic Press, New York.

[46] **Barstow, D. A., Clarke, A. R, Chia W. N.** (1986). Cloning, expression and complete nucleotide sequence of the *Bacillus stearothermophilus*. *Gene*, ***46***, 47–55.

[47] **Hart, K. W., Clarke, A. R., Wigley, D. B., Chia, W.N., Barstow, D. A., Atkinson, T, Holbrook, J. J.,** (1987). The importance of arginine 171 in substrate binding *Bacillus stearothermophilus* lactate dehydrogenase. *Biochem. Biophys. Res. Commun.*, ***146***, 346-53.

[48] **Clarke, A. R., Wigley, D. B., Barstow, D. A., Chia, W. N., Waldman, A. D. B., Hart, K. W., Atkinson, T., Holbrook, J. J.** (1987). Site-directed mutagenesis of *Bacillus stearothermophilus* lactate dehydrogenase. *Biochem. Soc. Transac.*, ***15***,152-3.

[49] **Luyten, M. A., Gold, M., Friesen, J. D., Jones, J.B.** (1989). On the effects of replacing the carboxylate-binding arginine-171 by hydrophobic tyrosine or tryptophan residues in the L-lactate dehydrogenase from *Bacillus stearothermophilus*. *Biochemistry*, **28**, 6605-10.

[50] **Clarke, A. R., Smith, C. J., Hart, K. W., Wilks, H. M., Lee, T. V., Chia, W. N., Birktoft, T.V., Banaszak, J. J., Barstow, L. J., Atkinson, D. A., Holbrook, J. J.** (1987). Rational construction of a 2-hydroxy acid dehydrogenase with new substrate specificity, *Biochemical and Biophysical Research Communications*, ***148***, 15-23.

[51] **Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R. & Holbrook, J. J.** (1988). A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework. *Science*, ***242***, 1541-1544.

[52] **Clarke, A. R., Holbrook, J. J.** (1989). The rational construction of new enzymes: design and synthesis of a dehydrogenase which accepts an unnatural substrate. *Sci. Prog.*, ***73***,117-31.

[53] **Wilks, H. M., Halsall, D. J., Atkinson, T., Chia, W. N., Barstow, D. A., Clarke, A. R., Holbrook, J. J.** (1990). Designs for a broad substrate specificity keto acid dehydrogenase. *Biochemistry*, ***29***, 8587–8591.

[54] **Dunn, C. R., Wilks, H. M., Halsall, D. J., Atkinson, T., Clarke A. R., Muirhead, H., Holbrook, J. J.** (1991). Design and synthesis of new enzymes on the lactate dehydrogenase framework. *Philosophical Transactions: Biological Sciences*, ***332***, 177-184.

[55] **Wilks, H. M., Moreton, K. M., Halsall, D. J., Hart, K. W., Sessions, R. B., Clarke, A. R., Holbrook, J. J.** (1992). Designs of a phenyllactate dehydrogenase by peptide loop exchange on the *Bacillus stearothermophilus* lactate dehydrogenase framework. *Biochemistry*, ***31***, 7802–7806.

[56] **Sakowicz, R., Kallwass, H. K., Parris, W., Kay, C. M., Jones, J. B., Gold, M.** (1993). Threonine 246 at the active site of the L-lactate dehydrogenase of *Bacillus stearothermophilus* is important for catalysis but not for substrate binding. *Biochemistry, **32***, 12730-5.

[57] **Nicholls, D. J., Davey, M., Jones, S. E., Miller, J., Holbrook, J. J., Clarke, A. R., Scawen, M.D., Atkinson ,T., Goward, C.R.** (1994). Substitution of the amino acid at position 102 with polar and aromatic residues influences substrate specificity of lactate dehydrogenase. *J. Protein Chem., **13***, 129-33.

[58] **Hogan, J. K., Pittol, C. A., Jones, J. B., Gold, M.** (1995). Improved specificity toward substrates with positively charged side chains by site-directed mutagenesis of the L-lactate dehydrogenase of *Bacillus stearothermophilus. Biochemistry, **34***, 4225-30.

[59] **El Hawrani, A. S., Sessions, R. B., Moreton, K. M., Holbrook, J. J.** (1996). Guided evolution of enzymes with news substrate specificities. *J. Mol. Biol., **264***, 97-110.

[60] **Allen, S. J., Holbrook, J. J.** (2000). Production of an activated form of *Bacillus stearothermophilus* L-2-hydroxyacid dehydrogenase by directed evolution. *Protein Eng., **13***, 5-7.

[61] **Karagüler, N. G., Sessions, R. B., Holbrook, J. J.** (2001). Role of glutamate–52 in the mechanism of L-lactate dehydrogenase from *Bacillus stearothermophilus*. *Biotechnology Letters, **23***, 395 - 399.

[62] **Sinclair, R., Reid, G. A., Chapman, S. K.** (1998). Re-design of *Saccharomyces cerevisiae* flavocytochrome b2: introduction of L-mandelate dehydrogenase activity. *Biochem. J., **333***, 117-120.

[63] **Qiagen**, *A handbook for high-level expression and purification of 6xHis tagged proteins*, Qiagen, 2008, USA.

[64] **Camm, E. L., Towers, G. H. N.** (1973). Phenylalanine ammonia lyase. *Phytochemistry, **12***, 961-973.

[65] **Shaw, N. M, Bolwell, G. P, Smith, C.,** (1990). Wound-induced phenylalanine ammonia-lyase in potato (*Solanum tuberosum*) tuber discs. Significance of glycosylation and immunolocalization of enzyme subunits. *Biochem J., **267***, 163-70.

[66] **Calabrese, J. C., Jordan, D. B., Boodhoo, A., Sariaslani, S., Vannelli, T.** (2004). Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. *Biochemistry, **43***, 11403-11416.

[67] **Abell, C. W, Shen, R. S.** (1987). Phenylalanine Ammonia-Lyase from the Yeast *Rhodotorula-Glutinis*. *Methods in Enzymology, **142***, 242-248.

[68] **Langer, B., Rother, D., Retey, J.** (1987). Identification of essential amino acids in phenylalanine ammonia-lyase by site-directed mutagenesis. *Biochemistry, **36***, 10867-10871.

[69] **Röther, D., Poppe, L., Morlock, G., Viergutz, S., Rétey, J**. (2002). An active site homology model of phenylalanine ammonia-lyase from *Petroselinum crispum*. *Eur. J. Biochem., **269***, 3065–3075.

[70] **Ambrus, C. M., Ambrus, J. L., Horvath, C., Pedersen, H., Sharma, S., Kant, C., Mirand, E., Guthrie, R., Paul, T.** (1978). Phenylalanine depletion for the management of phenylketonuria: use of enzyme reactors with immobilized enzymes. *Science, **4358**,* 837-9.

[71] **Evans, C. T., Choma, C., Peterson, W., Misawa, M.** (1987). Bioconversion of trans-cinnamic acid to L-phenylalanine in an immobilized whole cell reactor. *Biotechnol Bioeng., **30**,* 1067-72.

[72] **D'Cunha, G. B., Satyanarayan, V., Nair, P. M.** (1996). Purification of phenylalanine ammonia lyase from *Rhodotorula glutinis. Phytochemistry, **42**,* 17-20.

[73] **Evans, C. T, Conrad, D., Hanna, K., Peterson, W., Choma, C., Misawa, M.** (1987). Novel stabilization of phenylalanine ammonia lyase catalyst during bioconversion of *trans*-cinnamic acid to l-phenylalanine. *Appl. Microbiol. Biotechnol., **25**,* 399-405.

[74] **Orndorff, S. A, Constantino, N., Stewart, D., Durham, D. R.** (1988). Strain improvement of *Rhodotorula graminis* for production of a novel l-phenylalanine ammonia lyase. *Appl. Environ. Microbiol., **54**,* 996-l002.

[75] **Fritz, R. R., Hodgins, D. S., Abell, C. W.** (1976). Phenylalanine ammonia lyase: induction and purification from yeast and clearance in mammals. *J. Biol. Chem., **251**,* 4646-4650.

[76] **Yamada, S., Nabe, K., Izuo, N., Nakamichi, K., Chibata, I.** (1981). Production of L- phenylalanine from *trans*-cinnamic acid with *R. glutinis* containing l-phenylalanine ammonia lyase activity. *Appl. Environ. Microbiol., **42**,* 773-778.

[78] **D'Cunha, G. B., Satyanarayan, V., Nair, P. M.** (1994). Novel direct synthesis of l-phenylalanine methyl ester by using *Rhodororula glutinis* phenylalanine ammonia lyase in an organic-aqueous biphasic system. *Enzyme Microb. Technol., **16**,* 318-322.

[79] **Ogata, K., Uchiyama, K., Yamada, H.** (1967). Metabolism of aromatic amino acid in microorganisms. Part I: formation of cinnamic acid from phenylalanine. *Agric. Biol. Chem., **31**,* 200-206.

[80] **Hodgins, D. S.** (1971). Yeast phenylalanine ammonia lyase. Purification, properties, and the identification of catalytically essential dehydroalanine. *J. Biol. Chem., **246**,* 2977-2985.

[81] **Calabrese, J. C., Jordan, D. B., Boodhoo, A., Sariaslani, S., Vannelli, T.** (2004). Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. *Biochemistry, **43**,* 11403-11416.

[82] **Kyndt, J. A., Meyer, T. E., Cusanovich, M. A., Van Beeumen, J. J.** (2002). Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein. *FEBS Lett., **512**,* 240-244.

[83] **Liu, W.** (1999). Synthesis of optically active phenylalanine analogues using *Rhodotorula graminis*. *US Patent,* No: 5, 981,239.

[84] **Evans, C. T., Hanna, K., Payne, C., Conrad, D., Misawa, M.** (1986). Biotransformation of *trans*-cinnamic acid to l-phenylalanine: optimization of reaction conditions using whole yeast cells. *Enzyme Microb. Technol.*, ***9***, 417-421.

[85] **Brosius, J., Holy, A.** (1984). Regulation of ribosomal RNA promoters with a synthetic lac operator. *Proc. Natl. Acad. Sci.*, ***81***, 6929–6933.

[86] S**temmer, W. P. C.** (1994). Rapid evolution of a protein in vitro by DNA shuffling, *Nature*, ***370***, 389–391.

[87] **Zhao, H., Arnold, H. F.** (1997). Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res.*, ***25***, 1307–1308.

[88**] Invitrogen,** *Gene Tailor™ Site-Directed Mutagenesis System*, USA.

[89] **InsightII,** *Molecular Modeling Software*, 2000, Accelrys, Inc., San Diego, USA.

[90] **Binay, B., Karagüler, N. G.** (2007). Attempting to remove the substrate inhibition of l-lactate dehydrogenase from *Bacillus stearothermophilus* by site directed mutagenesis. *Appl. Biochem. Biotechnol.*, ***141***, 265–272.

[91] **Sambrook, J., Fritsch, E. E., Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York, USA.

[92] **Bradford, M. M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, ***72***, 248-254.

[93] **Leaterbarrow, R. J.** (2001). GraFit version 5. Erytacus Software Ltd, Staines, UK.

[94] **Rowles, I.** (2009). *Development of Novel Ammonia-Lyase Biocatalysts for the Production of Unnatural Amino Acids for Industry.* (P.hD. thesis) University of Manchester, Manchester, UK.

[95] **Stratagene**, *QuikChange® Site-Directed Mutagenesis Kit* Catalog# 20518.

[96] **Kuchner, O., Arnold, F. H.** (1997). Directed evolution of enzyme catalysts. *TrendsBiotechnol.*, ***15***, 523–530.

[97] **Chen, R., Greer, A., Dean, A. M.** (1996). Redesigning secondary structure to invert coenzyme specificity in isopropylmalate dehydrogenase. *Proc. Natl. Acad. Sci.*, ***93***, 12171–12176.

[98] **Getzoff, E. D., Cabelli, D. E., Fisher, C. L., Parge, H. E., Viezzoli, M. S., Banci, L., Hallewell, R. A.** (1992). Faster superoxide-dismutase mutants designed by enhancing electrostatic guidance. *Nature*, ***358***, 347–351.

[99] **Zhang, J. H., Dawes, G., Stemmer, W. P. C.** (1997). Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening. *Proc. Natl. Acad. Sci.*, ***94***, 4504–4509.

[100] **Garvie, E. I.** (1980). Bacterial lactate dehydrogenase. *Microbiol. Rev.*, ***44***, 106–139.

[101] **Shoemark, D. K., Cliff, M. J., Sessions, R. B., Clarke, A. R.** (2007). Enzymatic properties of the lactate dehydrogenase enzyme from *Plasmodium falciparum*. *FEBS J.*, ***274***, 2738–2748.

[102] **Chapman, A. D. M., Cortés, A., Dafforn, T. R., Clarke, A. R., Brady, R. L.** (1999). Structural basis of substrate specificity in malate dehydrogenases: crystal structure of a ternary complex of porcine cytoplasmic malate dehydrogenase, α-ketomalonate and tetrahydo NAD. *J. Mol. Biol.*, ***285***, 703–712.

[103] **Cameron, A. D., Roper, D. I., Moreton, K. M., Muirhead, H., Holbrook, J. J., Wigley, D. B.** (1994). Allosteric activation in Bacillus stearothermophilus lactate dehydrogenase investigated by an X-ray crystallographic analysis of a mutant designed to prevent tetramerization of the enzyme. *J. Mol. Biol.*, ***238***, 615–625.

[104] **Binay, B., Shoemark, D. K., Sessions, R. B., Clarke, A. R., Karaguler, N. G**. (2009). Increasing the substrate specificity of *Bacillus stearothermophillus* lactate dehydrogenase by DNA shuffling. *Biochemical Engineering Journal*, ***48***, 118-123.

[105] **Schar, H. P., Zuber, H., Rossmann, M. G.** (1982). Crystallization of lactate dehydrogenase from *Bacillus stearothermophilus*. *J. Mol. Biol.*, ***154***, 349-353.

[106] **Parker, D. M., Holbrook, J. J.,** (1977). *Pyridine nucleotide-dependent dehdrogenases*. In Sund H. Walter, (Edt.), de Gruyter and Co., New York.

[107] **Clarke, A. R., Wigley, D. B., Chia, W. N., Barstow, D. A., Atkinson, T., Holbrook, J. J.** (1986). Site-directed mutagenesis reveals role of mobile arginine residue in lactate dehydrogenase catalysis. *Nature*, ***324***, 669-702.

[108] **Bur, D., Clarke, A. T., Friesen, J. D., Gold, M., Hart, K. W., Holbrook, J. J., Jones, J. B., Luyten, M. A., Wilks, H.** M. (1989). On the effect on specificity of Thr246----Gly mutation in L-lactate dehydrogenase of *Bacillus sterothermophilus*. *Biochem. Biophys. Res. Commun.*, ***161***, 59-63.

[109] **Grau, U. M., Trommer, W. E., Rossmann, M. G**. (1981). Structure of the active ternary complex of pig heart lactate dehydrogenase with S-lac-NAD at 2.7 A resolution. *J. Mol. Biol.*, ***151***, 289-307.

[110] **Eisenthal, R., Cornish-Bowden, A.** (1974). Direct Linear Plot-New Graphical Procedure for Estimating Enzyme Kinetic-Parameters. *Biochemical Journal*, ***139***, 715-720.

[111] **Orndorff, S. A., Durham, D. R.** (1988). Isolation of phenylalanine ammonia-lyase producing *Rhodotorula* graminis mutant strain for use in phenylalanine manufacture. *US Patent*, No: 4,757,015.

[112] **Schwede, T. F., Retey, J., Schulz, G. E.** (1999). Crystal structure of histidine ammonialyase revealing a novel polypeptide modification as the catalytic electrophile. *Biochemistry*, ***38***, 5355-5361.

[113] **Smith, T. A., Cordelle, F. H., Abeles, R. H.** (1967). Inactivation of histidine deaminase by carbonyl reagents. *Archives of Biochemistry and Biophysics*, **120**, 724-725.

[114] **Schuster, B., Retey, J.** (1994). Serine-202 Is the Putative Precursor of the Active-Site Dehydroalanine of Phenylalanine Ammonia-Lyase - Site-Directed Mutagenesis Studies on the Enzyme from Parsley (*Petroselinum-Crispum L*). *Febs Letters*, ***349***, 252-254.

[115] **Klee, C. B., Kirk, K. L., Cohen, L. A.** (1979). 4-Nitro-L-Histidine as a Substrate for Histidine Ammonia-Lyase-Role of Beta-Hydrogen Acidity in the Rate-Limiting Step. *Biochemical and Biophysical Research Communications*, ***87***, 343-348.

[116] **Schuster, B., Retey, J.** (1995). The Mechanism of Action of Phenylalanine Ammonia- Lyase - the Role of Prosthetic Dehydroalanine. *Proceedings of the National Academy of Sciences of the United States of America*, ***92***, 8433-8437.

[117] **Retey, J.** (2003). Discovery and role of methylidene imidazolone, a highly electrophilic prosthetic group. *Biochimica Et. Biophysica Acta-Proteins and Proteomics*, ***1647***, 179-184.

[118] **Poppe, L., Retey, J.** (2005). Friedel-Crafts-type mechanism for the enzymatic elimination of ammonia from histidine and phenylalanine. *Angewandte Chemie-International Edition*, ***44***, 3668-3688.

[119] **Montavon, T. J., Christianson, C. V., Festin, G. M., Shen, B., Bruner, S. D.** (2008). In design and characterization of mechanism-based inhibitors for the tyrosine aminomutase SgTAM, *9th Tetrahedron Symposium on Challenges in Organic and Bioorganic Chemistry*, Berkeley, CA, USA, Jul 22-25.

[120] **Christianson, C. V., Montavon, T. J., Festin, G. M., Cooke, H. A., Shen, B., Bruner, S. D.** (2007). The mechanism of MIO-based aminomutases in beta-amino acid biosynthesis. *Journal of the American Chemical Society*, ***129***, 15744-15745.

[121] **Bartsch, S., Bornscheuer, U. T.** (2009). A Single Residue Influences the Reaction Mechanism of Ammonia Lyases and Mutases. *Angewandte Chemie-International Edition*, ***48***, 3362-3365.

APPENDICES

APPENDIX A: Vectors.

APPENDIX B: Unstained Standard Protein Weight Marker (Fermentas Inc.).

APPENDIX C: Media, solutions, and reagents.

APPENDIX D: List of amino acids and their abbrevations.

APPENDIX A: Vectors

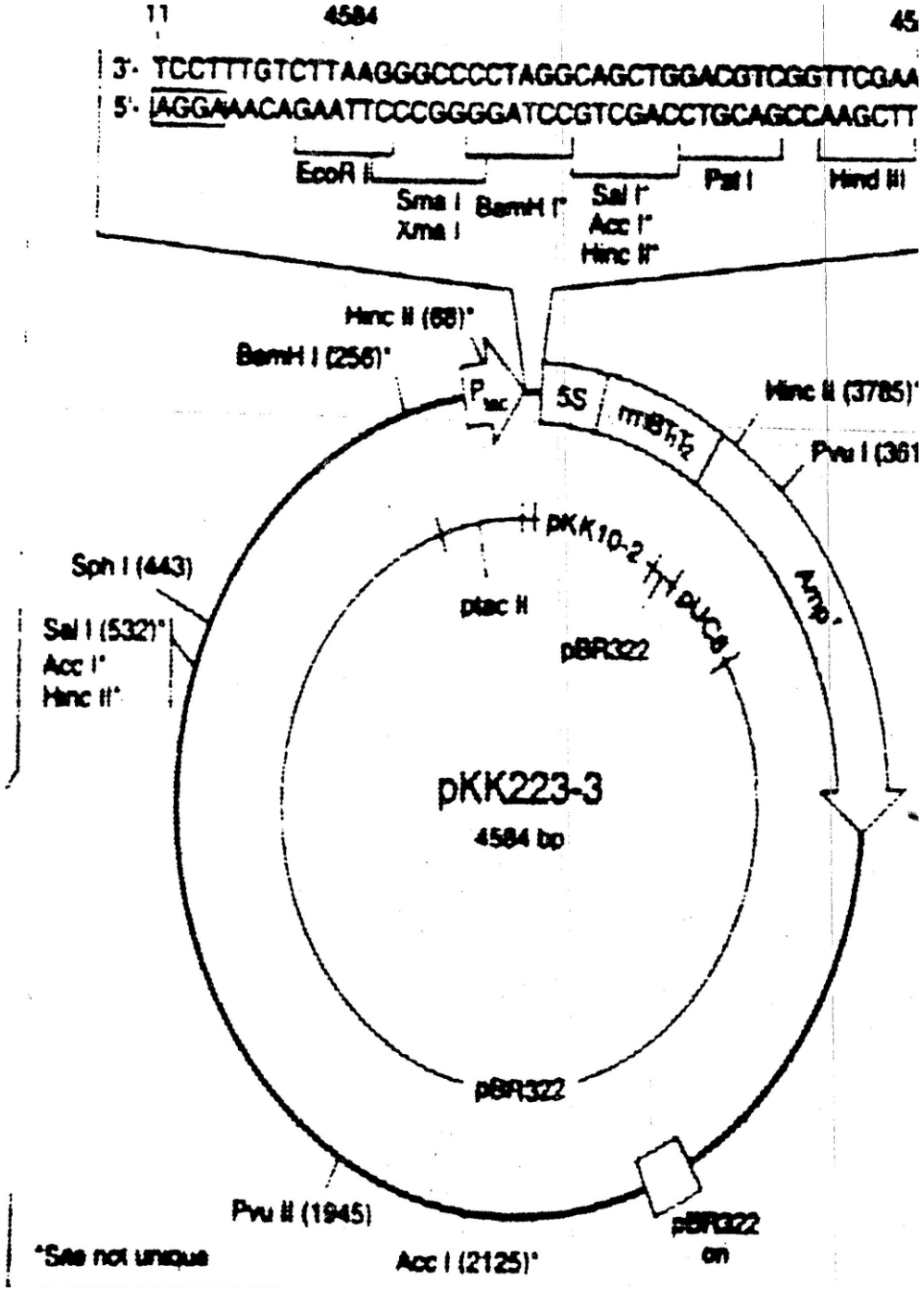


Figure A.1 : Plasmid map of pKK 223-3 vector.

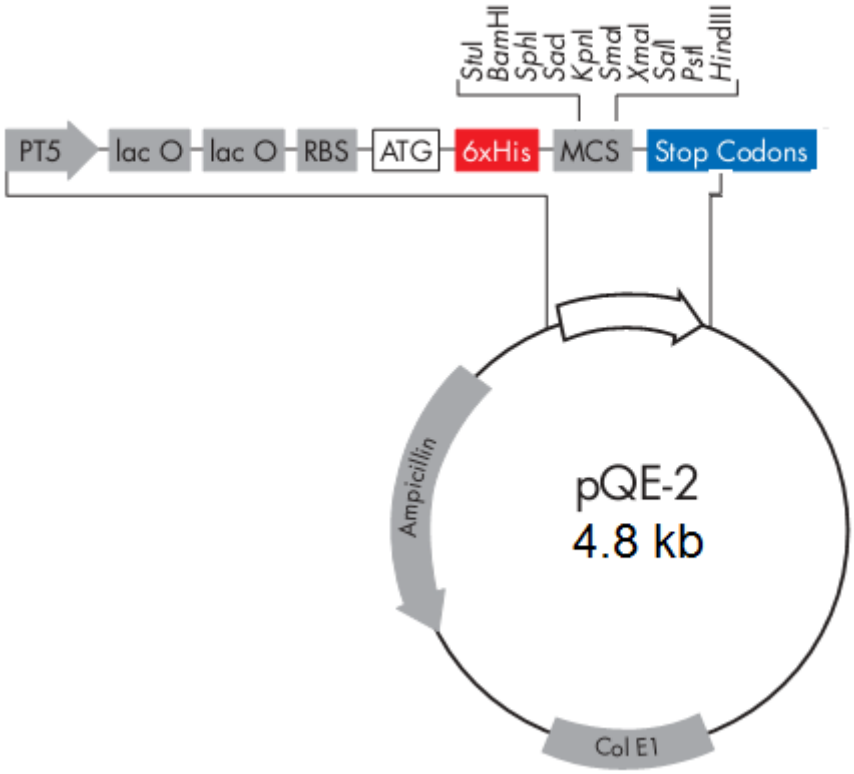


Figure A.2 : Plasmid map of pQE-2 vector.

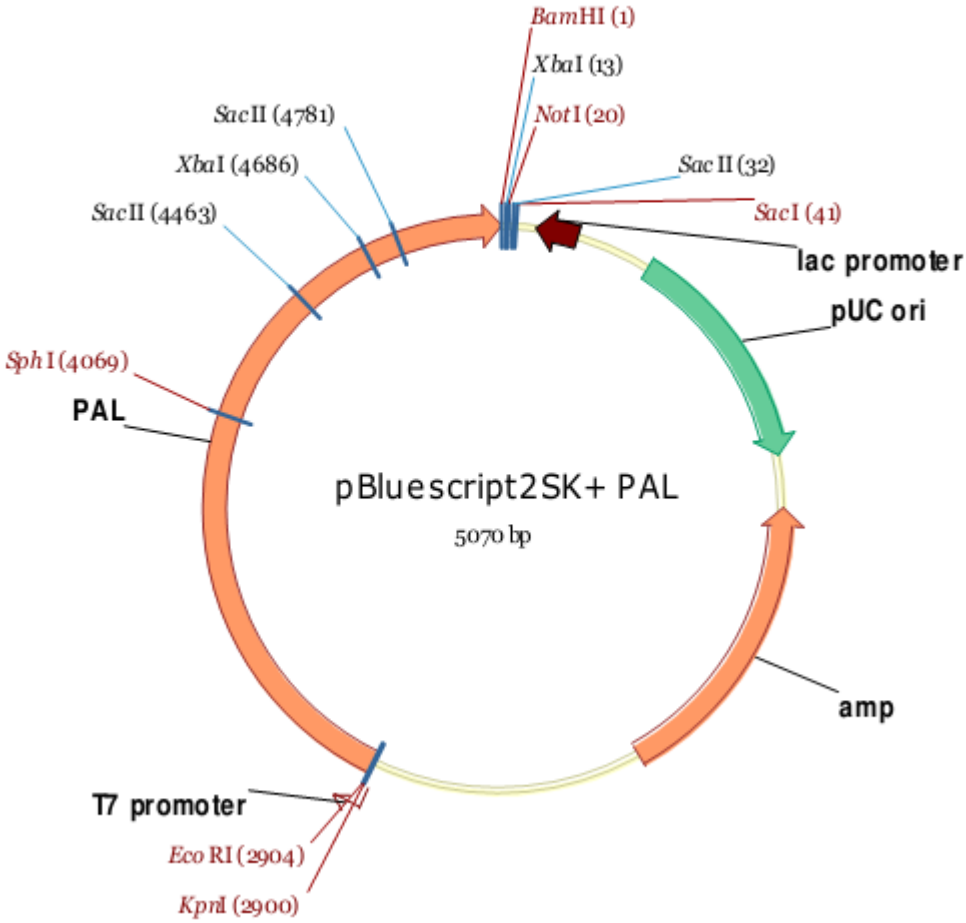


Figure A.3 : PAL expression vector.

APPENDIX B: Unstained Standard Protein Weight Marker (Fermentas Inc.)

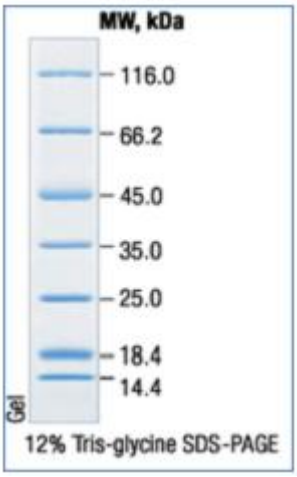


Figure B.1 : Unstained Standard Protein Weight Marker on 12% polyacrylamide gel.

APPENDIX C: Media, solutions and reagents

LB medium: 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl.

LB agar: LB medium containing 15 g/liter agar.

Ampicillin stock solution: 100 mg/ml in H_2O, sterile filter, store in aliquots at –20°C.

IPTG (1 M): 238 mg/ml in H_2O, sterile filter, store in aliquots at –20°C.

50 mM TrisHCl: 6.05 g Tris base (MW 121.1 g/mol) in 1 liter H_2O.

5x SDS-PAGE sample buffer: 0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT.

SDS-PAGE running buffer: 3.02 g Tris base, 14.4 g glycine in 1 liter H_2O.

Lysis buffer: 50 mM NaH_2PO_4, 6.90 g N $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol), 10 mM imidazole 0.68 g imidazole (MW 68.08 g/mol) in H_2O then adjust pH to 8.0 using NaOH and finally to 1 liter H_2O volume.

Wash buffer: 50 mM NaH_2PO_4, 6.90 g NaH2PO4·H_2O (MW 137.99 g/mol), 300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol), 20 mM imidazole 1.36 g imidazole (MW 68.08 g/mol) in H_2O then adjust pH to 8.0 using NaOH and finally to 1 liter H_2O volume.

Elution buffer: 50 mM NaH_2PO_4, 6.90 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol), 300 mM NaCl 17.54 g NaCl (MW 58.44 g/mol), 250 mM imidazole 17.00 g imidazole (MW 68.08 g/mol) in H_2O then adjust pH to 8.0 using NaOH and finally to 1 liter H_2O volume.

APPENDIX D: List of amino acids and their abbrevations

Table D.1: List of amino acids and their abbrevations

One - letter code	Three - letter-code	Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

CURRICULUM VITAE

Name Surname: Barış BİNAY

Place and Date of Birth: Kemah, 03/04/1979

Address: Istanbul Technical University,
Faculty of Letter and Science,
Department of Molecular Biology-Genetics and Biotechnology, 34469,
Maslak, Istanbul.

Tel: (W) +90 212 285 7250-51

(Gsm) +90 533 231 36 14

E-Mail: binay@itu.edu.tr

B.Sc.: University of Ankara, Chemistry.

M.Sc.: Istanbul Technical University, Advanced Technologies Program -Molecular Biology-Genetics and Biotechnology.

Professional Experience

- **Oct.2009-Oct.2010**-Visitor PhD student, Ammonia Lyase Biocatalysts: Cloning, Mutagenesis, Screening, and Engineering (by my official PhD co-superviser: N.J. TURNER, University of Manchester, Manchester, UK, supported by TUBITAK and BBSRC).
- **May.2005-Aug.2005**- Visitor M.Sc. student, Application of rational design strategies to *candida methylica* FDH and *bacillus stearothermophilus* LDH (Supervisor: Nevin Gül Karagüler and Sessions RB, Clarke AR., University of Bristol, UK).
- **Sept.2003-Aug.2005**-M.Sc. Protein engineering applications on NAD^+-dependent L-lactate dehdrogenase from *Bacillus stearothermophilus* (Supervisor:Nevin Gül Karagüler, supported by DPT).
- **Jan.2001- Jun.2001**-B.Sc. Thesis, Lipaz enziminin gıda endüstrisinde kullanımı (Supervisor: Sibel Sungur).

Experience in Teaching/Studying

- **Aug.2005-Present**-Research Assistant, in Molecular Biology-Genetics and Biotechnology, Istanbul Technical University, Istanbul.
- **Sept.2003-Present**-Some lab./courses Assistant, in Molecular Biology-Genetics and Biotechnology, ITU, Istanbul.
- **Sept.2000-June2001**-Student Assistant, Chemistry, Ankara University, Ankara.

- **June2000-Sept.2000-**BAYER Turk, Quality Control Departments, Topkapı, Istanbul (Training Period).

Awards

- TUBITAK_2214 Research Scholarship (8 months).
- University of Manchester, "Producing unnatural amino acids by novel mutant PAL enzyme", Biotechnology and Biological Sciences Research Council (BBSRC), UK (4 months).
- TUBITAK scholarships for publications and conferences.

Publications From M.Sc.

- **Binay B.**, Karagüler N.G. (2007). Attempting to remove the substrate inhibition of L-lactate dehydrogenase from *Bacillus stearothermophilus* by site-directed mutagenesis. *Appl. Biochem. Biotechnol,* 141, 265-72.
- **Binay B.,** Karagüler, N.G. (2004). Removing the substrate inhibition of L-lactate dehydrogenase from *Bacillus stearothermophilus* by site-directed mutagenesis. *6th International conference on Protein Stabilization ProtStab2004*, 26-29 Sept 2004. Bratislava, Slovakia.

Patents/Publications/Presentations From Thesis

- **Binay, B.,** Shoemark, D.K., Sessions, R.B., Clarke, A.R. and Karaguler, N.G. (2009). Increasing the substrate specificity of the lactate dehydrogenase from *Bacillus stearothermophilus* by DNA shuffling. *Biochemical Engineering Journal*, 48, 118–123.
- Karagüler, N.G., **Binay, B.,** Ordu, E.B., Clarke, A.R. (2007). Protein engineering applications of industrially exploitable enzymes: *Geobacillus stearothermophilus* LDH and *Candida methylica* FDH. *Biochem Soc Trans.,* 35:1610-5.
- **Binay, B.,** Sessions, R.B., Gül-Karagüler N. A double mutant of highly purified *Bacillus stearothermophilus* lactate dehydrogenase recognises mandelic acid as a substrate. *Submitted to Enzyme and Microbial Technology.*
- I. Rowles, **B. Binay,** K. Malone, I. Fotheringham, G. Jenkins and N.J. Turner, for the mutant PAL enzyme applied to Europe Patent Office by AMRI Global (Patent Application).
- Rowles, I., **Binay, B.** Malone, K.I., Fotheringham, G.J., Turner, N.J. Ammonia Lyase Biocatalysts: Cloning, Mutagenesis and Screening. *In preparation.*
- **Binay, B.,** Rowles, I., Turner, N. J., and Karaguler, N.G. (2012). Protein Engineering Applications for Increasing Substrate Specificity of PAL and LDH Enzymes. *9th International Conference on Protein Stabilization ProtStab2012*, 2-5 May 2012, Lisbon, Portugal.

- Rowles, I., **Binay, B.,** Malone, K., Fotheringham, I., Jenkins G., and Turner, N. J. (2010). Ammonia Lyase Biocatalysts: Cloning, Mutagenesis and Screening. *5th International Congress on Biocatalysis 2010 (Biocat2010)*, August 29 - September 2, 2010, Hamburg, Germany.
- Rowles, Karagüler, N. G., Ordu, E., **Binay, B.,** Yelboğa, E., Par, G., Session, R.B., Clarke, A.R. (2009). Nanobiyokatalizör geliştirlmesinde Protein Mühendisliği Uygulamaları. *İTÜ Nano-Bilim ve Nano-Teknoloji Çalıştayı,* 12-13 Şubat 2009, İstanbul, Türkiye.
- **Binay, B.,** Session, R.B., Clarke, A.R., Karagüler, N. G. (2008). Introducing malate dehydrogenase based on rational design to the lactate dehydrogenase from *Bacillus stearothermophilus* by DNA shuffling. *23rd Annual Symposium of The Protein Society*, 5-9 August 2008, Boston, USA.
- **Binay B.,** Özboyacı, M., Sessions R. B., Gül-Karagüler N. (2008). Construction of 246Gly and 240Ala mutant for changing the substrate specificity of the *bacillus sterothermophillus* Lactate dehydrogenase. *ESF Research Conference on Protein Design and Evolution for Biocatalysis*, 25 - 30 October 2008, Saint Feliu de Guixols, Spain.
- **Binay, B.,** Şanlı, K., Ordu, E., Karagüler, N.G. (2008). Application of computational methods to design thermostable *Candida methylica* FDH. *International Enzyme Engineering Symposium-IEES'08-*,01-05 October 2008, Kusadasi, Turkey.
- Doluca O., **Binay B.,** Bıçakçı E., Kısakesen H.I., Session R.B., Clarke A.R., Tamerler C., Gül-Karagüler N. (2008). Effects of cysteine/cysteine engineering on thermal stability of *Candida methylica* FDH. *33rd FEBS Congress and 11th IUBMB Conference*, 28 June-3 July 2008, Athens, Greece.
- Yelboga E., Candan Z., **Binay B.,** Bıçakçı E., Session R. B., Clarke A. R., and Gül-Karagüler N. (2008). Crucial residues in $NAD^+/NADP^+$ specificity of *Candida methylica* FDH. *33rd FEBS Congress and 11th IUBMB Conference*, 28 June-3 July 2008, FEBS Journal, 2008, Volume: 275 Pages: 234-234, Athens, Greece.
- **Binay B.,** Sezerman O. U., (2008). Predict Permissive Sites of Protein for Insertion Domains. *International Symposium on Health Informatics and bioinformatics, HIBIT'08*, 18-20 May 2008, Istanbul, Turkey.
- Yelboga E., **Binay B.,** Bıçakçı E., Session R. B., Clarke A. R., Gül-Karagüler N. (2007). Alteration the coenzyme specificity of cm FDH. *8th International conference on Protein Stabilization ProtStab2007,* Exeter, UK.
- Doluca O., Bıçakçı E., **Binay B.,** Session R. B., Clarke A. R., Tamerler Behar C., Gül-Karagüler N. (2007). Enhancing thermal stability of the *cm*FDH by cysteine-cysteine bridge. *8th International conference on Protein Stabilization ProtStab2007,* Exeter, UK.
- **Binay B.,** Session R. B., Clarke A. R., Gül-Karagüler N. (2007). Changing the substrate specificity of the *bs*LDH. *8th International conference on Protein Stabilization ProtStab2007,* Exeter, UK.

- **Binay, B.,** Karagüler, N. G. (2006). Increasing the substrate specificity of *bacillus stearothermophillus* lactate dehydrogenase by DNA shuffling. *31st FEBS Congress*, 24-29 June 2006, FEBS Journal, 2006, Volume: 273, Pages: 336-336.
- Akkor, M., **Binay, B.,** Karagüler, N. G., Karabay, A. (2006). Relations of microtubules with LDH: clue for neurodegenerative diseases. *31st FEBS Congr*ess, 24-29 June 2006, FEBS Journal, 2006, Volume: 273, Pages: 241-241. Istanbul, Turkey.