ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

Q102R AND Q203L DOUBLE MUTANT EFFECTS ON SUBSTRATE SPECIFICITY AND ACTIVATOR REQUIREMENT IN *bs*LDH ENZYME

M.Sc. THESIS

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*bs*LDH ENZİMİNDE Q102R VE Q203L ÇİFT MUTANTININ SUBSTRAT ÖZGÜLLÜĞÜ VE AKTİVATÖR GEREKSİNİMİNE ETKİLERİ

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Ceren SAĞDIÇ, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID 521121133, successfully defended the **thesis** entitled "**Q102R AND Q203L DOUBLE MUTANT EFFECTS ON SUBSTRATE SPECIFICTY AND ACTIVATOR REQUIREMENT IN *bs*LDH ENZYME**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Jury Members :	**Prof. Dr. Ayten KARATAŞ** İstanbul Technical University	……
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FOREWORD

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August 2015 Ceren SAĞDIÇ

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ABBREVIATIONS

amp : Ampicilin
bla : β- lactamase
bs : *Bacillus stearothermophilus*
bp : Base pair
CAT : Chloramphenicol Acetyl Transferase
CBM : Carbohydrate Binding Module
Da : Dalton
dsDNA : Double Stranded DNA
EDTA : Ethylenediaminetetraacetic Acid
FBP : Fructose 1,6 Bisphosphate
h : Hour
His-tag : Poly Histidine tagged
IDA : Iminodiacetic Acid
IPTG : Isopropyl β-D-1-Thiogalactopyranoside
ISM : Iterative Saturation Mutagenesis
kb : Kilobase
K_{cat} : Catalytic Constant
Km : Michaelis Constant
LDH : Lactate Dehydrogenase
min : Minute
M : Molar
MWCO : Molecular Weight Cut Off
mL : Mililiter
mM : Milimolar
mV : Milivolt
NAD(H) : Nicotinamide Adenine Dinucleotide
Ni-NTA : Nitrilotriacetic Acid-Nickel
nm : Nanometers
RBS : Ribosomal Binding Site
RT : Room Temperature
PCR : Polimerase Chain Reaction
[S] : Substrate Concentration
sec : Second
SDS : Sodium Dodecyl Sulfate
SOC : Super Optimal Broth with Catabolite Repression
TAE: : Tris-acetic acid-EDTA
TCA : Tricarboxylic Acid
Tm : Melting Temperature
uL : Microliter
WT : Wild Type
V : Velocity

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SUMMARY

Enzymes are biological catalysts that play role in chemical transformations, thus they are important for several branches of industries.
The L-lactate dehydrogenase (LDH) from *Bacillus stearothermophillus(bs)* is substantial for the synthesis of pharmaceuticals and agrochemicals as it provides the production of chiral building blocks. It enables the reaction that generates hydroxy acids from their corresponding oxoacids. Moreover, because *Bacillus stearothermophillus* is a thermophilic bacterium, the enzyme is highly stable to heat. However, the enzyme has a limited substrate specificity.
On the other hand, the enzyme is activated by an expensive activator, fructose 1,6 bisphosphate (FBP). FBP also shows undesirable co-factor complications for industrial processes.
Besides, *bs*LDH activity is inhibited by excess substrate (pyruvate), which is once more a deleterious feature owing to its industrial use. All these characteristics make *bs*LDH an applicable target for protein engineering.

Previous studies have provided significant developments over these unwanted properties by using rational and randomized mutagenic methods. For instance, by Wilks et al., a Q102R substitution (found in the naturally occurring malate dehydrogenases) on the *bs*LDH sequence has been shown to convert the substrate specificity from lactate to malate by 3 orders of magnitude.

In another research, Allen and Holbrook succeeded in producing a mutant (6A) which is almost fully activated in the absence of FBP. This 6A mutant had three amino acid replacements: R118C, Q203L and N307S, resulting in a 70-fold activation. Normally FBP activates the enzyme by enabling structural rearrangements which turns the protein into tetrameric form from dimeric form. Because the Q203L is located at the dimer-dimer interface, this substitution is thought to have realized the substrate switch by altering the dimer-tetramer equilibrium.

By our laboratory, similar protein engineering applications have yielded significant effects on *bs*LDH as well. For example, by D38R replacement, using PCR based overlap extension mutagenesis, the substrate inhibition was decreased threefold by Binay and Karaguler. In another study, recombinant colonies, formed by random mutagenesis were screened and a more efficient malate dehydrogenase, compared to Q102R variant, was produced.

Our aim in this study is forming a double mutant form of *bs*LDH enzyme and investigate its effect both on the activator need and on substrate specificity. In order to achieve this, Q102R and Q203L mutants were constituted by site-directed

mutagenesis in *bs*LDH sequence which was found in pQE2 vector. To be used in the PCR reaction, appropriate oligonucleotides were designed to generate Q102R and Q203L mutations. Following the PCR with these oligos, mutant dsDNAs were selected by Dpn I digestion. After that, mutated DNA was transformed into *E.coli* strain BL21 *E. coli genotype fhuA2 [lon] ompT gal [dcm] ΔhsdS* competent cells. Plasmid DNA was purified following the transformation and samples were sequenced to ensure to have achieved the correct mutations. According to the data obtained, Q102R and Q203L mutations are formed in *bs*LDH sequence. Mutated DNA was amplified in large-scale *E.coli* culture and the culture was precipitated in order to obtain protein from cells. Desired protein with 6xHis-tag was sorted from the protein pool by Ni-NTA resin system which selectively binds His-tagged proteins. Thereafter, by Sodium Dodecyl Sulphate Gel Electrophoresis (SDS-PAGE), the protein was visualized at the region of its molecular mass (35kDa) and have ensured to obtain the enzyme. By ultrafiltration, smaller proteins and other contaminants were removed and a purer enzyme was attained.

With different concentrations of pyruvate as substrate, wild type and mutant enzyme kinetic measurements were realized in the absence and presence of FBP. Wild type data were found quite consistant with the previous studies. But we could not get detectable results in mutants with pyruvate. Actually, this phenomenon agrees with the Q102R mutant feature, which gives rise to a malate dehydrogenase upon mutation. Further experiments will be made with malate and oxaloacetate substrates to more precisely elucidate the double mutant effect.

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ÖZET

Enzimler, organizmada meydana gelen pek çok reaksiyonun katalizini gerçekleştiren biyolojik aktivatörlerdir. Enzimler, girdikleri reaksiyonun yönünü değiştirmezler; ancak tepkimenin aktivasyon enerjisini düşürerek kimyasal dengeye gelme sürecini hızlandırırlar. Bu olgu, enzimlerin "aktif bölge" olarak adlandırılan kısımlarında substratlarıyla kompleks oluşturma özelliklerinden kaynaklanır.

Enzimler biyomedikal araştırmalar, ilaç sanayi ve pek çok ticari ürün eldesinde yaygın olara kullanılmaktadır. Günümüzde, deterjanlardan fermente içecek yapımına kadar pek çok uygulamada mikroorganizmalar tarafından büyük ölçekli olarak üretilen enzimler rol alır. 2000 yılı verilerine göre tahmini endüstriyel enzim pazarı değeri yaklaşık 1.5 milyar dolardır.

Kimyasal katalizörlerle karşılaştırıldığında yenilenebilir kaynaklardan elde edilmeleri, biyolojik olarak çözünebilmeleri, daha elverişli pH ve sıcaklık koşullarında çalışabilmeleri enzimleri çok daha tercih edilir duruma getirmiştir. Öte yandan artan nüfus ve azalan doğal kaynak baskısı, daha az kirlilik meydana getiren üretim yöntemlerini tercih etme gerekliliğini ortaya koymuştur.

Klasik kimyasal katalizörlere karşı üstünlükleri, enzimleri giderek daha önemli endüstriyel ürünler haline getirse de, çoğu enzim ticari uygulamalar açısından gerekli özellikleri barındırmaz. İstenen koşullarda, daha yüksek verimle, daha fazla substrat kullanarak çalışabilen enzimler üretmek protein mühendisliği uygulamalarıyla mümkün hale gelmiştir. Bu uygulamalarda rasyonel dizayn ve yönlendirilmiş evrim olmak üzere iki ana yöntem ile bu ikisinin olumlu özellikleri alınarak elde edilmiş yarı-rasyonel dizayn sistemi kullanılmaktadır. Rasyonel dizayn, bilgisayar programlı modelleme ve biyoinformatik bilgisi ışığında proteinin yapısal ve işlevsel özelliklerini ortaya koyma ve buradan hareketle hedeflenen bölgelerde mutasyon yapma esasına dayanır. Yönlendirilmiş evrim ise doğal bir süreç olan evrim mekanizmasını laboratuvar ortamında ve moleküler seviyede taklit eder. Bu yöntemde, ilgili protein dizisi üzerinde rastgele mutasyonlar oluşturulur, daha sonra mutant bireyler, proteinin değiştirilmek istenen karakterini taşıyıp taşımamalarına göre ayırt edilir. Yarı-rasyonel dizayn ise bir yandan rasyonel dizayndaki yapı ve fonksiyon bilgisi gerekliliğini, diğer yandan yönlendirilmiş evrimdeki çok sayıda birey tarama zorluğunu ortadan kaldırır.

Laktat dehidrogenaz (LDH) enzimi, glikolizin son adımında piruvatı laktata dönüştüren enzimdir. NAD-bağımlı 2-hidroksikarboksilat dehidrogenazlar ailesinin üyesidir. Piruvatın laktata çevrilmesi reaksiyonunu terisinir olarak katalizlerken

kofaktörü olan NADH'yi de NAD^+'a yükseltger. Hemen her organizmadan izole edilen LDH, oksijenli ve oksijensiz solunum yolaklarında kullanılabilen bir ara ürün olan piruvatı üretimi/tüketimi bakımından önemlidir. Prokaryotik ve ökaryotik olmak üzere, bilinen iki tip LDH vardır.

Bacillus stearothermophillus bakterisinden elde edilen L-laktat dehidrogenaz enzimi (*bs*LDH), eczacılık ve zirai kimyasallarda kullanılmak üzere kiral yapıtaşları elde etme işleminde ticari yönden oldukça önemlidir. Her ne kadar yüksek stereo uygunluk seviyesiyle kiral hidroksiasitlerin sentezine olanak sağlasa da, *bs*LDH dar substrat özgüllüğüyle endüstriyel üretim açısından dezavantaj oluşturmaktadır. Termofil bir bakteriden elde edilen ve bu yüzden ısıya karşı oldukça stabil olan bu enzim substrat kullanımını artırmak için oldukça uygun bir hedeftir.

Öte yandan, *bs*LDH enzimi fruktoz 1,6 bisfosfat (FBP) tarafından aktive edilmektedir. Aktivasyon, dimerik formda düşük substrat afinitesi gösteren enzimin, FBP bağlanmasıyla çok daha aktif olan tetramerik forma geçmesiyle gerçekleşir. Bu aktivatör pahalı olmakla beraber, endüstriyel süreçte istenmeyen ko-faktör problemlerine yol açar. Protein mühendisliği yöntemleriyle, enzimin aktivatör kullanımını azaltmak hedeflenmektedir.

Günümüze kadar yapılan çalışmalar, rasyonel ve randomize mutajenik metotlar kullanarak, enzimin substratlarının artırılmasında ve özgüllüğünün değiştirilmesinde önemli yol kat etmeyi sağlamıştır. Örneğin, Wilks ve arkadaşları, *bs*LDH dizisinde Q102R mutasyonuyla, substrat kullanımını laktat-piruvat çiftinden oksaloasetat-malat çiftine dönüştürmeyi, yani LDH gen dizisinde değişiklik yaparak malat dehidrogenaz işlevi gören bir enzim tasarlamayı başarmıştır.

Başka bir çalışmada Allen ve arkadaşları, üç dizi randomize mutagenez ve tarama yöntemiyle FBP yokluğunda da aktif, üç amino asit mutasyonu taşıyan (R118C, Q203L, N307S) 6A mutantı üretmiştir. Mutantın sözü edilen aktivasyon özelliğini göstermesinde etkili bölge olarak dimer-dimer arayüzünde bulunan Q203L mutasyonu gösterilmektedir. Yabanıl tip *bs*LDH için FBP varlığında K_{Mpyr} 5mM iken, FBP ile aktive edildiğinde K_{Mpyr} 0.05 mM'a düşer. 6A mutantında ise FBP yokluğunda K_{Mpyr} 0.07 dir. Bu da FBP'li yabanıl tip değerine oldukça yakındır.

Bu çalışmada amaçlanan, *bs*LDH enziminin, sözü edilen Q102R ve Q203L mutantlarını aynı dizide oluşturarak çift mutant elde etmek ve bunun FBP gereksiniminin yanı sıra substrat özgünlüğünü araştırmaktır. Bunu gerçekleştirmek için, pQE-2 klonlama vektörü içinde bulunan *bs*LDH gen dizisinde, bölgeye özel mutagenez sistemi kullanılarak Q102R ve Q203L mutasyonları oluşturulmuştur. Öncelikle Polimeraz Zincir Reaksiyonunda (PZR) kullanılmak üzere uygun oligonükleotitler tasarlanmıştır. PZR'yi takiben, mutant çift sarmal DNA'ların seçimi Dpn I yıkımıyla gerçekleştirilmiştir. Sonrasında BL21 kompetent *E. coli* genotip *fhuA2 [lon] ompT gal [dcm] ΔhsdS* hücrelerinde transformasyon yapılmış ve plazmit DNA'sı saflaştırılmıştır. Örnekler dizilemeye tabi tutulmuş ve doğru mutantların elde edildiği, yabanıl tip dizi ile mutant dizi karşılaştırılarak teyit edilmiştir. Elde edilen veriye göre *bs*LDH dizisinde Q102R ve Q203L mutasyonları gerçekleşmiştir. Mutant diziye sahip DNA, *E. coli* hücrelerinde çoğaltılmış, besi yerleri çöktürülerek protein izolasyonu için hücre eldesi sağlanmıştır. 6-His-tag'le işaretlenen LDH proteini, bu diziyi seçici olarak tanıyan ve ortamdan izole edilmesini sağlayan Ni-NTA agaroz ile ayrıştırılmıştır. Sodyum dodesil sülfat – poliakrilamid jel elektroforeziyle (SDS-

PAGE) kendi moleküler ağırlığına karşılık gelen bölgede görüntülenerek proteinin elde edildiği doğrulanmıştır. Ardından, ultrafiltrasyon yöntemiyle LDH dışındaki tüm proteinler ortamdan uzaklaştırılmış, daha saf bir enzim eldesi sağlanmıştır.
Yabanıl tip ve mutant enzimlerin kinetik ölçümleri FBP varlığında ve yokluğunda substrat olarak değişen konsantrasyonlarda piruvat kullanılarak gerçekleştirilmiştir. Bunun sonucunda yabanıl tip LDH kinetik ölçümleri FBP varlığında, literatürdekine çok yakın değerler vermiştir. Öte yandan, mutant enzimin piruvat ile kinetik ölçümlerinden saptanabilir bir veri elde edilememiştir. Bu durum, Q102R mutasyonu ile malat dehidrogenaz işlevi gösteren enzim oluşturulması durumuyla örtüşmekte ve yapılan mutasyonun doğru çalıştığına işaret etmektedir. Benzer kinetik deneyler, malat ve oksaloasetat substratları ile tekrar edilecek, çift mutant etkisi daha kesin şekilde aydınlatılacaktır.

1. INTRODUCTION

1.1. Lactate Dehydrogenase

Lactate dehydrogenases (LDHs) belong to a wide group of 2-ketoacid : NADP-dependent dehydrogenases. They catalyze the interconversion of 2-hydroxyacids (pyruvate) to the corresponding 2-ketoacids (L-lactate) (Holbrook et al., 1975) and meanwhile NADH is used as a coenzyme as shown in Figure 1.1. LDH is one of the most extensively studied enzymes and plays role in the last step of anaerobic glycolysis. Many LDHs have been isolated and characterized from a variety of organisms. Its catalytic mechanism has been described in detail. It includes conformational rearrangements of the structure upon substrate binding and release (Gerstein, M. & Chothia, C., 1991). When the competent catalytic state is reached, the enzyme catalyzes the direct transfer of a hydride ion from the pro-R face of NADH to the C2 carbon of pyruvate (Burgner & Ray, 1984; Deng et al., 1994).

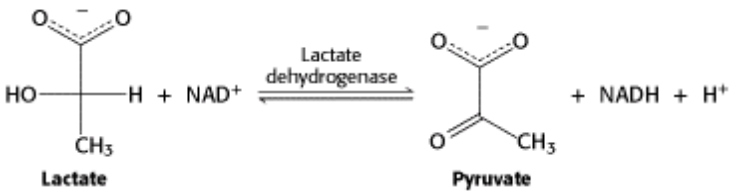

Figure 1.1: The reaction catalysed by NAD^+-dependent lactate dehyrogenases.

There are 2 types of LDHs identified so far: the prokaryotic-type and the eukaryotic-type enzymes. Although the latter is studied more in detail, they are structurally related. While vertebrate LDHs do not need to be activated by an allosteric effector (non-allosteric enzyme), some prokaryotic LDHs are activated by fructose-1,6-bisphosphate (FBP) molecule. The *Bacillus stearothermophilus* LDH (*bs*LDH) is a typical prokaryotic type enzyme, sequence of which is shown in Figure 1.2.

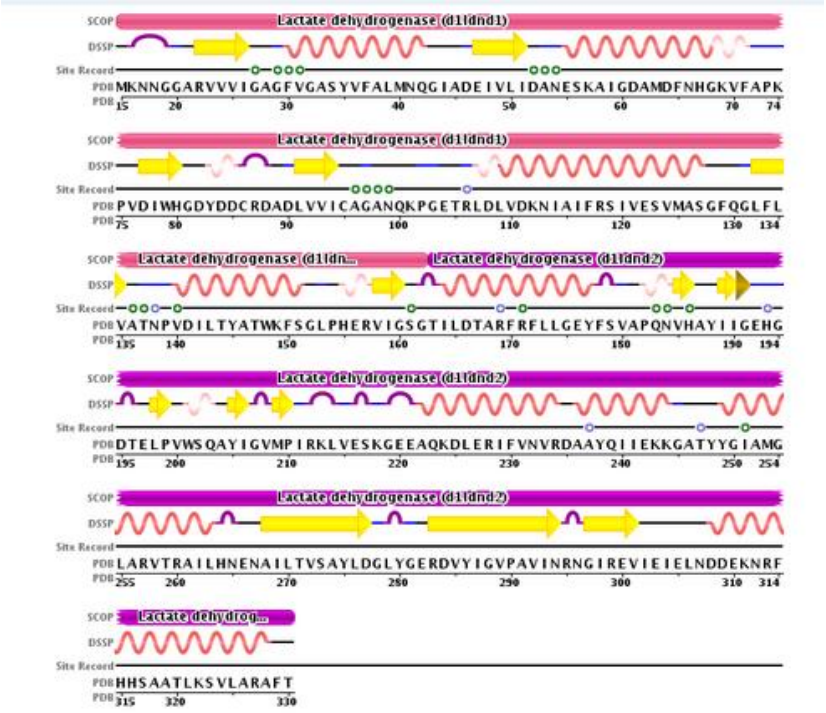

Figure 1.2: Domains of *bs*LDH.

Interconversion of pyruvate and lactate by LDH is realized in different tissues by isozymic forms of the enzyme. LDH is composed of a tetramer of two kinds of 35-kd subunits encoded by similar genes. In 1964, the first X-ray diffraction results for M4 isozyme for pig were declared. After then, the enzyme was isolated from many species as well. The family of these isozymes is composed of the two isozymic polypeptide chains for this enzyme: the H isozyme highly expressed in heart and the M isozyme found in skeletal muscle (Fine et al., 1963). The amino acid sequences are 75% identical. These subunits combine to form the tetramers: H4, H3M1, H2M2, H1M3, and M4. The H4 isozyme (type 1) has higher affinity for substrates than does the M4 isozyme (type 5) and, unlike M4, is allosterically inhibited by high levels of pyruvate. The other isozymes have intermediate properties, depending on the ratio of the two kinds of chains. H4 is designed to oxidize lactate to pyruvate, which is then utilized as a fuel by the heart through aerobic metabolism. Indeed, heart muscle never functions anaerobically. In contrast, M4 is optimized to operate in the reverse direction, to convert pyruvate into lactate to allow glycolysis to proceed under anaerobic conditions (Berg et al., 2002, pp 421-422). A third homotetramer of X-subunits is only present in adult male testicular tissue. Isozymes and isozyme profile changes are shown in Figure 1.3.

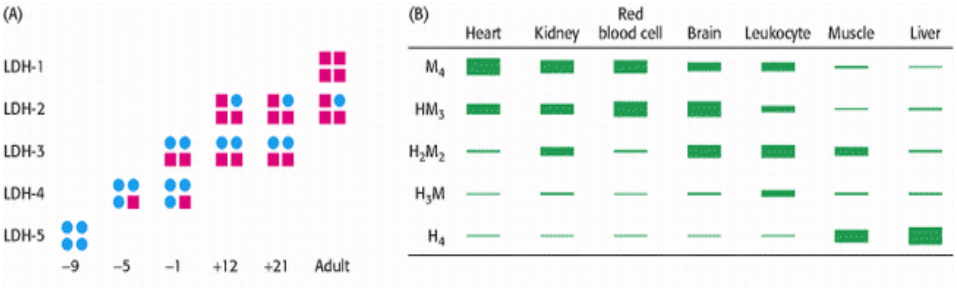

Figure 1.3: Isozymes of lactate dehydrogenase. (A) The rat heart LDH isozyme profile changes in the course of development. The H isozyme is represented by squares and the M isozyme by circles. The negative and positive numbers denote the days before and after birth, respectively. (B) LDH isozyme content varies by tissue (Berg et al., 2002, p.422).

The appearance of some isozymes in the blood is indicative of tissue damage and can be used for clinical diagnosis. For instance, an increase in serum levels of H4 relative to H3M is an indication that a myocardial infarction, or heart attack, has occurred.

The structures of the ternary complexes of LDH were deduced from crystallographic studies of the binding of NAD-pyruvate. *bs*LDH was crystalised and the three dimensional crystal structures of the enzyme were solved in the apo and holo forms (Birktoft et al., 1982).

1.2. NAD-Binding of Dehydrogenases

Glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase, and lactate dehydrogenase have in common a domain for NAD^+ binding, demonstrated in Figure 1.4. This nucleotide-binding region is made up of four α helices and a sheet of six parallel β strands. This common structural domain, is called a Rossmann fold after Michael Rossmann, who first recognized it. This fold represents an aboriginal dinucleotide-binding domain that is found in the dehydrogenases of glycolysis and other enzymes indicating their descent from a common ancestor (Berg et al., 2002, p. 655).

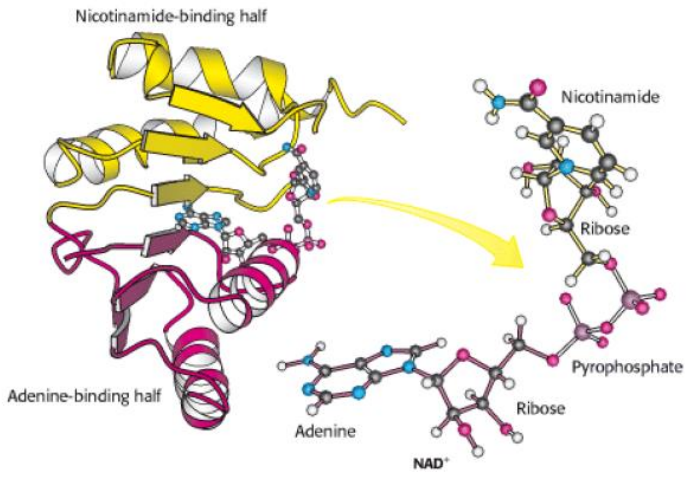

Figure 1.4: NAD^+ binding region in dehydrogenases (Berg et al., 2002, p.666)

1.2.1 NADH binding of LDH

Nicotinamide adenine dinucleotide (NADH) is a coenzyme composed of ribosylnicotinamide 5'-diphosphate coupled to adenosine 5'-phosphate by pyrophosphate linkage. It is involved in numerous enzymatic reactions in which it serves as an electron carrier by being alternately oxidized (NAD^+) and reduced (NADH). These forms are shown in Figure 1.5.

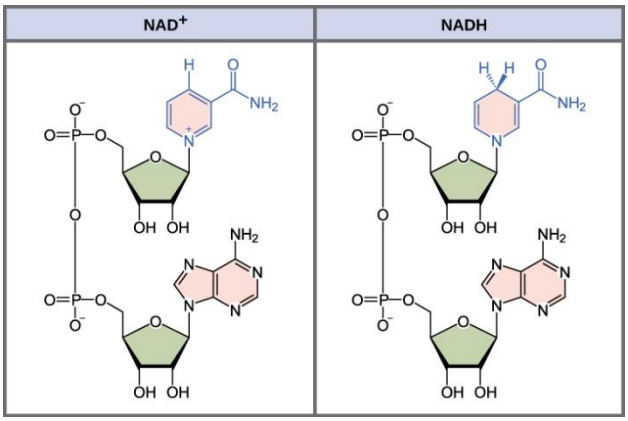

Figure 1.5: Structure of NAD^+ and NADH.

Directed by the rotation of glycosidic bond, the nicotinamide ring can be positioned in two orientations (Arnott and Hukins, 1972, p.453). It is adjusted due to the display of the A and B-side of the ring to the substrate site.

According to the quantum chemistry calculations, the binding mechanism involves the donation of a hydride ion H-, from the C2 carbon of lactate to the pro-R, re face of the nicotinamide moiety of the coenzyme combined with the transfer of a proton

from the O3 oxygen of lactate to the active-site histidine His-195 (Wilkie and Williams, 1992; Andres et al., 1993).

The interaction mechanism between the coenzyme and the protein remained unclear for a while. Previously, it was believed that the hydroxyl of Ser-163 residue (conserved in all known LDH sequences) made hydrogen bonding with the nitrogen atom of the carboxyamide side-chain of the coenzyme. Later on, this amino acid residue was found to be unsignificant for the binding (Wigley et al., 1987). On the other hand, researches highly support the presence of the hydrogen bonding between the carboxamide side chain of NADH and the enzyme. In such a study, it was found that NADH affinity of the LDH is more than its affinity for 3-acetyl pyridine, an anolog of NADH (Fine et al., 1963, p.116). Latter studies have suggested that it is very likely there is a hydrogen bond between the nitrogen atom and the main-chain carboxyl group of residue 138. Residues important in binding are indicated in Figure 1.6.

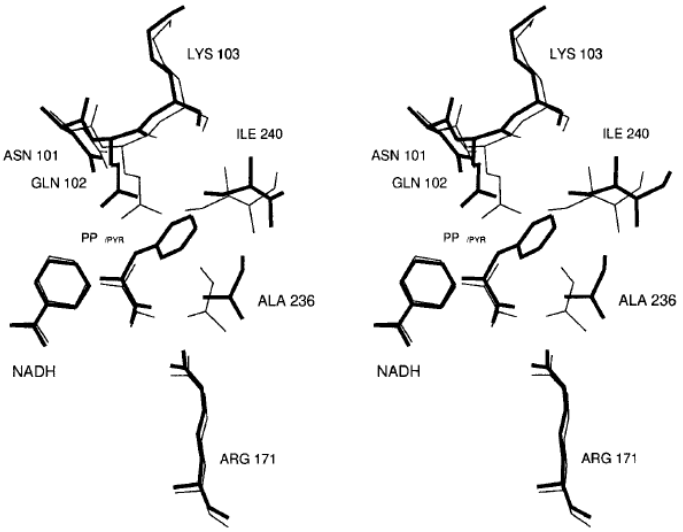

Figure 1.6: Binding residues in *bs*LDH sequence. Two stereo views comparing the wild-type-*bs*LDH structure complexed with either pyruvate (thin lines) or phenylpyruvate (thick lines). (El Hawrani, 1996).

1.3 Active Site of the Enzyme

The active site of an enzyme is the region that binds the substrate and contributes the amino acid residues that directly participate in the making and breaking of chemical bonds.

Sequences and conformations of LDHs are different in spite of the same substrate, as they are derived from different sources. Residues 98 to 110 consists of the active site in *bs*LDH and the conformation of this loop determines substrate.

1.3.1 Substrate (pyruvate) binding of LDH

Pyruvate is the anion of pyruvic acid. It is a crucially important compound as it is the end product of glycolysis. The structure of pyruvate is shown in Figure 1.7. Pyruvate stands at the junction between aerobic and anaerobic pathways. In anaerobic respiration, pyruvate is used as the starting point for fermentation, yielding either ethanol or lactate. For aerobic respiration, pyruvate is transported to the mitochondria to be used in the TCA cycle.

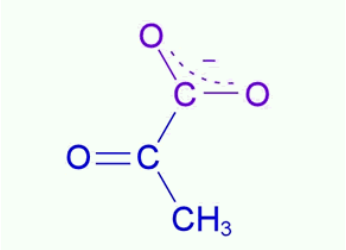

Figure 1.7: Structure of pyruvate.

In the LDH: NAD-pyruvate ternary complex, the position of the substrate binding site has been determined by the pyruvate.

Thr-246 seems to be an important residue for *bs*LDH. It is found in all LDHs. In a mutant enzyme study, the role of this residue for substrate binding was revealed. By the replacement of Thr-246 by glycine, a malate dehydrogenase could be designed (Wilks et al., 1988).

A salt bridge is formed between the carboxylate of the substrate and the side chain of Arg-171. The imidazole ring of the His-195 generates a hydrogen bond with the carbonyl group. While His-195 orients pyruvate, it also stabilizes the negative charge of the substrate formed during reduction (Clarke et al., 1988).

In addition, when NAD^+ is bound, Asn-140 becomes accessible for hydrogen bonding to the carbonyl of pyruvate. When reduced, however, NAD^+ coenzyme loses its charge and this charge is transferred to His-195 (Hart et al., 1987).

Here, His-195 behaves like a redox enzyme to realize the reaction.

Site-directed mutagenesis studies of the LDH active site have shown that Arg-109 (Clarke et al., 1986) and Asp-168 are substantial in the rate enhancement produced by the enzyme. Arg-109 causes a polarization of the carbonyl bond of the oxidized substrate, pyruvate (Clarke et al., 1986; Deng et al., 1994). Asp-168 is responsible for modulating the pKa of His-195 (Clarke et al., 1988). Likewise studies have revealed that Arg-171 is a vital residue in correctly binding the substrate carboxylate to the enzyme. (Hart et al., 1987).

LDH:NAD-pyruvate ternary complex is isomorphous with respect to the LDH:NAD:oxalate and further form the coenzyme than the pyruvate.

1.4 The Activator (Fru-1,6-P_2) Binding of LDH

Fructose 1,6-bisphosphate (FBP), also known as Harden-Young ester, is fructose sugar phosphorylated on carbons 1 and 6, as shown in Figure 1.8. The β-D-form of this compound is abundantly found in cells. The vast majority of glucose and fructose entering a cell is converted to fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate plays role in the glycolysis metabolic pathway and is produced by phosphorylation of fructose 6-phosphate. It is, in turn, broken into two compounds: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. It is an allosteric activator of pyruvate kinase.

It has been shown that two FBPs bind per *bs* LDH. In many bacteria (e.g., *Thermus, Lactobacili, Bacili*) LDH activity is markedly regulated by FBP (Clarke et al., 1986).

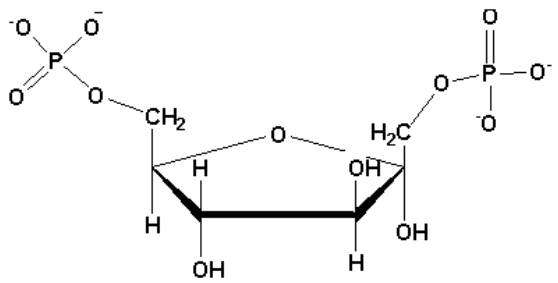

Figure 1.8: Structure of fructose-1,6-bisphosphate (url-1).

*bs*LDH is allosterically activated by FBP. This activation is formed by the FBP induced tetramerization of the dimeric enzyme. Upon this reaction, a structural rearrangement of key active site residues is realized and the enzyme becomes activated (Clarke et al., 1987). The non-activated form has a Km for pyruvate of 5 mM. However, in the presence of FBP, the Km for pyruvate drops to 0.05 mM.

In fact, dimeric form of the enzyme shows substrate affinity to a certain extent. But, the liganded tetramer and to a lesser extent the unligated tetramer have high affinity.
The two FBP molecules are located in a central hole in the enzyme and connect to the P-axis with their phosphate groups. This conformation stabilizes the assembled tetramer. In the absence of activator, the subunit interface on the P-axis is less stable ; the protein segregates and give dimers . These subunits are united by the Q axis which is stable. Along the 163-195 residues, both the substrate and the activator binding (anion group) sites are located. The binding of the FBP anion site to the His-188 and Arg-173 leads to a change in the relative His-195 and Arg-171, which in turn bridges the βG/βH-2αF structure. While the enzyme catalyzes the reaction, 195 and 171 residues hold carbonyl and carboxylate groups of pyruvate (Holbrook et al., 1975). From this point, we can conclude that their orientation must be important to regulate the firmness of the substrate binding.

1.5 Enzyme Kinetics

Living systems depend on chemical reactions which, on their own, would occur at extremely slow rates. Enzymes are catalysts which reduce the needed activation energy so these reactions proceed at rates that are suitable for the cell.
For many enzymes, the rate of catalysis V_0, which is defined as the number of moles of product formed per second, depends on the substrate concentration [S]. The rate of catalysis goes up linearly as substrate concentration increases. When reached a maximum, it no more rises with higher substrate concentrations and begins to fall.
The Michaelis-Menten model complies with the kinetic features of many enzymes. The rate V_0 of formation of product is given by the Michaelis-Menten equation:

$$v_0 = \frac{V_{\max}[\mathrm{S}]}{K_M + [\mathrm{S}]} \qquad \textbf{(1.1)}$$

Here, V max is the reaction rate when the enzyme is fully saturated with substrate and K_M (Michaelis constant), is the substrate concentration at which the reaction rate is half of the maximal rate. The maximal rate, V max, is equal to the product of k_2 or k_{cat} and the total concentration of enzyme. The kinetic constant k cat, called the turnover number, is the number of substrate molecules converted into product per unit time at a single catalytic site when the enzyme is fully saturated with substrate. Turnover numbers for most enzymes are between 1 and 104 per second. The ratio of k_{cat}/K_M provides a penetrating probe into enzyme efficiency. In Figure 1.9, Michaelis-Menten graph is indicated.

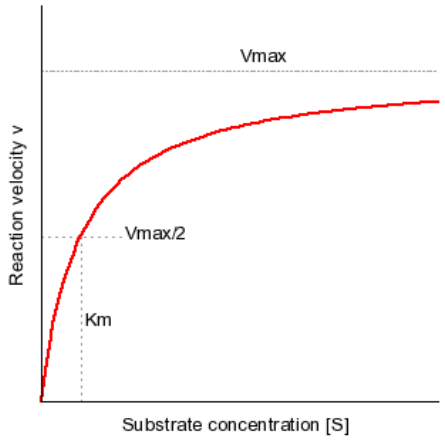

Figure 1.9: Michaelis-Menten kinetics.

A plot of $1/V_0$ versus 1/[S], called a Lineweaver-Burk or double-reciprocal plot, yields a straight line with an intercept of 1/V max and a slope of K_M/V max, as shown Figure 1.10. The intercept on the x-axis is $-1/K_M$.

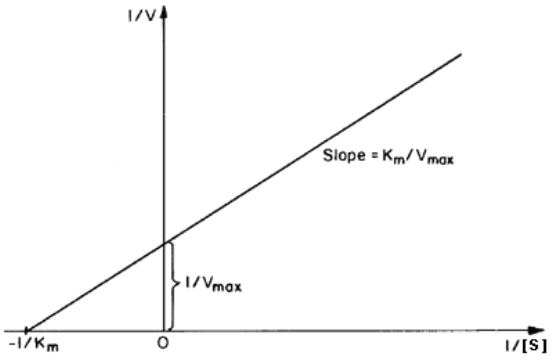

Figure 1.10: Lineweaver-Burk plot.

1.5.1 Kinetic mechanism of LDH

The kinetic mechanism of LDH is evaluated as ordered; because unless the coenzyme is bound to enzyme, lactate or pyruvate can not bind (Clarke & Daffron, 1998). Crystallographic studies show that coenzyme binding leads to a conformational change which moves 98-110 residues to close up the active site (Adams, 1968; White, 1976).

At neutral pH, lactate and NAD^+ is preferred by the reaction equilibrium. While pyruvate dissociates, the equilibrium is shifted through the products and all bound NAD^+ molecules become reduced. The rate-limiting step starts with the slower dissociation of NADH, in the steady state (Figure 1.10). As mentioned before, binding of the activator FBP stabilizes the tetrameric form and in this manner, the enzyme binds pyruvate 50 times more strongly than the dimeric structure does. In addition, NAD^+ regeneration is induced by the formation of the glycolytic intermediate Fru-1,6-P_2 (Bizzari and Kishi, 2003). The kinetic mechanism of *bs*LDH is indicated in Figure 1.11.

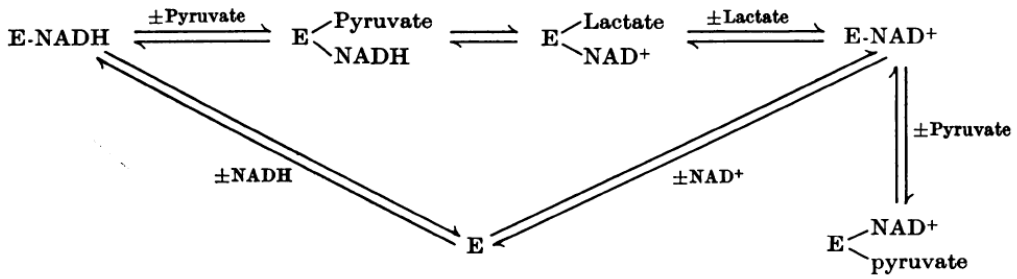

Figure 1.11: Kinetic mechanism of *bs*LDH

1.6 Substrate Inhibition of LDH

It has been shown that mammalian and bacterial dehydrogenases are inhibited by high concentrations of pyruvate during the enzyme catalysis. This is due to the formation of a covalent abortive adduct . The adduct is formed between pyruvate and NAD^+, just before the release of oxidized cofactor (NAD^+) (Fromm, 1961; Gutfreund et al., 1968; Coulson & Rabin, 1969). The adduct binds tightly to the enzyme meanwhile it is produced by the naturally occuring reaction, and in turn it inhibits the further redox catalysis (Everse et al., 1971).

By previous studies, it has been demonstrated that this substrate inhibition can be removed by S163L mutation in *Bacillus stearothermophilus* and human muscle LDHs (Eszes et al., 1996; Hewitt et al., 1997). Later, it is also shown that the same mutation is effective in human heart isoform, the most strongly substrate-inhibited LDH known (Hewitt et al., 1999).

Binay and Karaguler (2007), also showed that the substrate inhibition of *bs*LDH is decreased three-fold by substituting the conserved aspartic acid residue at position 38 by arginine. This is due to the weakining of NAD^+ binding of the enzyme; longer basic arginine residue instead of aspartic acid changes the binding properties of the coenzyme and thus decreases the formation of the abortive NAD^+-pyruvate complex.

1.7 Industrial Importance of LDH

For the "greening" of chemical manifacture, biocatalytic applications are being more and more attractive (Allen and Holbrook, 2000).

The reaction catalyzed by LDH- interconversion of pyruvate (oxo acid) and lactate (hydroxy acid) is important in various sections of industry. For producing several pharmaceuticals such as semi-synthetic penicillin (from S-α-hydroxyisocaproic acid) and antihypertensives (from S-α-hydroxyl-4-phenyl butanoate), obtaining chirally pure α-hydroxy acids from their corresponding achiral α-oxo acids is of great value. They are used in medicine as well, to diagnose phenylketonuria by detecting S-ketoisohexanoic acid (Karaguler et al., 2007).

One important α-hydroxy acid is lactic acid that is produced by *bs*LDH. Lactic acid is a colorless liquid organic acid and is miscible with water or ethanol. It exists as a racemate mixture, made up of stereoisomers. Global lactic acid production is estimated to be more than 100,000 tons per year and approximately 75% of the lactic acid produced is used in the food industry as an acidulant for flavor or as an antimicrobial agent (Raber, 1998). More recent uses for lactic acid have been for ecological interest and involve the nonchlorinated solvent ethyl lactate and the biodegradable plastic polylactic acid production (Skory, 2000).

On the other hand, working with thermostable enzymes has advantages over the non-thermostables. They are robust, have good stability and are relatively tolerant to organic solvents. Reactions can be run at higher temperatures thus minimizes the risk

of microbial contamination, eases solubility and viscosity (Allen and Holbrook, 2000). Together with other dehydrogenases, LDH shows significant promise for industry as it may constitute a key step in various synthesis works.

1.8 Protein Engineering

Many microorganisms produce several enzymes naturally. However, to use these natural enzymes in industry has some limitations such as low catalytic efficiency, low activity and low stability. In order to overcome these limitations, many protein engineering applications have been developed. These applications rely on two main strategies: rational design and directed evolution. Rational design is the earliest approach. It requires information of the structural properties of the enzyme active site and its role in the functioning of the protein. More recently, directed evolution has become a popular tool, as it allows relatively rapid engineering of enzymes without requiring detailed information about structure-function relationship, unlike rational design (Chica et al., 2005). Finally, semi-rational approach has got on the stage and proven to be especially advantageous as it has the virtues of both strategies. It does not need high-throughput screening method and a smarter library can be formed in comparison to the random whole-gene mutagenesis approach (Dalby, 2003).
Some new methods have been developped that stand between the directed and rational approaches. These techniques are summarized in Figure 1.12. Also, advantages and disadvantages of mentioned techniques are stated in Table 1.1.

1.8.1 Directed evolution

Directed evolution is a sweeping term that includes a variety of methods for improving or changing the function of enzymes using a nature-inspired twofold strategy of mutagenesis followed by selection. Through a user-defined aim, it mimics the natural selection process in order to produce more efficient nucleic acids or proteins, as indicated Figure 1.13. It is widely accepted as the most effective and useful application in modifying enzymes, especially to increase catalytic performance (Hida et al., 2007).

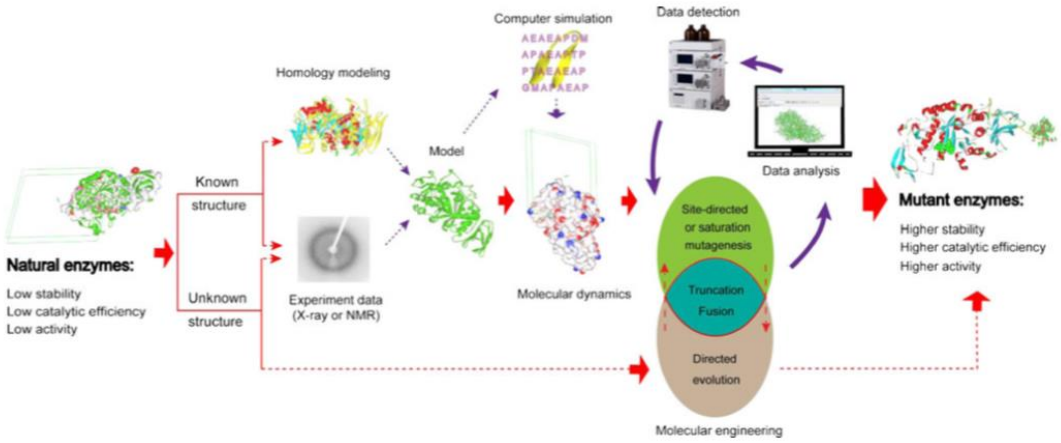

Figure 1.12: Molecular engineering methods and procedures to improve the performance of industrial enzymes (Yang et al., 2014).

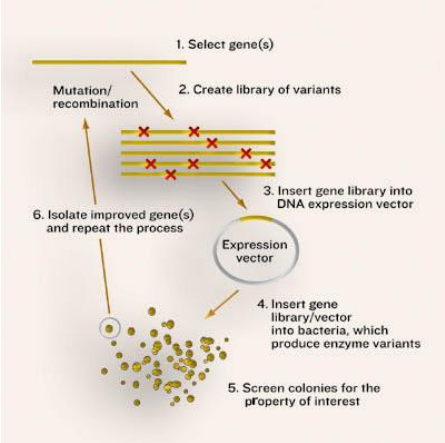

Figure 1.13: An overview of directed evolution.

1.8.2 Rational design

The first approach in this stage, rational design relies on the basis of predicting how an alteration in the structure of a molecule will affect its function. It gets line on computational programs which enable scientists to make complex modelling and calculations about a molecule's framework. Thus, unlike directed evolution, in this approach, having knowledge about the conformation and proceeding on the assumption of how it would likely to change its behaviour are indispensible.

Site directed mutagenesis : It is a rational design application which entails producing single and combinational mutations in order to modify enzyme genes. It is based on analyses of the structure, function, catalytic mechanism, and catalytic residues of enzymes.

This method is especially utile when a specific residue of a protein is important in the structure or function. This could be realized, for instance, removing the target amino acid completely or substituting it with a different residue. Alanine (alanine scanning) is frequently used for this purpose as its side chain is non-bulky, non-charged, chemically unreactive and is compatible with several structural motif formantion. This type of engineering can be accepted "blind" via transposition of all backbone residues, or can be more targeted (e.g. the replacement of a specific amino acid believed to be within an enzyme's active site in order to map the active site more efficiently).

Table 1.1: Advantages and disadvantages of different molecular engineering methods (Yang et al., 2014)

Factors	Advantages	Disadvantages
Directed Evolution	Mimic natural evolution	Random mutation library is large; low efficiency
Site-directed mutagenesis	Directed; high efficiency; mutation library is small; simplify methods for mutagenesis	The structure-function relationship of the target enzyme should be clear
Saturation mutagenesis	Easy; the screening effort drastically	Mutation library is large
Iterative saturation mutagenesis	High-quality libraries; more efficient and faster than others (e.g. saturation mut.)	Mutation library is large
Truncation	High efficiency	The relationships between domains and properties should be clear
Fusion	The structure-function relationship of the target enzyme is not necessary	There is not a clear criterion of the fusion sequence

1.8.3 Semi-rational design

Semi-rational design can be defined as a combination of random methods of directed evolution and the virtues of rational enzyme adjustment. Thus, the limitations of both directed evolution and rational design can be overcome. By this method, "smart"

libraries (similar to directed evolution) that are more likely to render positive results can be formed as it is already targeted to particular residues which are determined prior to mutation by use of structural and functional knowledge (similar to rational design) (Chica et al., 2005).

Site-saturation mutagenesis: Site-directed saturation mutagenesis is used to quickly evolve the protein in laboratory conditions. In this system, each amino acid of a protein is exchanged with each of the other 19 naturally occuring amino acids. “Hotspots” of enzymes are the targets for saturation mutagenesis and enhanced thermostability or catalytic efficiency can be obtained by variants with single amino acid changes (Chen et al. 2012). A special form of this method is named as “iterative saturation mutagenesis” (ISM). It involves

- Randomized mutation of proper sites of residues
- Screening of the initial mutant libraries for properties such as catalytic efficiency, stereoselectivity, and thermal robustness
- Use of the best individual in a library as a template for saturation mutagenesis at other sites
- Continuation of the process until the desired degree of enzyme improvement has been reached (Gumulya et al. 2012).

Truncation: As some domains of proteins are not essential for enzyme activity, truncation can be used to enhance or change the properties of enzymes. This may be realized by random or directed truncation. In random mutation, mutants with desired properties are screened through a truncation library. On the other hand, by means of site-directed truncation, truncated enzymes can be directly obtained (Yang et al., 2014).

Fusion: Latterly, the building of new chimeric enzymes has become a novel and important phenomenon for enzyme engineering, especially to improve the catalytic quality. By fusing the catalytic and substrate binding domains of different enzymes, most chimeric proteins are obtained. Carbohydrate-active enzymes, for example, are good targets for fusion strategy. They contain two distinct modules, a catalytic module and a carbohydrate-binding module (CBM), that are discrete structural and functional units usually connected by a flexible linker. There are 54 defined families of CBMs based on amino acid similarities (Han et al.2013; Linke et al. 2012), and

these CBMs display significant diversity in ligand specificity. Therefore, different CBMs usually interact with different carbohydrates. Therefore, CBMs are usually fused with other enzymes to create new chimeric enzymes to improve catalytic quality.

A chart about how to choose the right strategy to engineer a protein is given in Figure 1.14.

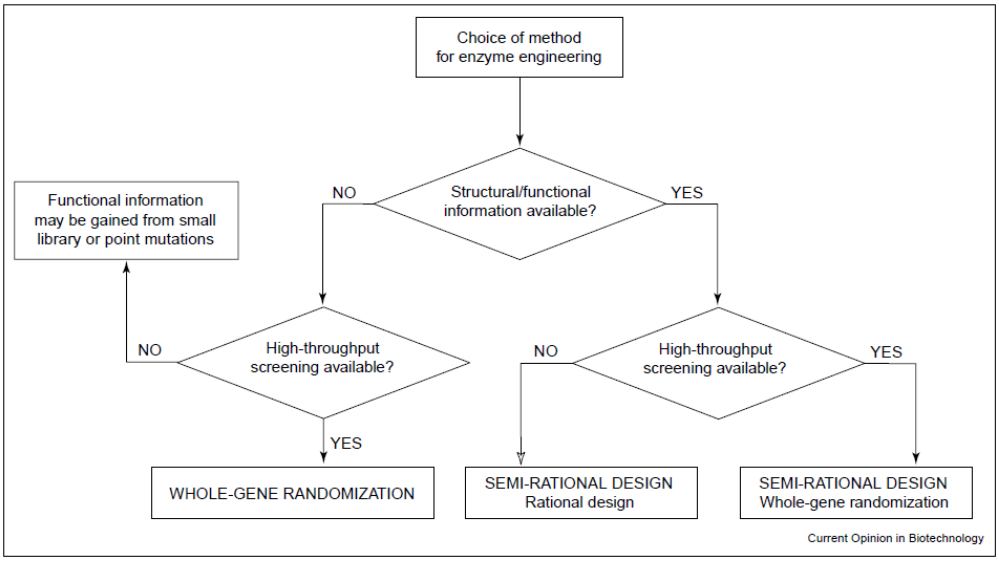

Figure 1.14: Selection of the preferred experimental approach for enzyme engineering based on the availability of tools and prior knowledge of structure and function.

2. MATERIALS & METHODS

2.1. Materials

2.1.1. Laboratory equipment

Equipments used are given in Table 2.1.

Table 2.1: Equipments used in the study.

Equipment	**Supplier company**
Centrifuge	Beckman Coulter Microfuge®18, Beckman Coulter Allegra 25R, Beckman Coulter J-30 I
High pressure steam sterilizer	TOMY SX-700E Tuttnauer Systec 2540 mL
Magnetic stirrer	Heidolph MR Hei-Standard
pH meter	Hanna Instruments HI 221 Microprocessor pH meter
Precision scale	Precisa XB 220 A Precisa BJ 610 C
Power supply	Biometra PowerPack P25T
Vortex	Heidolph
Ice machine	Scotsman AF 10
Microwave Oven	White Westinghouse MicroMagic
Thermal cycler	Biometra Professional
Shaker	Heidolph Duomax 1030
Freezers	SANYO Biomedical Freezer (-20oC), Electrolux SpacePlus (+4 oC), New Brunswick Scientific (-80 ^{o}C)
Spectrophotometer	Shimadzu UV 1601 UV visible spectrophotometer
Microplate Spectrophotometer	BioRad Benchmark Plus
Microfuge tubes	1.5ml, 2ml, Axygen
Centrifuge tubes	15ml, 50ml ISOLAB
Micropipettes	2.5µl, 10µl, 100µl, 200µl, Eppendorf 1000µl, Finnpipette Thermo
Spectrophotometer cuvettes	Plastibrand 1.5 mL semi-micro
Pure water system	TKA Wasseraufbereitungssysteme
Electrophoresis equipment	Whatman, Inc. Horizon 20-25
Thermoshaker	Biometra
Incubator	GFL
Centrifugal ultrafiltration tube	Milipore

2.1.2. Chemicals

Chemicals used are listed in Table 2.2.

Table 2.2: Chemicals used in the study.

Chemical	**Supplier company**
Sodium chloride (for analysis)	Merck
Yeast extract powder	Lab M Limited
Agar Bacteriological	Oxoid
Pepton from casein	Merck
Potassium chloride	GPR^{TM} BDH Laboratory supplies
Sodium pyruvate	Fluka
Ammonium persulfate for electrophoresis	Sigma
Tris (Hydroxymethyl)- aminomethan	Merck
EDTA (Ethylenediaminetetra-acetic acid Disodium salt)	GPR^{TM} BDH Laboratory supplies
Unstained protein marker	Fermentas SM0431

2.2. Methods

2.2.1. Cloning studies

In this study, pQE2 vector is used as cloning vector. In Figure 2.1, vector map of pQE2 is shown. This vector is 4800 bp. long.

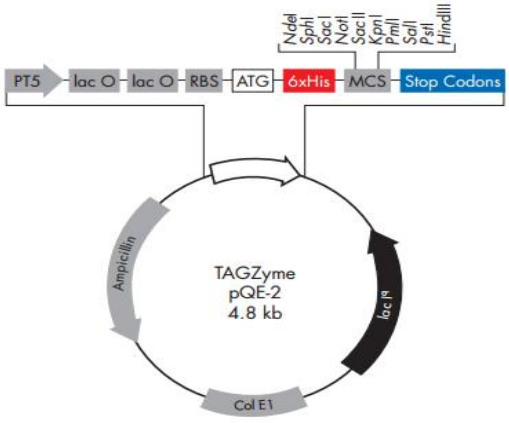

Figure 2.1: pQE-2 vector map

High-level expression of 6xHis-tagged proteins in *E. coli* using pQE vectors is based on the T5 promoter transcription–translation system. pQE plasmids belong to the pDS family of plasmids and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DHFRS (Bujard et al., 1987). These low-copy plasmids have the following features:

• Optimized promoter–operator element consisting of phage T5 promoter (recognized by the E. coli RNA polymerase) and two lac operator sequences which increase lac repressor binding and ensure efficient repression of the powerful T5 promoter

• Synthetic ribosomal binding site, RBSII, for high translation rates • 6xHis-tag coding sequence either 5' or 3' to the cloning region

• Multiple cloning site and translational stop codons in all reading frames for convenient preparation of expression constructs

• Two strong transcriptional terminators: t0 from phage lambda, and T1 from the rrnB operon of E. coli, to prevent read-through transcription and ensure stability of the expression construct

• β-lactamase gene (bla) conferring resistance to ampicillin at 100 μg/ml. The chloramphenicol acetyl transferase gene (CAT) present between t0 and T1 has no promoter and is not normally expressed. Depending on the bacterial strain and insert, low CAT activities may be detectable.

• ColE1 origin of replication.

2.2.2. Primer design

Primer design is accomplished by applying the instructions recommended in the QuikChange® II XL Site-Directed Mutagenesis Kit. According to this kit, primers are prepared by the following considerations:

• Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

• Primers should be between 25 and 45 bases in length, with a melting temperature (*T*m) of ≥75°C. Primers longer than 45 bases may be used, but using longer primers increases the likelihood of secondary structure formation, which may affect the efficiency of the mutagenesis reaction.

The following formula is commonly used for estimating the *T*m of primers:

$$T\text{m} = 81.5 + 0.41(\%\text{GC}) - 675/N - \%\ \text{mismatch} \qquad \textbf{(2.1)}$$

For calculating *T*m:

• *N* is the primer length in bases.

• values for %GC and % mismatch are whole numbers.

For calculating *T*m for primers intended to introduce insertions or deletions, use this modified version of the above formula:

$$Tm = 81.5 + 0.41(\%GC) - 675/N \quad \textbf{(2.2)}$$

where N does not include the bases which are being inserted or deleted.

• The desired mutation (deletion or insertion) should be in the middle of the primer with ~10–15 bases of correct sequence on both sides.

• The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases.

Applying these recommendations, the following primers are designed by the help of OligoEvaluator™ Sigma-Aldrich system.

• Sequence (5'-3'): CGG TCT GGA GCC AGG CTT ATA TCG GCG

F Primer: CGG TCT GGA GCC TGG CTT ATA TCG GCG

R primer: GCC AGA CCT CGG ACC GAA TAT AGC CGC

Analysis result of this primer pair is given in Figure 2.2.

1. Analysis Results

Sequence: 5' CGGTCTGGAGCCTGGCTTATATCGGCG 3'

Base Count	Molecular Weight	Extinction Coefficient	Oligo Type	µg/OD at 260 nm	Length (bp)	Tm (°C)	GC%	GC Clamp	Run Length (bp)	Secondary Structure	Primer Dimer	BLAST
A = 3, U = 0, G = 10, C = 7, T = 7, I = 0, Total = 27	8323.5	248.2	No Mod	33.5	27	78.9	63.0	5	2	Moderate	No	Sequence

Figure 2.2: Analysis result of the Tm 78.9 primer pair.

• Sequence (5'-3'): CCC GGT CTG GAG CCA GGC TTA TAT CGG CG

F primer: CCC GGT CTG GAG CCT GGC TTA TAT CGG CG

R primer: GGG CCA GAC CTC GGA CCG AAT ATA GCC GC

Analysis result of this primer pair is given in Figure 2.3.

1. Analysis Results

Sequence: 5' CCCGGTCTGGAGCCTGGCTTATATCGGCG 3'

Base Count	Molecular Weight	Extinction Coefficient	Oligo Type	µg/OD at 260 nm	Length (bp)	Tm (°C)	GC%	GC Clamp	Run Length (bp)	Secondary Structure	Primer Dimer	BLAST
A = 3, U = 0, G = 10, C = 9, T = 7, I = 0, Total = 29	8901.9	262.6	No Mod	33.9	29	82.1	65.5	5	3	Moderate	No	Sequence

Figure 2.3: Analysis result of the Tm 82.1 primer pair.

2.2.3. Polymerase chain reaction (PCR)

PCR is a method which enables the synthesis of new DNA strand complementary to the related template strand. It uses the enzyme DNA polymerase which has the ability to add nucleotides to 3'-OH group of the DNA. Thus, a primer is required to which add the first nucleotide. This situation also allows to select a particular region to amplify. As a consequence of PCR, a large number of copies of the desired sequence is produced.

In order to introduce the desired mutation to the *bs*LDH gene in pQE vector, the PCR reaction shown in Table 2.3. is set.

Table 2.3: PCR reaction for *bs*LDH Q203L mutation.

Content	Volume
Plasmid DNA template (119,9 ng/µl)	0,4 µl
Forward primer (10µM)	1 µl
Reverse primer (10µM)	1 µl
dNTP mix	1 µl
Quick solution mix	3 µl
Pfu polymerase	1 µl
10x reaction buffer	5 µl
ddH_20	37,6 µl
Total reaction volume	50 µl

To ensure that the PCR reaction is accomplished properly, a control reaction is also set to compare them in agarose gel electrophoresis. The reaction is set as shown in Table 2.4.

Table 2.4: Control PCR reaction for *bs*LDH Q203L mutation.

Content	Volume
Plasmid DNA template	2 µl
Forward primer	1,25 µl
Reverse primer	1,25 µl
dNTP mix	1 µl
Quick solution mix	3 µl
Pfu polymerase	1 µl
10x reaction buffer	5 µl
ddH_2O	35,5 µl
Total reaction volume	50 µl

PCR program for amplification is set as shown in Table 2.5.

Table 2.5: PCR program for amplification.

Segment	Cycles	Temperature	Time
1	1	95	1 min.
2	18	95	50 sec.
		60	50 sec.
		68	5 min
3	1	68	7 min.

2.2.4. Agarose gel electrophoresis

To separate DNA fragments by their sizes agarose gel electrophoresis is used. In this project, 1 % agarose gels were used to estimate the size of DNA molecules following PCR reaction and restriction enzyme digestion.

To prepare 1 % agarose gel :

1g agarose was dissolved in 50 ml 1X TAE (Tris/Acetic Acid/EDTA) buffer. The agarose was solubilized by heating until the agarose was completely dissolved. Gel was cooled to ≤ 50ºC, then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel. Approximately 15 min later the gel was

solidified and then placed into an electrophoresis tank, where the gel was covered with 0,5X TAE buffer. The DNA was mixed with 6X Ez
vision dye in the proportion of 5:1 and the sample was placed into a well on the agarose gel. For the fragment size control, Lambda DNA/EcoRI+Hind III Marker, 3 and Lambda DNA/Hind III Marker 2 were used. Electrophoretic separation was achieved by constant current at 100 mV for 1 h.

DNA within agarose gels were visualized under UV light. The size of the DNA was determined by comparing their mobility with the fragments of the DNA ladder.

2.2.5. Dpn I digestion of the amplification products

Methylation of adenine and cytosine is part of the restriction modification system of many bacteria, in which specific DNA sequences are methylated periodically throughout the genome. A methylase is the enzyme that recognizes a specific sequence and methylates one of the bases in that sequence. Foreign DNAs (which are not methylated by this way) that are introduced into the cell are degraded by sequence-specific restriction enzymes and cleaved, while bacterial genomic DNA is not recognized by these restriction enzymes. The methylation of native DNA allows bacteria to protect themselves from infection by bacteriophages, thus acts as a sort of primitive immune system. *E. coli* DNA adenine methyltransferase (Dam) is an enzyme of ~32 kDa that does not belong to a restriction/modification system. *E. coli* Dam recognizes and targets GATC sequence, as the methylation occurs at the N6 position of the adenine in this sequence (G mATC). The three base pairs flanking each side of this site also influence DNA–Dam binding. Dam plays several significant roles in bacterial processes, including mismatch repair, the timing of DNA replication, and gene expression. As a result of DNA replication, the status of GATC sites in the *E. coli* genome changes from fully methylated to hemimethylated.

Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5′-Gm6ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to Dpn I digestion.

- 1 µl of the Dpn I restriction enzyme was added (10 U/µl) directly to each amplification reaction.
- Each reaction mixture was thoroughly mixed by pipetting the solution up and down several times. Reaction mixtures were spinned down in microcentrifuge for 1 minute, then the reactions were immediately incubated at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

2.2.6. Bacterial strains

BL21 competent *E. coli* genotype fhuA2 [lon] ompT gal [dcm] ΔhsdS were used in the study.

2.2.7. Bacterial culture media

LB medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 liter distilled water. The media was sterilized by autoclaving at 121ºC for 10 min. After sterilization, Amp. antibiotic was added to the LB medium as 0,1% concentration to the total volume in order to make selection media.

LB- agar plate was prepared by adding 15 g/L of agar to LB medium and sterilized by autoclaving as described in LB medium.

SOC medium was used to cultivate *E. coli* cells for 1 hour after heat shock during transformation. It was prepared by dissolving 2 g tryptone, 5 g yeast extract, 0.058 g NaCl, 0.0186 g KCl, 0.095 g MgCl2, 0.23g MgSO4 and 0.36 g glucose in 100 ml distilled water and sterilized at 121 °C with autoclaving for 10 min.

2.2.8. Transformation of competent cells

Both XL10-Gold ultracompetent cells (provided with the QuikChange® II XL Site-Directed Mutagenesis Kit) and laboratory-made (BL21 competent *E. coli* genotype fhuA2 [lon] ompT gal [dcm] ΔhsdS) competent cells were used to compare the efficiencies.

Transformation is performed according to the QuikChange® II XL Site-Directed Mutagenesis Kit manual:

Both strains were thawed gently on ice.

For each control and sample reaction to be transformed, 45 µl of the ultracompetent cells were aliquotted to a prechilled Falcon® 2059 polypropylene tube. 2 µl of the β-ME mix (provided with the kit) was added to the 45 µl of cells. Contents of the tube

were swirled gently. Cells were incubated on ice for 10 minutes, swirling gently every 2 minutes. 2 µl of the *Dpn* I-treated DNA from each control and sample reaction were transferred to separate aliquots of the ultracompetent cells. Heat-pulse was applied to the tubes in a 42°C thermoshaker for 30 seconds. Tubes were incubated on ice for 2 minutes. 0.5 ml of preheated (42°C) SOC medium was added to each tube, then the tubes were incubated at 37°C for 1 hour with shaking at 250 rpm. 50 µl of each transformation reaction was plated on agar plates containing the Ampicilin antibiotic for the plasmid vector.

For the mutagenesis and transformation controls, cells were spreat on LB–ampicillin agar plates containing 80 µg/ml X-gal and 20 mM IPTG.

Transformation plates were incubated at 37°C for 16 hours.

Process up to the plating of DNA samples are summarized in Figure 2.4.

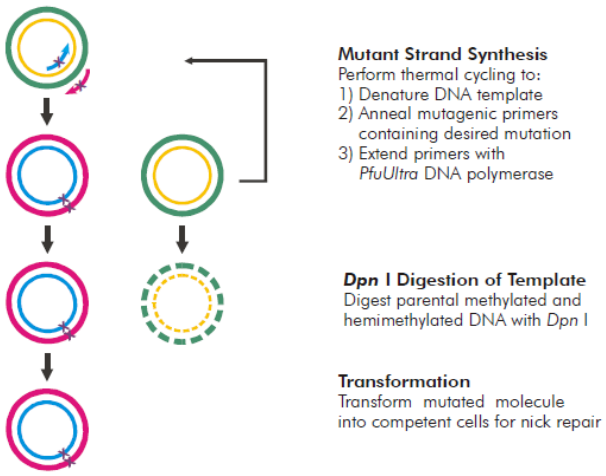

Figure 2.4: An overview of Quikchange site-directed mutagenesis method.

2.2.9. Plasmid purification from bacterial cultures

The StrataPrep plasmid miniprep kit was used to purify plasmid DNA from bacterial cultures. The method employs a modification of the alkaline method of cell lysis and a microspin cup with a silica-based fiber matrix that binds DNA in the presence of a chaotropic salt. The sample is combined with a ribonuclease-containing solution, a cell lysis solution, and a DNA-binding solution and is then transferred to a microspin cup that is seated inside a receptacle tube. The plasmid DNA binds to the fiber matrix in the microspin cup. The contaminants are then washed from the microspin cup with a wash buffer. The purified plasmid DNA is eluted from the fiber matrix with a low-ionic-strength buffer and captured in a microcentrifuge tube. The result is

purified plasmid DNA that is ready for restriction digestion, ligation, and sequencing reactions. Following steps are realized in order to obtain this purified plasmid:

A culture medium of 5ml with a single bacterial colony was inoculated and 5 µl Amp. was added. Cell culture was incubated 20 hours at 37°C with vigorous shaking. 1.5 ml of the cell culture was aliquotted into a 1.5-ml microcentrifuge tube. Tubes were spinned in microcentrifuge at maximum speed for 1 minute. After centrifugation, supernatant was removed and discarded. 100 µl of solution 1 was added to the microcentrifuge tubes. The tubes was vortexed to resuspend the cell–solution 1 mixture and completely disperse the cells. 100 µl of solution 2 was added to the microcentrifuge tubes. The contents were mixed by inverting the tube several times. 125 µl of solution 3 was added to the microcentrifuge tubes. Contents were mixed by inverting the tubes several times. Tubes were spinned in a microcentrifuge at maximum speed for 5 min. Supernatants were decanted. The caps of the 2-ml receptacle tubes were snapped onto the top of the microspin cup. Pellets and the microcentrifuge tubes were discarded. The samples were spinned in a microcentrifuge at maximum speed for 30 sec. The caps of the 2-ml receptacle tubes were opened, microspin cups were removed from the receptacle tubes, and the liquids were discarded. Microspin cups were replaced in the receptacle tube.

Endonucleases from the samples were washed according to the following protocol:

750µl of nuclease-removal buffer was added to the microspin cup. The cap of the2ml receptacle tube was snapped onto the top of the microspin cup. The sample was spinned in a microcentrifuge at maximum speed for 30 sec. The filtrate in the microcentrifuge tube was discarded. The microspin cup was placed back in the 2-ml receptacle tube and snap the cap of the receptacle tube onto the top of the microspin cup. Sample was spinned in a microcentrifuge at maximum speed for an additional 30 seconds. The microspin cup was removed and the filtrate was discarded. 750 µl of 1× wash buffer (prepared with adding the equal amount of 100% ethanol to the stock washing buffer) was added to the microspin cup. Sample was spinned in a microcentrifuge at maximum speed for 30 seconds. The microspin cup was removed and the filtrate was discarded. The sample was spinned in a microcentrifuge at maximum speed for an additional 30 sec. The microspin cup was transferred to a

fresh 1.5 ml tube. 50 µl of elution buffer was loaded directly on top of the fiber matrix at the bottom of the microspin cup. The sample was incubated at room temperature for 5 minutes. Then, the sample was in a microcentrifuge at maximum speed for 30 seconds. Microspin cup was discarded and the eluted DNA was obtained.

2.2.10. Determination of DNA concentration

Recovery, purity and concentration of nucleic acids were determined spectrophotometrically with NanoDrop 2000. The ratio of absorptions at 260 nm vs 280 nm is commonly used to evaluate the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acids) tends to absorb light at 280 nm. The ratio of absorbance (A260/A280) of a pure DNA solution is between 1.8 and 2.0. As protein contamination increases, the ratio decreases.

2.2.11.Identifying the mutated regions

DNA samples obtained from bacterial cell recovery are subjected to DNA sequencing.

2.2.12. Obtaining the WT and mutated cells from colonies

Glycerol stocks had been prepared from small scale cultures (10 mL) previously.
To obtain small scale culture again, 10ul glycerol stock ingredient was added to 10 ml LB+Amp. This mixture was growth overnight at 37^{o}C. The following day, this culture was added to 1L. LB+Amp. After ~ 3 h. later, when the OD_{600} value of the culture reached 0.6, the gene expression of the bacteria was induced by 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).
IPTG is an analog of lactose that inactivates the *lac* repressor. IPTG serves as a mimic of the naturally occurring allolactose that is an inducer of the *lac* operon. IPTG induction is commonly used in protein expression.
After 4h. of IPTG induction, samples were centrifuged at 5000 rpm for 20 min. to extract cells from growth medium.

2.2.13. Enzyme purification from bacterial cells

Both WT and mutated cells obtained by centrifugation were dispersed in lysis buffer + lysozyme mixture (1mg lysozyme per 1ml lysis buffer). This mixture was chilled on ice for 20 min. After that, the samples were subjected to sonication for 2 min. by 10 sec on/20 sec off intervals. To remove the cellular debris, samples were centrifuged at 10.000 g for 10 min. To each supernatant (obtained from 250 mL culture), 500uL Ni-NTA resin was added to attach our His-tagged protein.

2.2.14. The 6xHis tag purification of enzyme

The most commonly used tag for collecting large amounts of highly purified protein is a poly-histidine tag (His-tag). It comprises 6 – 14 histidines and is typically fused to the N- or C-terminal end of a target protein. In some cases, the tag can also be inserted into an exposed loop of the target protein. The imidazole side chains of a His-tag can engage inreversible coordinative bonds to certain transition metal ions, such as Ni2+, Co2+, or Zn2+, respectively.

Ni2+ shows the highest affinity and selectivity for His-tags and is therefore preferred. Using certain chelators covalently linked to a matrix, Ni2+ can be immobilized in a way that still allows the Ni2+ ion to interact with histidine side chains. When His-tagged proteins are applied to this Ni2+ matrix, they specifically bind to the resin, while most untagged proteins do not. Bound proteins can be released from the matrix using mild conditions. Imidazole competes for coordination sites on Ni2+ displacing His-tagged proteins from the matrix. Alternatively, lowering pH will protonate His-tags and hence also elute the His-tagged proteins.

Ni-NTA system

Nitrilotriacetic acid (NTA), is a tetradentate chelating adsorbent developed at Hoffmann-La Roche that overcomes these problems. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag as shown in Figures 2.5 and 2.6. NTA binds metal ions far more stably than other available chelating resins (Hochuli, 1989) and retains the ions under a wide variety of conditions, especially under stringent wash conditions. The NTA matrices can therefore bind 6xHis-tagged proteins more tightly than IDA matrices, allowing the purification of proteins from less than 1% of the total protein preparation to more than 95% homogeneity in just one step (Janknecht et al., 1991).

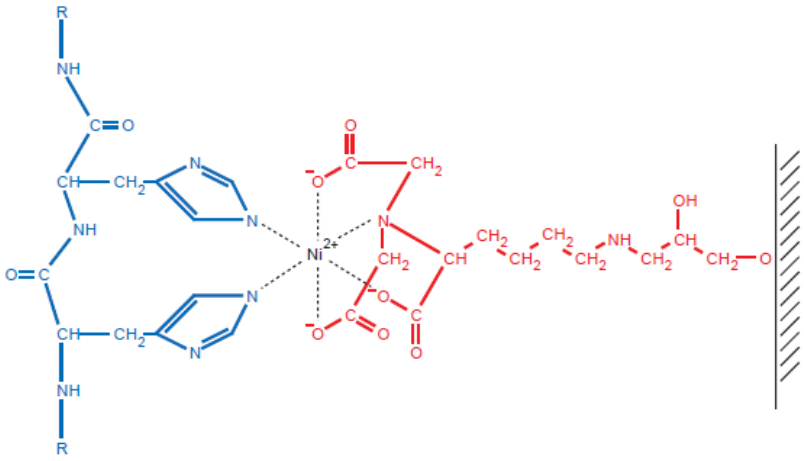

Figure 2.5: Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix.

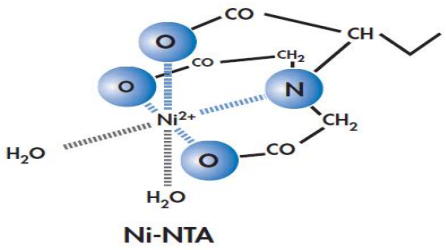

Figure 2.6: Interaction of NTA chelate matrice with nickel ion.

Ni-NTA agarose added samples were incubated at 4ºC for 40 min. After 40 min, samples were centrifuged at 4000 g for 5 min. The supernatant was taken to be analysed in SDS-PAGE. The resin-binded protein was washed 3 times with 750 uL wash buffer , in each step centrifuging at 4000 g for 1 min. These washed samples were collected and labeled as W1, W2 and W3, again to analyse in SDS-PAGE.

After washing steps, elution was realized 3 times by 250 uL elution buffer centrifuging at 4000 g for 1 min every time. after each step, the supernatants were taken and labeled as E1, E2 and E3.

Lysis buffer (1 liter):

50 mM NaH_2PO_4 6.90 g NaH2PO4·H2O (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)

pH adjusted to 8.0 using NaOH.

Wash buffer (1 liter):

50 mM NaH2PO4 6.90 g NaH2PO4·H2O (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)

pH adjusted to 8.0 using NaOH.

Elution buffer (1 liter):

50 mM NaH2PO4 6.90 g NaH2PO4·H2O (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)

pH adjusted to 8.0 using NaOH.

2.2.15. SDS-PAGE analysis of the purified protein

SDS-PAGE separates proteins primarily by mass because the ionic detergent sodium dodecyl sulfate (SDS) denatures and binds to proteins to make them evenly negatively charged. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel toward the positively charged electrode. Proteins with less mass travel more quickly through the gel than those with greater mass because of the sifting effect of the gel matrix. Once separated by electrophoresis, proteins can be detected in a gel with various stains, transferred onto a membrane for detection by Western blotting and/or excised and extracted for analysis by mass spectrometry.

To obtain optimal resolution of proteins, a stacking gel is cast over the top of the resolving gel. The stacking gel has a lower concentration of acrylamide, lower pH and a different ionic content. This allows the proteins in a loaded sample to be concentrated into a tight band during the first few minutes of electrophoresis before entering the resolving portion of a gel. A stacking gel is not necessary when using a gradient gel, as the gradient itself performs this function.

The SDS gels were prepared as shown in Table 2.6.

Table 2.6: Ingredients of SDS gel used in the study.

	Resolving gel (12%)	**Stacking gel (5%)**
dH_2O	2 mL	1.4 mL
40% Acrylamide	1.5 mL	0.25 mL
1.5 M Tris (pH 8,8)	1.3 mL	-
1 M Tris (pH 6,8)	-	0.25 mL
10% SDS	0.05 mL	0.02 mL
APS (10%)	100 uL	50 uL
TEMED	10 uL	5 uL

Samples were loaded into the gel with 5X Laemmli buffer (1uL laemmli buffer per 5 uL sample). Proteins were denatured at 95ºC for 15 min, and the marker for 5 min. Fermentas Unstained Protein Molecular weight marker (SM0431) was used to determine protein range.

The gel was run at 120 V for 1 h. Then, the gel (resolving part) was taken into a container with Coomassie blue for staining and incubated at RT on a shaker overnight.

The day after, the gel was conveyed to destaining solution and incubated on the shaker at RT for 45 min. When destaining was complete, the gel was visioned using densitometer.

2.2.16. Ultrafiltration of the eluted protein

Ultrafiltration is a method to concentrate protein or other macromolecules through a membrane with defined pores. The membranes used in this system have a molecular weight cut-off (MWCO) which is the limit of size (or range) of a protein that can pass through the membrane. Each membrane also has a chemical characteristic for binding proteins and stability in various solvents. There are two main methods to force the protein containing fluid through the membrane, pressure from an inert gas

or centrifugal force. Larger volumes are more suited to the pressure stirred cells. For smaller volumes the centrifugal devices are easier and more practical to use. In centrifugation, the hydrostatic pressure during centrifugation forces the fluid through the membrane. Proteins larger than the MWCO are retained and the smaller molecules, like the salts, buffers, water and small proteins pass through.
Ultrafiltration was realized using centrifugal ultrafiltration tubes of MWCO size 30.000 Da. Eluted protein (E3) was solved in 10 mL 50mM Tris-HCl Buffer pH 7.4. To obtain ~1000 uL ultrafiltered protein, the mixture was centrifuged at 2000 rpm for 20 min. Then, the protein was aliquotted into 2 mL centrifuge tubes for further use.

2.2.17. Indication of enzyme concentration

Enzyme concentration was measured using Microplate Spectrophotometer. Bovine Serum Albumin (BSA) of determined concentrations were used to assess the unknown concentrations. To realize this, 5 uL of protein and 195 uL Bradford reagent were used for each well.

2.2.18. Kinetic measurements

All steady-state kinetic parameters were measured from the decrease in extinction at 340 nm at 25^{o}C in 50 mM Tris buffer pH 7.4. Various substrate concentrations were used both in WT and mutant enzymes. 0.2 mM NADH and 5 nM *bs*LDH were used in each reaction in the absence or presence of FBP (5 mM).

3. RESULTS & DISCUSSION

3.1. Cloning the insert into pQE2 vector

To form the Q203L mutation in *bs*LDH gene sequence, PCR was realized with the appropriate primers. In order to ensure that the plasmid carries the desired insert, Dpn I-digested and non-digested samples were run by agarose gel electrophoresis. Electrophoresis result is shown in Figure 3.1. DNA fragment of interest is visualized at ~5600 bp region, as the pQE vector is 4.8 kbp and *bs*LDH sequence is ~800 bp long.

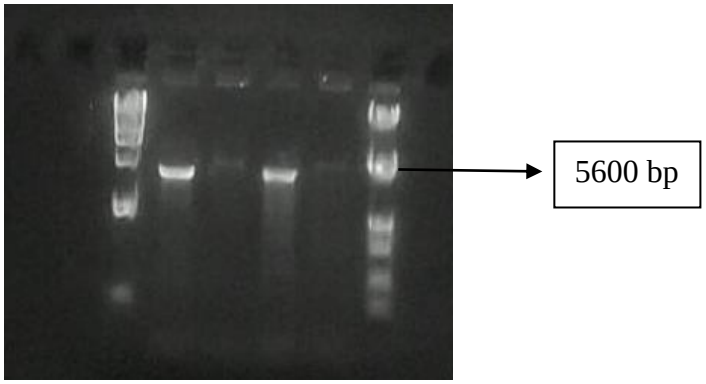

Figure 3.1: Agarose gel image of both Dpn I digested and non-digested samples.

3.2. Obtaining colonies following transformation

Following transformation of Dpn I digested and control DNA samples, plates were incubated at 37^{o}C overnight. 16h. later, the plates are taken from 37^{o}C. As shown in Figure 3.2 , transformation with XL10-Gold® ultracompetent cells was more effective than transformation with BL-21 strain, as higher amounts of colonies are obtained from β-mer plate.

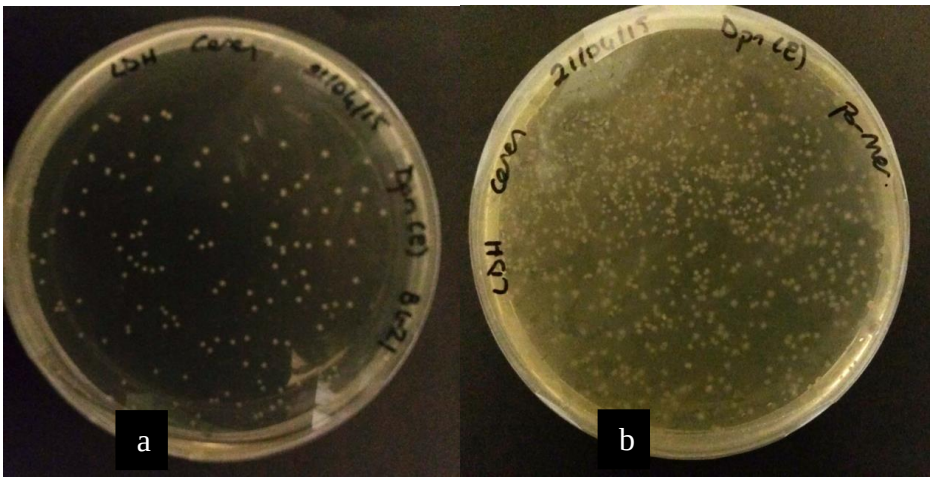

Figure 3.2: Colonies formed following transformation of Dpn I digested DNA
a: in BL-21 competent cell strain, b: in XL10-Gold® ultracompetent cells.

3.3. Sequencing the plasmid with *bs*LDH gene to demonstrate the correct mutations

After obtaining colonies from both plates, plasmid DNA purification was realized. According to the sequencing data, the mutations were verified to be correct as shown in Figures 3.3, 3.4 and 3.5.

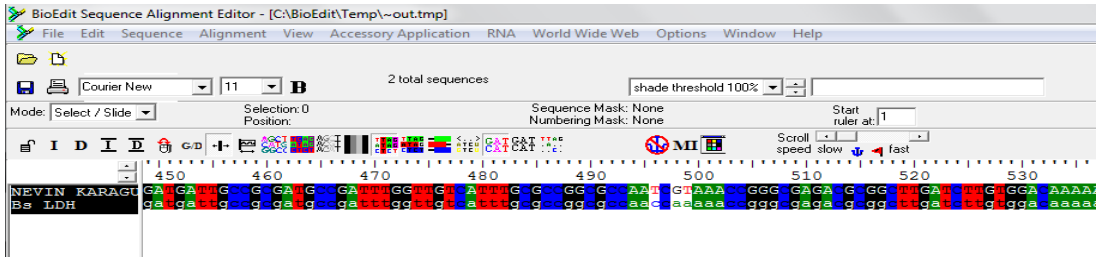

Figure 3.3: Mutation in Q102R position in *bs*LDH.

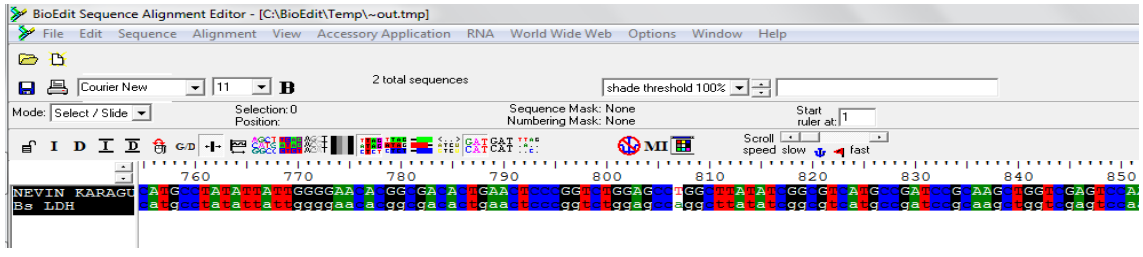

Figure 3.4: Mutation in Q203L position in *bs*LDH.

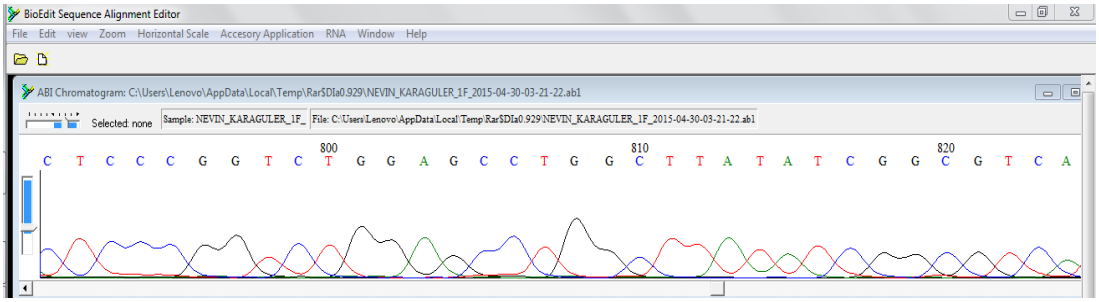

Figure 3.5: Absorbance data of Q203L mutation in *bs*LDH.

3.4. Determination of the Purified Enzyme

Following lysis, washing and elution steps, obtained protein mix was subjected to SDS-PAGE analysis in order to determine the presence of *bs*LDH enzyme which is 35 kDa in molecular mass. WT and mutant proteins were investigated for both wash and elution steps. SDS-PAGE image of the WT and mutant enzymes are given in Figure 3.6 and 3.7 respectively.

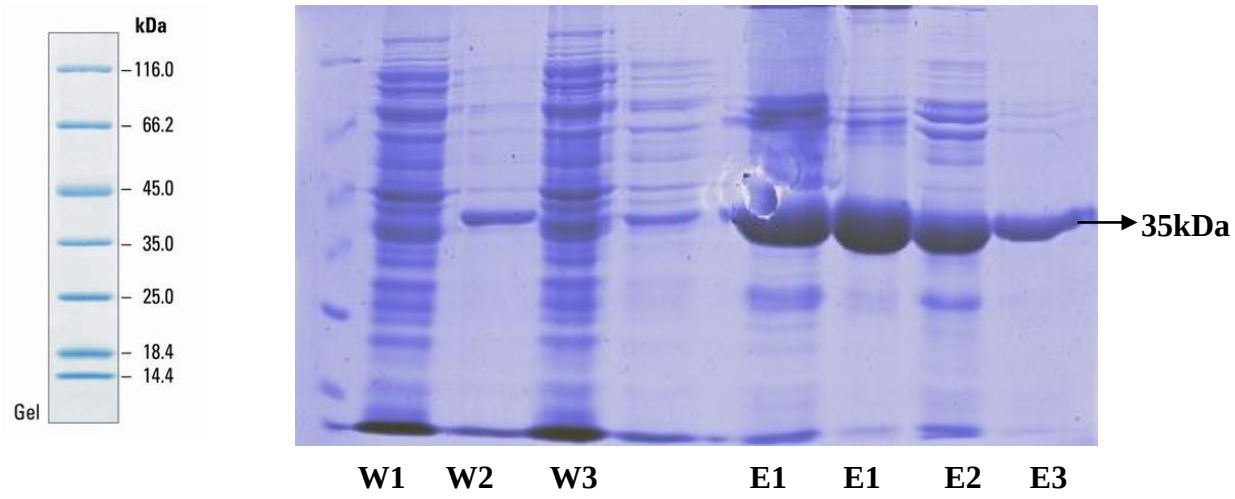

Figure 3.6 : SDS-PAGE result of the wild type *bs*LDH (W: successive washing steps ; E : succesive elution steps).

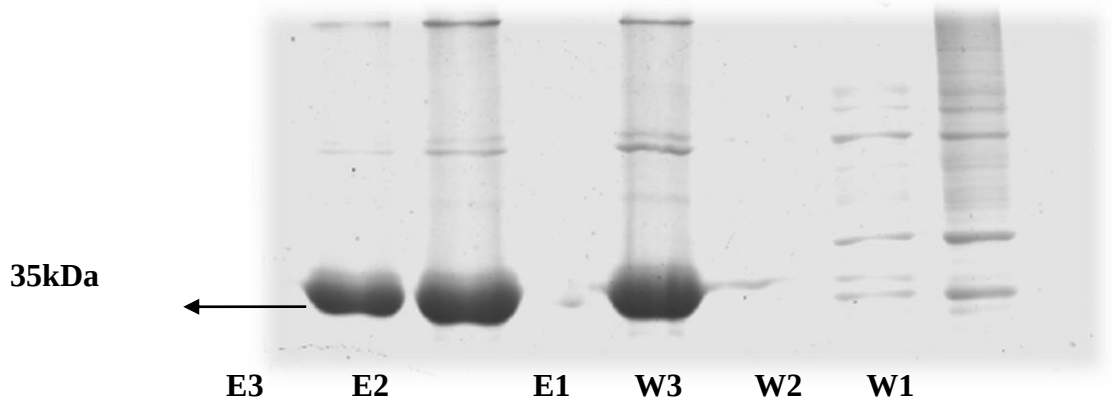

Figure 3.7: SDS-PAGE result of the mutant *bs*LDH (W: successive washing steps; E : succesive elution steps).

3.5. Indication of Enzyme Concentration After Ultrafiltration

Enzyme concentrations were found out to be 0.538 mg/mL for WT and 0.217 mg/mL for mutant proteins. Ingredients were calculated as to correspond to 0.2 mM NADH, 5 nM *bs*LDH and 5mM FBP in a total reaction volume of 1mL.

3.6. Kinetic Measurements

Absorbance data at 340 nm of WT and mutant enzymes are given in Table 3.1. Rates were calculated subtracting the t_1 (1.min) value from t_0 (data recorded as soon as placing the reaction cuvette) value.

The steady-state catalytic properties of WT and mutant enzymes were measured at 25^{o}C by spectrophotometer in the absence and presence of FBP. Vmax and Km values were calculated by GraFit 7 software programme along with the Michaelis-Menten and Lineweaver-Burk plots.

Table 3.1: Rates of WT and mutant enzymes according to various pyruvate concentrations

	WT		Mutant	
Pyruvate concentration	- FBP	+ FBP	- FBP	+ FBP
0,01mM	0,0310	0,0120	0,0741	0,0978
0,02 mM	0,0223	0,1428	0,0280	0,0442
0,04 mM	0,0257	0,1491	0,0259	0,0873
0,06 mM	0,2038	0,1439	0,0005	0,0702
0,08 mM	0,0036	0,0745	0,0007	0,0014
0,1 mM	0,1233	0,2225	0,0027	0,0029
0,15 mM	0,2340	0,0120	0,0104	0,2165
0,2 mM	0,2883	0,2937	0,0000	0,0168
0,25 mM	0,2225	0,2774	0,0145	0,0595

Kinetic data of *bs*LDH native and mutants with different substrates are shown in Table 3.2. In this table, Vmax, Km and Kcat values of wild type and single mutants are given from the reference studies. Also, the double mutant data, which is the subject of our work is included with its correlated WT to comparatively evaluate both the effect of FBP and the how the function of the enzyme alters as shifted from native enzyme to various mutant types. Because the Q203L mutation causes the enzyme to work almost in a fully activated way in the absence of FBP, but not an increase in substrate specificity, there is no data for Q203L in oxaloacetate and α-keto malonate sections. Here, the Vmax, Km and Kcat values obtained in our work for WT and mutant enzymes, using pyruvate, the natural substrate of the enzyme, are shown in "WT *bs*LDH *** with FBP" part. As we did not observe any activity without FBP, this part is stated as ND, not detected. Regions indicated as ---, belongs

to the ongoing experiments made by using oxaloacetate and α-ketomalonate. By completion of these data, the kinetic behaviour of *bs*LDH Q102R and Q203L double mutant through diversified substrates and FBP need will be elucidated.

As an example for WT with FBP k_{cat} was calculated as follows:

Km = 0.0597 mM Vmax = 0.3559 sec^{-1}

$A = \varepsilon . C . I$ **(3.1)**

I = 1 cm $\varepsilon_{NADH} = 6220 \rightarrow 0.3559 = 6220 . C . 1$ $\Delta C = 6 \times 10^{-5}$

$k_{cat} = 6 \times 10^{-5} / 5 \times 10^{-9} = 12000\ sec^{-1}$

Similar calculations will be performed for ongoing experiments with oxaloacetate and α-keto malonate substrates.

Table 3.2: Kinetic parameters of native, Q102R mutant, Q203L mutant and Q102R&Q203L double mutant enzymes with various substrates in the absence/presence of FBP.

Enzyme	Substrate											
	Pyruvate						Oxaloacetate					
	+FBP			-FBP			+FBP			-FBP		
	Vmax	Km (mM)	K cat (s^{-1})	Vmax	Km (mM)	K cat (s^{-1})	Vmax	Km (mM)	K cat (s^{-1})	Vmax	Km (mM)	K cat (s^{-1})
Wt *bs*LDH *		0.062 ±0.004	140±3		2.7±0.17	240±3		3.42±0.02	6.3±0.05		4.1±0.2	6.1±0.3
Q102R *		2.1±0.01	1.1±0.3		1.9±0.1	0.89 ±0.06		0.06 ±0.003	253±3		0.062 ±0.003	234±2
Q203L **		0.05	30		0.07	36						
Q102R +Q203L ***	ND	ND	ND	ND	ND	ND	---	---	---	---	---	---
Wt *bs*LDH ***	0.3559	0.0597	12000	ND	ND	ND	---	---	---	---	---	---

* data taken from Binay et al., 2009
** data taken from Allen and Holbrook, 2000
*** data obtained by this work

Table 3.2 (cont'd): Kinetic parameters of native, Q102R mutant, Q203L mutant and Q102R&Q203L double mutant enzymes with various substrates in the absence/presence of FBP.

Enzyme	Substrate					
	α -ketomalonate					
	+FBP			-FBP		
	Vmax	Km (mM)	K cat (s^{-1})	Vmax	Km(mM)	K cat(s^{-1})
Wt *bs*LDH *		70±2	2.13±0.07		18.8±1.4	1.5±0.01
Q102R *		3.2±0.2	244±4		3.0±0.2	223±3
Q102R +Q203L ***	---	---	---	---	---	---
Wt *bs*LDH ***	---	---	---	---	---	---

* data taken from Binay et al., 2009
*** data obtained by this work

4. CONCLUSION

*bs*LDH is a commercially substantial enzyme owing to its use in industry to produce agrochemicals and pharmaceuticals. The technical enzymes including these groups comprise 65% of the worldwide industrial enzyme market. On the other hand, enzymes are being more and more popular against their synthetic equivalents by their advantages of biodegradability, being derived from renewable sources and ability to work under milder pH and temperature conditions. Considering these factors together, *bs*LDH is worth being interested in from the protein engineering point of view.

Moreover, *bs*LDH has some limiting features which makes it difficult to use in industry such as:

- Narrow substrate range
- Activation by an expensive activator, FBP
- Substrate inhibition : being inhibited by excess amount of pyruvate/lactate

All these disadvantegous properties, from the other side, make bsLDH enzyme an appropriate target for protein engineering.

Previous studies on *bs*LDH sequence showed that Q102R single residue mutation gives rise to a switch in substrate specificity by three orders of magnitude from lactate to malate (Wilks et al., 1988).

On the other hand, a *bs*LDH mutant carrying 3 amino acid substitutions, one of which is Q203L alteration, was found out to be almost fully activated in the absence of the natural activator, FBP (Allen and Holbrook, 2000). This shift is most likely be caused by this particular residue as it is found in the dimer-dimer interface of the gene sequence.

In light of this information, by this work, it is aimed to investigate the double mutant (Q102R and Q203L) effect on both substrate specificity and activator requirement and to inquire into the kinetic behaviour of the newly formed enzyme by using rational design methods. In order to accomplish the work, Q102R and Q203L mutants were constituted by site-directed mutagenesis in *bs*LDH sequence which

was found in pQE2 vector. To be used in the PCR reaction, appropriate oligonucleotides were designed to generate Q102R and Q203L mutations. Following the PCR with these oligos, mutant dsDNAs were selected by Dpn I digestion. . After that, mutated DNA was transformed into *E.coli* competent cells. Plasmid DNA was purified following the transformation and samples were controlled to ensure to have achieved the correct mutations. After confirming to form Q102R and Q203L mutations in *bs*LDH sequence, mutated DNA was amplified in large-scale *E.coli* culture. The culture was precipitated in order to obtain protein from cells. Desired protein with 6xHis-tag was sorted from the protein pool by Ni-NTA resin system which selectively binds His-tagged proteins. Thereafter, by SDS-PAGE, the protein was visualized at the region of its molecular mass (35kDa) and have ensured to obtain the enzyme. By ultrafiltration, smaller proteins and other contaminants were removed and a purer enzyme was attained.

Completing kinetic measurements, the Km value of the WT enzyme in the presence of FBP was found to be 0,0597 mM using pyruvate as substrate consistent with the mentioned data (Allen & Holbrook, 2000; Km = 0,05mM). However, no detectable data could be obtained from our double mutant when subjected to kinetic measurements with pyruvate. Actually this phenomenon is consistent with the Q102R mutant enzyme feature : functioning as a malate dehydrogenase. Moreover, obtaining the WT data similar to the reference study implies that the mutant works correctly.

To further investigate the double mutant effect (Q102R and Q203L) on *bs*LDH sequence, additional studies will be carried on analyzing the kinetic parameters using oxaloacetate and alpha keto malonate as substrates both in the absence and presence of FBP. Also, these information will enlighten the contribution of the mutation to the activator requirement.

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APPENDICES

APPENDIX A: Amino asid abbreviations

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

Figure A.1: Amino asid abbreviations.

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