ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

PROTEIN ENGINEERING APPLICATIONS ON NAD^+ -DEPENDENT L- LACTATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

M.Sc. Thesis by
Barış BİNAY, B.Sc.
(521031206)

Tezin Enstitüye Verildiği Tarih : 11 Ağustos 2005
Tezin Savunulduğu Tarih : 04 Ağustos 2005

Tez Danışmanı : **Assist. Prof. Dr. Nevin GÜL KARAGÜLER**
Diğer Jüri Üyeleri : **Yard. Doç. Dr. Müşerref ÖZEREN**
Yard. Doç. Dr. Çenk SELÇUKİ

AĞUSTOS 2005

Acknowledgements

I am grateful to my supervisor Assistant Prof. Dr. Nevin Gül KARAGÜLER for her guidance, patience, continued advice, supports and discussions in every stage of my master thesis. She shared all her sources, materials and the most importantly her valuable time with me. It was a great opportunity for me to work with her and to participate in her research project.

Special thanks to lab mates Volkan DEMİR, Anıl CEBECİ, and Sibel ÇETİNEL for their continued help and support within my laboratory hours. I sincerely thank all of my teachers and friends for sharing their laboratory experiences.

I am greatly indebted to my family; my father Ali BİNAY and my mother Hatice BİNAY, my brother Baykal BİNAY and my friends; Fatma ÖZBAY, Düzgün DOĞAN 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), Advanced Technologies in Engineering, Istanbul Technical University are gratefully acknowledged.

AUGUST 2005 BARIŞ BİNAY

CONTENTS

4. PROTEIN PURIFICATION

ABBREVATIONS

A	Adenine
A	Absorbance
´	Angstrom
Amp	Ampicilin
Amp^R	Ampicilin resisitance genes
APS	Ammonium persulfate
ATP	Adenosine 5' – triphosphate
bs	*Bacillus stearothermophilus*
bp	Base pair
Bis	N'N – methylene bisacrylamide
β – gal	β – galactosidase
°C	Degrees celsius
C	Cytosine
c	centi
d	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxytimine triphosphate
dNTPs	Deoxyribonucleotides 5' - triphosphate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
E. coli	Escherichia coli
EtBr	Ethidium bromide
FBP	Fructose - 1, 6 - bisphosphate
g	gram
G	Guanine

K	Degrees Kelvin
k	Kilo
k_{cat}	Turnover number of an enzyme
kD	Kilo Dalton
K_i	Inhibitor constant
K_M	Apparent Michaelis constant
l	Liter
lac	Lactate
LB	Luria - Bertani
ldh	Lactate dehydrogenase gene
LDH	Lactate dehydrogenase
µ	Micro
M	Molar
mA	miliampere
m	Milli
m	meter
min	Minute
mol	Mole
MCS	Multiple cloning site
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
Pyr	Pyruvate
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute

SDS	Sodium dodecyl sulfate
Sec	Seconds
ssDNA	Single stranded DNA
T	Thymine
TBE	Tris borate EDTA buffer
TEA	Triethanolamine buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
tRNA	Transfer RNA
U	Units of enzyme activity
U	Uracil
UV	Ultraviolet light
V_{max}	Maximum rate of catalysed by enzyme
Vol	Volume

Amino Acids

	One - letter code	Three - letter-code	Name
1	A	Ala	Alanine
2	C	Cys	Cysteine
3	D	Asp	Aspartic Acid
4	E	Glu	Glutamic Acid
5	F	Phe	Phenylalanine
6	G	Gly	Glycine
7	H	His	Histidine
8	I	Ile	Isoleucine
9	K	Lys	Lysine
10	L	Leu	Leucine
11	M	Met	Methionine
12	N	Asn	Asparagine
13	P	Pro	Proline
14	Q	Gln	Glutamine
15	R	Arg	Arginine
16	S	Ser	Serine
17	T	Thr	Threonine
18	V	Val	Valine
19	W	Trp	Tryptophan
20	Y	Tyr	Tyrosine

LIST OF THE TABLES

LIST OF THE FIGURES **Page Number**

ÖZET

BACILLUS STEAROTHERMOPHİLUS'TAN İZOLE EDİLEN NAD^+ BAĞIMLI L- LDH ENZİMİNE PROTEİN MÜHENDİSLİĞİNİN UYGULAMALARI

Bacillus stearothermophilus' tan izole edilen NAD^+ bağımlı L - LDH enzimi saf kiral hidroksi bileşiklerin üretilmesindeki endüstriyel potansiyelinden dolayı katalitik ve yapısal özellikleri açısından yoğun bir şekilde araştırılmaktadır. Kiral alfa hidroksi asitlerin şimdiki endüstrideki üretimi 10 ton olup, 0.1 M dan daha fazla miktarda substrat (piruvat) kullanımını sağlayabilecek herhangi bir gelişme endüstriyel açıdan oldukça karlı olacaktır. Pirüvatın laktata dönüşümündeki reaksiyon, oxo-asit substratın, karbonil grubunun koenzim olarak kullanılan NADH' ten alınan hidrojen iyonu ile indirgenmesi şeklinde gerçekleşir. LDH'lerin dezavantajları substrat olarak kullanılan pirüvatın aşırı konsantrasyonunda inhibe olması ve geniş bir substrat spesifikliğinin olmayışıdır.

LDH'ın endüstriyel kullanımını kısıtlayan bu dezavantajlarının uzaklaştırılmasında protein mühendisliği umut verici bir uygulamadır. Proje süresince, istenilen özelliklerdeki LDH'i üretmek için bölgeye özel mutasyon ve DNA suffling olmak üzere 2 farklı strateji uygulandı.

.

SUMMARY

PROTEIN ENGINEERING APPLICATIONS ON NAD^+-DEPENDENT L-LACTATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHİLUS*

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. 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.

In order to make LDH more suitable enzyme for its industrial applications, protein engineering is a promising approach. Two routes are used in this project to produce LDH enzyme with the desired properties. These are site directed mutagenesis and DNA shuffling.

1. Introduction

1. 1. Lactate Dehydrogenase

L-lactate dehydrogenases (LDH) (EC 1.1.1.27, LDH) are members of the family of NAD-dependent 2-hydroxycarboxylate dehydrogenases.LDHs are tetrameric enzymes catalyzing the last step of glycolysis in which pyruvate is converted to L-lactate by using NADH as a coenzyme (Figure1.1)[1].

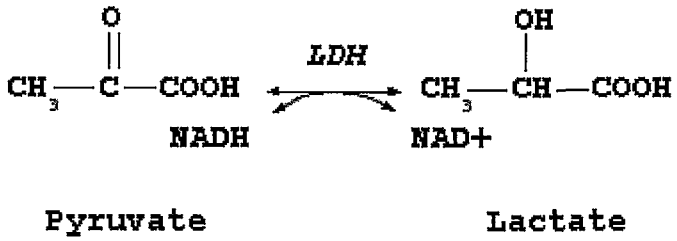


Figure 1. 1. The reaction catalysed by NAD^+-dependent lactate dehydrogenases.

The major biological function of this reaction is in sustaining the flux of sugars through glycolysis. The oxidative processes in this series of glycolysis reactions provide ATP as a source of energy, converting NAD^+ to NADH in the process. To enable glycolysis to continue, NADH must be reoxidised and this is achieved by conversion of pyruvate to lactate in the LDH reaction. The lactate is usually excreted from the cell.

1. 1. 1. Molecular Properties of LDH

The enzyme has been isolated from many species. The first X-ray diffraction results were reported in 1964 for pig M4 enzyme that is a tetramer of Mr 140000 Da. The two forms of the enzyme, the H, predominating in heart muscle, and the M, predominating in skeletal muscle, give rise to family isozymes[2]. There are five main LDH isoenzymes made up of tetramers formed from the combination of two subunit M (muscle) and H (heart) in different rates in many organisms. A third homotetramer of X-subunits is present only in adult male testicular tissue. The amino acid sequence difference between the subunits results in the homotetrameric enzymes LDH-M4,-H4

and -X4 having significantly different enzyme properties particularly with regard to kinetic parameters[3].

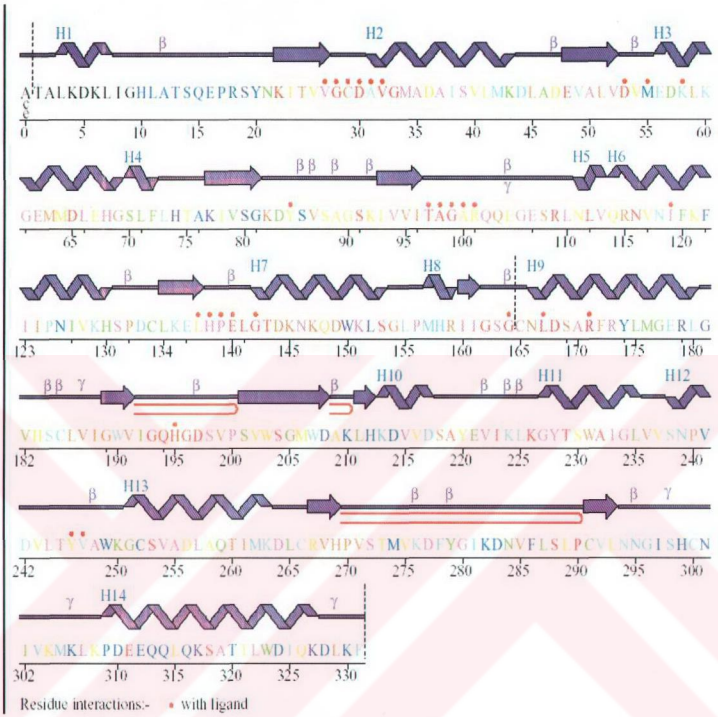
The NAD^+ binding region which of the protein makes up four α-helices and six strands of parallel β-sheets, is very similar in a variety of dehydrogenases. The conformation of NAD^+ bound to L-LDH, alcohol, malate, D-lactate, glyceraldehydes 3-phosphate and to glutamate dehydrogenase is also nearly the same. The adenosine moiety of NAD^+ is bound in a hydrophobic crevice. In contrast, the nicotinamide unit is bound so that the reactive side of the ring is in a polar environment, whereas the other side makes contact with hydrophobic residues of the enzyme.

Some lower organisms contain D-lactate specific LDH. In a survey of Anthropoida, Mollusca, Annelida, and Coelenterata, Lang and Kaplan showed that while all species have LDH, they never have both D-and L-specific forms. Also in these some lower organisms, some results showed that active molecule is only a dimer[4].

Two types of LDH are known: the prokaryotic-type enzymes and the more intensively studied, but structurally related, eukaryotic type enzymes. The *Bacillus stearothermophilus* (*bs*) enzyme is a typical prokaryotic type enzyme. Vertebrate LDHs are non-allosteric, but some bacterial LDHs are activated by an allosteric effector, fructose-1,6-bisphosphate (FBP). L-2-hydroxyisocaproate dehydrogenases and tetrameric LDH-like MDHs are also included in this group. The amino acid sequence of thermophilic LDH from *Bacillus stearothermophilus* (*bs* LDH) was obtained by purification of the enzyme and amino acid sequencing. The amide assignments were made from the gene derived amino acid sequence. The polypeptide chain of thermophilic *bs* LDH consists of 317 amino acid residues. There are no disulphide bridges on LDH and it does not have any metals (Figure1.2.) [1, 5].

1. 1. 2. The structure of the enzyme

The structures of the ternary complexes of LDH were deduced from crystallographic studies of the binding of NAD-pyruvate[6]. Lactate dehydrogenase from *Bacillus stearothermophilus* was crystalliased in 1982 and the three-dimensional crystal structures of the enzyme were solved in in the apo and holo forms (Figure1.3) [7].



Conservation colouring: Low 1 2 3 4 5 6 7 8 9 High

Figure 1. 2. The domains of the *bs* LDH[8].

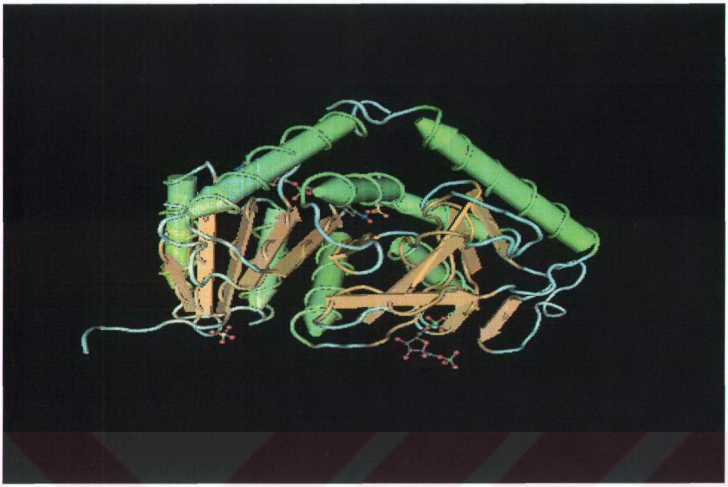


Figure 1. 3. The structure of the *bs* LDH[8].

1. 1. 3. Active site

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.

Enzymes convert substrates to products by breaking and making chemical bonds and so seem logical that at some point in proceedings enzyme and substrate are chemically bound together briefly in an enzyme-substrate complex at the active site.

Because of the different sources of LDHs, sequences and conformations of LDHs are different in spite of the same substrate. In *bs* LDH residues 98 to 110 consist of the active site. Conformation of this loop determines substrate.

In bacterial enzyme the loop adopts more conformations, with the tip of the loop being some 2 Å further away from Tyr-237 that represents the lip of the active site pocket resulting in the widening of a hole at the top of the active site. This hole is made even more accessible because of the smaller bulk of the side-chain at the tip of the loop that would obscure the hole in the eukaryotic enzymes. This residue

(Glu-105 in most eukaryotic enzymes) is replaced by a proline residue in the bacterial enzymes, which tightens the turn at the tip of the loop structure (compared to the dogfish enzyme) between Asn-101 and the main chain carbonyl group of residue 108. These bonds serve to"pin-pack" the upturned loop and restrain it in the more open conformation. It is known that *bs* enzyme shows a broader specifity for substrates than do the eukaryotic enzymes, and will accept substrates with larger chains. The specifity is reduced further when Pro-105 is replaced by the even less bulky serine residue, which suggests that this hole is important for the design of L-hydroxy-acid dehydrogenase with a broad specifity[9].

1. 1. 4. NADH-binding site

Nicotinamide adenine nucleotide has a primary role in biological oxidations and reductions within cells. It can exist in an oxidized form (NAD^+) that is converted to a reduced form (NADH) when it accepts electrons (in association with hydrogen) (Figure1.4).

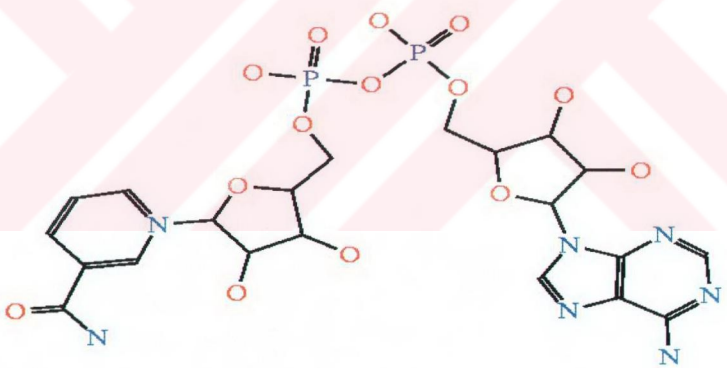


Figure 1. 4. The structure of the NADH.

There are two permitted orientations for the nicotinamide ring that can be obtained by rotation about the glycosidic bond[10, 11]. These correspond to exposing the A and B-side of the ring to the substrate site[1].

The interactions of the coenzyme with the protein that produce the correct conformation needed for catalysis were designed. Hydrogen-bonding requirements of the coenzyme are satisfied by contacts with the protein as well as with a number of

water molecules. The major difference between the binary and ternary structures concerns the proposed hydrogen bond between the nitrogen atom of the carboxamide side-chain of the coenzyme and the hydroxyl of Ser-163. This was measured to be 2.9 Å in the binary complex, yet is 3.6 Å in both the dogfish M4 and pig M4 enzyme/NADH/oxamate structures. In all of the ternary complexes: this distance is too great to constitute a hydrogen bond of any significance. This is supported by the observation that a site-directed mutant of LDH in which Ser-163 is changed to alanine shows wild-type properties[12]. In particular, the affinity of the apo-enzyme for NADH is unaltered by the mutation. This suggests that a hydrogen bond between Ser-163 and the coenzyme is not important for binding of the coenzyme to LDH[10]. However, the carboxiamide side-chain of NADH is in contact with the enzyme in the ternary complex. Rather than being hydrogen bonded through the nitrogen atom to Ser-163, there appears to be a hydrogen bond between the nitrogen atom and the main-chain carboxyl group of residue 138. This distance is 3.0 ´ in the *bs* LDH terneray complex compared to 3.3 ´ in the binary complex. This same hydrogen bond is 2.8 ´ in the dogfish ternary complex. The utilization of hydrogen bonds via main-chain rather than side-chain atoms makes good evolutionary sense, as the enzyme is less susceptible to spontaneous mutations. The affinity of LDH for 3-acetyl pyridine analogue of NADH is less than that for NADH[13]. This suggests that there is indeed a hydrogen bond between the carboxamide side-chain of NADH and the enzyme but we believe this to be with the main-chain carbonyl group of residue 138 rather than with the side-chain of Ser-163 (Figure1.5).

Ser-163 is conserved in all known LDH sequences, but is not present in the closely homologous malate deydrogenase enzymes. However, the function of this residue in LDH remains unclear, through, together with the main-chain carbonyl of residue 164; it is involved in binding water molecule in the active site that appears to be close enough to hydrogen bond to the oxygen-atom of the carboxyamide side-chain of the coenzyme. It is not known whether this water molecule is also present in the Ala-163 mutant enzyme, or what the role of this water molecule might be[9].

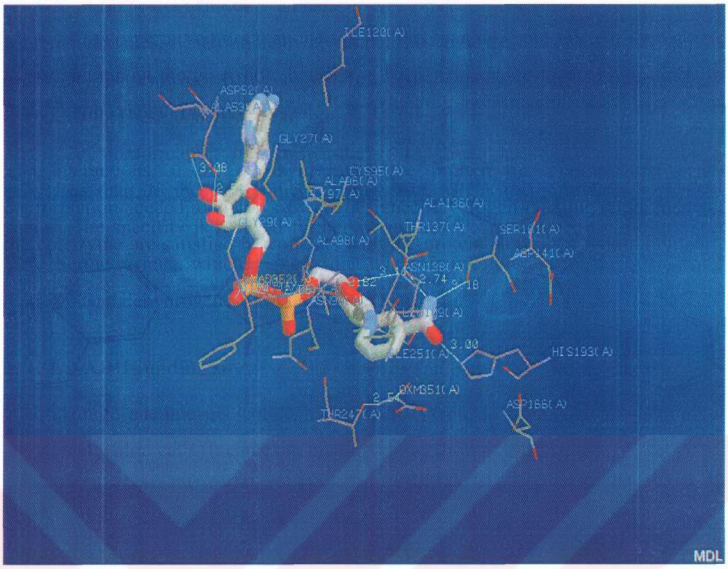


Figure 1. 5. The binding of the NADH to the *bs* LDH[8].

The 400-fold better binding of NADH over NAD^+ is not well understood in terms of structure at this time. An alternation of interaction between Lysine-250 and the nicotinamide moiety contribute to this change[1].

1. 1. 5. The substrate binding site

In the LDH:NAD-pyruvate ternary complex, the position of the substrate binding site has been determined by the pyruvate. Pyruvate is the anionic form of the three-carbon organic acid, pyruvic acid. Pyruvate is a key intermediate in the glycolytic and pyruvate dehydrogenase pathways, which are involved in biological energy production. Pyruvate is widely found in living organisms. It is not an essential nutrient since it can be synthesized in the cells of the body. Pyruvate, however, is consumed in the diet. The average daily intake of this substance ranges between about 100 milligrams and one to two grams. Certain fruits and vegetables are rich in pyruvate. For example, an average-size red apple contains approximately 450 milligrams. Dark beer and red wine are also rich sources of pyruvate.

Recent research suggests that pyruvate in supraphysiological concentrations may have a role in cardiovascular therapy, as an isotropic agent. Supraphysiological amounts of this dietary substance may also have bariatric and ergogenic applications.

Pyruvate is also known as 2-oxopropanoate, alpha-ketopropionate, acetylformate and pyroracemate (Figure1.6). Pyruvate and pyruvic acid are frequently use interchangeably, even though pyruvate is the anion of pyruvic acid and the form that occurs in living organisms. Pyruvic acid is represented by the following chemical structure:

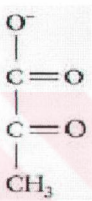


Figure 1. 6. The structure of the pyruvate.

LDH:NAD-pyruvate ternary complex is isomorphous with respect to the LDH:NAD:oxalate and further form the coenzyme than the pyruvate. All three compounds show clearly the carbonyl group of the substrate site between the nicotinamide and His-195, which binds the carbonyl group of the substrate. The oxalate forms salt bridges with Arg-109 and Arg-171, the residue that binds the carboxylate group of the substrate. This anion site is occupied by a sulfate ion in the apo-structure crystals grown in ammonium sulfate. It can be replaced by various anions but not oxamate[1]. The oxamate binding site in this structure is similar to that described for the dogfish ternary complexes. However one residue that seems to be very important in *bs* LDH was not noted in the dogfish structures. Thr-246 forms a good hydrogen bond with oxamate in this structure. Thr-246 is present in all LDHs, but is always smaller than in MDH and provides the affinity of the enzyme for pyruvate in a mutant enzyme in which Thr-246 has been replaced by a glycine residue. This amino acid substitution is proved to be very important for the design of a malate dehyrogenase from the LDH enzyme[14]. The general role of this residue in determining substrate specificity in LDH has been discussed by Bur[15]. The sulfate

ion is stabilized by Arg-171 and His-195 and can be displaced if the crystals are transferred to a solution of sodium citrate, oxamate or pyruvate (Figure1.7) [16].

The carboxylate of the substrate forms a salt bridge with side chain of Arg-171. The carbonyl group forms a hydrogen bond with the unprotonated imidazole ring of His-195. As well as orienting the substrate; His-195 stabilizes the negative charge that develops on the oxygen of pyruvate during reduction.

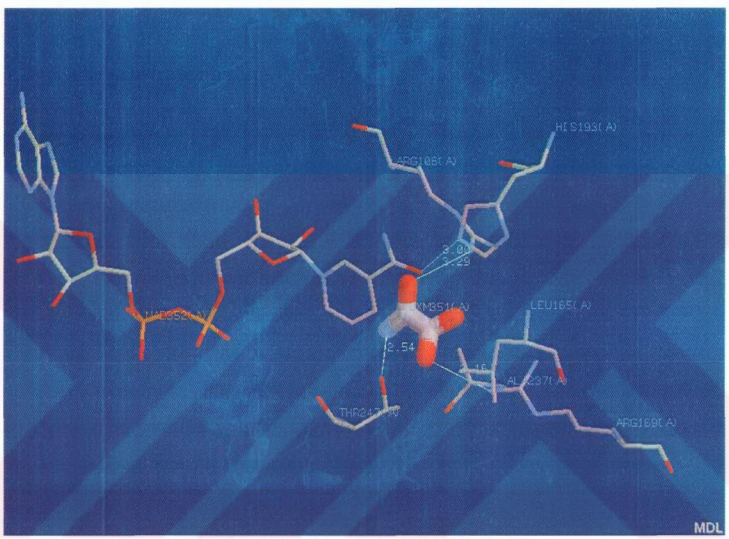


Figure 1. 7. The binding of the pyruvate to the *bs* LDH[8].

In the ternary complex the position of the loop excludes bulk water from the active site. The negative charge of the substrate needs to be neutralized[1]. Inspection of the model structure of the ternary complex suggests that substrates with larger side chains than pyruvate would extend into region occupied by Gln-102, Arg-109 and Thr-246. Two arginine residues 109 and 171 as well as His-195 are available to provide a partner for an ion-pair. Arg-109 that is involved in catalysis and polarizes the carbonyl bond of pyruvate is the epsilon-nitrogen. The terminal nitrogen is linked to a network at the break between helices α-1G and α-2G (Try-237, main chain nitrogen 236 and carbonyls 232 and 233) at the β-H/β-H turn Asp-197)[17].

If the inhibitor or substrate analog molecules, used in the crystallographic studies, were replaced in the model by the real substrates, then His-195 would be conveniently located to form a hydrogen bond with carbonyl of pyruvate or the hydroxyl of lactate. This hydrogen bond is not present in the NAD:pyruvate complex. The conformational changes of the loop bring Arg-109 close to the substrate side. The folded down loop encloses the active center, thereby increasing the already large number of charged and hydrophilic groups in this region of the subunit. These include Glu-107 and Asn-140, Asp-168, and Arg-109 and Arg-171, as well as His-195 and Glu-102. The roles of some of these residues have already been discussed. In addition, on binding NAD^+, Asn-140 is available for hydrogen bonding to the pyruvate carbonyl. On reduction, however the NAD^+ coenzyme loses its charge, which is transferred through the substrate to His-195. Asp-168 stabilizes the protonated His-195 in both ternary and binary complexes. When the Asp-168 was replaced by Asn in *bs* LDH, it showed that Asp-168 has no effect on pK of the histidine in the binary complexes[17].

The NAD-pyruvate is formed by the enzyme from NAD^+ and the enol form of pyruvate (the keto tautomer is the substrate for the normal reaction). His-195 in a manner similar to that of the oxidation of lactate catalyzes the mechanism for the reaction[18].

1. 1. 6. The Fru-1, 6-P_2 binding site

It has been demonstrated that only two fructose-1, 6-biphosphate (FBP, Fru-1, 6-P_2) (Figure1.8) bind per *bs* LDH[19].

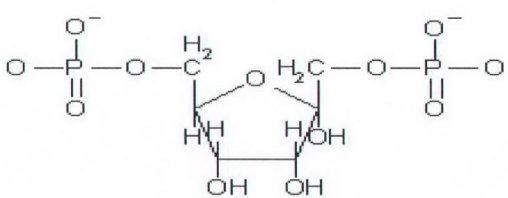


Figure 1. 8. The structure of the fructose-1, 6-biphosphate[8].

In many bacteria (e.g., *Thermus, Lactobacilli, streptococci* and *Bacilli*) lactate dehydrogenase activity is markedly regulated by fructose-1, 6-biphosphate [19].

Fru-1, 6-P_2 activation of tLDH leads to a change in the state of subunit association (dimer to tetramer)[20] and in the substrate affinity, subunit structure and effector. Dimeric form of the enzyme has allowed substrate affinity, while the liganded tetramer and to a lesser extent the unligated tetramer have a high affinity. One study of *streptococcal* bacteria has shown that an organism with fructose-1, 6-biphosphate-sensitive lactate dehydrogenase, when grown on excess glucose, produces large quantities of lactate and has a correspondingly high concentration of intracellular Fru-1, 6-P_2. When grown in conditions where glucose is limiting the same bacterium produces no lactate and has an almost undetectably low intracellular Fru-1, 6-P_2 level. Furthermore, the study demonstrated that a different strain of the same *Streptococcus species*, but having a LDH which is insensitive to Fru-1, 6-P_2 (the enzyme being always in the "activated state"), produced lactate whether glucose is limiting or not.

The two Fru-1, 6-P_2 molecules bound in a central hole in the molecule and bridge the P-axis with their phosphate groups, thus stabilizing the assembled tetramer. In the absence of activator the subunit interface on the P-axis is less stable and the protein molecule dissociates along the P-and R-axis to give dimers, the subunits of which are bound together by the stable Q-axis interface. The communication between the binding of activator and the binding of pyruvate is through the region of polypeptide consisting of residues 163-195 on which both the substrate and anion sites reside. The occupation of the anion sites, which contain the basic group's His-188 and Arg-173, could then cause a change in the relative position His-195 and Arg-171 that also span the βG/βH-2αF structure. The latter two residues, 195 and 171, hold the carbonyl and carboxylate groups, respectively, of pyruvate during catalysis[1]. It follows that their relative orientation must be critical in determining how tightly the substrate binds (Figure1.9) [19].

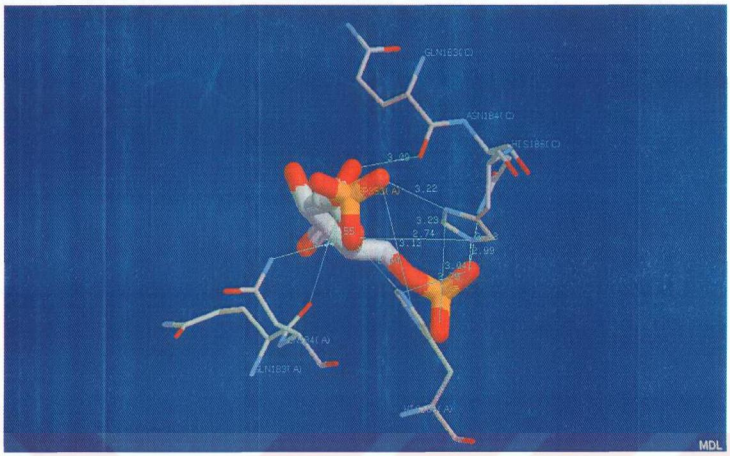


Figure 1. 9. The binding of the fructose-1, 6-biphosphate to the *bs* LDH[8].

It appears, therefore, that activation of LDH by Fru-1, 6-P_2 is both widespread among prokaryotes and an extremely important metabolic switch. The mechanism of action of this effector is, however, not well understood. In the case of the intensively studied LDH from *bs* the K_M for pyruvate (2-4 mM) is much higher than the cellular concentration range of the substrate, whilst the enzyme when fully saturated with Fru-1, 6-P_2 has a much-reduced K_M (about 50μM) (Figure1.10).

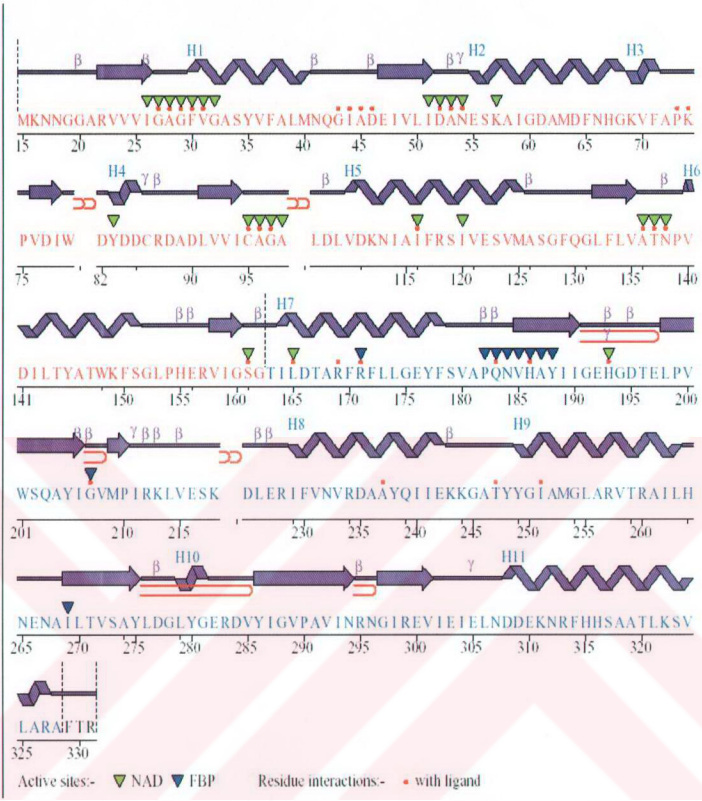


Figure 1. 10. The binding sites of the *bs* LDH[8].

1. 2. The Kinetic Mechanism

The enzyme binds lactate or pyruvate only in the presence of coenzyme[21]. Therefore, the mechanism is ordered, with the coenzyme binding first. Pertinent to this, is the observation from the crystallographic studies that coenzyme binding induces a conformational change in which residues 98 to 110 of the chain move through a relatively large distance to close over the active site in the ternary complex[22, 23].

Rapid reaction studies show that the mechanism differs somewhat from that of horse liver alcohol deydrogenase, although the dissociation of the enzyme-NADH complex is rate-determining in both reactions[24, 25]. The hydride transfer steps are very rapid in

the reactions of LDH and dissociation of pyruvate is slow, so that two ternary complexes equilibrate. Further the equilibrium position favors lactate and NAD^+ at neutral pH. When lactate is mixed with the haloenzyme at pH 7, 20% of the bound NAD^+ is reduced during the fourth millisecond of reaction, as the equilibrium between [E.NAD^+.lactate] and [E.NADH.pyruvate] is rapidly attained. As the pyruvate dissociates, the equilibrium is displaced toward products, so that all four bound NAD^+ molecules are reduced. There is then the slower dissociation of NADH; the rate-limiting step is in the steady state (Figure1.11). Coenzymes also bind independently to each site. In contrast, the enzyme from *bs* is allosterically regulated[26, 27]. A tetrameric form is stabilized by the binding the effector of Fru-1, 6-P_2 and binds pyruvate 50 times more tightly than the dimeric form does. The turnover numbers of tetramers and dimer are the same, and so they form a V-system. The build up of the glycolytic intermediate Fru-1, 6-P_2 under anaerobic conditions thus stimulates the regeneration of NAD^+ [28].

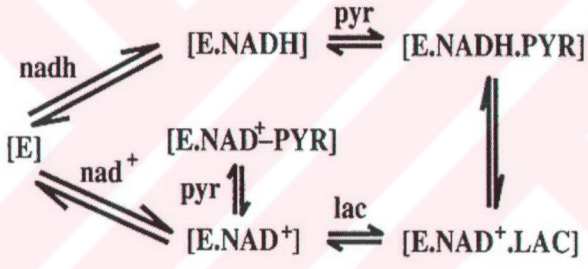


Figure 1. 11. The kinetic mechanism of the *bs* LDH.

Mammalian and bacterial LDHs show inhibition by high concentrations of substrate during the reduction of pyruvate to lactate. This inhibition is a consequence of the formation of a covalent adduct between pyruvate and the oxidized cofactor, NAD^+, before it is released from the enzyme[18]. The enzyme catalyses the formation of this adducts which then binds tightly to the protein, inhibiting further redox catalysis.

1. 3. LDH in Industry

The LDH enzyme from *Bacillus stearthermophilus* 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. α-Hydroxy acids are used in the production of various pharmaceuticals such as semi-synthetic penicillin (using S-α-hydroxyisocaproic acid), antihypertensive (using S-α-hydroxyl-4-phenylbutanoate), and in medical diagnostics (detecting S-phenylpyruvate and S-ketoisocaproic acid to diagnose phenylketoneuria and maple syrup urine diseases respectively).

One important of α-hydroxy acid is lactic acid or 2-hydroxypropionic that is produced by *bs* LDH. Lactic acid, $CH_3CHOHCO_2H$, is a colorless liquid organic acid (Figure1.12). It is miscible with water or ethanol. Lactic acid exists as a racemate mixture, made up of stereoisomers as shown below.

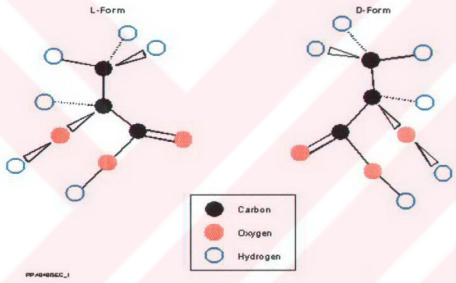


Figure 1. 12. Racemate forms of lactic acid.

World consumption of lactic acid was 120000-150000 metric tons in 2002, followed by lactic acid salts and esters at 50000-80000 metric tons. In China, the factual demand of lactic acid in the domestic is about 20000-30000 tons in the domestics. Such as Yancheng Sunny Int'l Trade Corp. Ltd. reported that we yearly produce DL-lactic acid 3000 tons, L-lactic acid 1000 tons, calcium lactate 2000 tons, sodium lactate 1000 tons, ethyl lactate 500 tons, ethyl caproic 500 tons, ethyl butyrate 500 tons, and surplus 60 kinds of alcoholic spices with an annual production value of approximately 60,000,000 Yuan. Lactic acid is widely used in, cosmetic, pharmacy, food and chemical industry. It has basically four functions; complexion agent and sequestrate, solvent, cleaning agent, and raw material for chemical synthesis[29].

Enzymes play a crucial role in chemical transformations important to the pharmaceutical, chemical, agriculture and food processing industries. They offer a number of advantages over conventional chemical catalysts. Methods have been developed in Bristol to synthesis 100% enantiomeric excess, chiral substituted-lactates using L-lactate dehydrogenase from *Bacillus stearothermophilus* both in its wild-type form and as versions that have been rationally designed for the efficient reduction of bulky α-ketoacids. However, in spite of having ability to produce chiral hydroxy acids from their oxo acids with high stereochemical fidelity, *bs* LDH is not widely used in industry. It has the disadvantages that one is not to have very wide substrate specifity and another is to be inhibited by its substrate. To make LDH a more suitable enzyme for production of chiral (S)-hydroxyacids in large scale, these disadvantages should be removed. Protein engineering is a promising approach which can be used to create LDH with the desired properties[29].

1. 4. Protein Engineering

Protein engineering can be defined as the use of genetic and chemical techniques to change the structure and function of a protein, thus producing a novel product with specific, desired, properties. Generally, the goals of protein engineering are to understand the function and mechanism of proteins and to improve proteins for applications. The reasons for engineering proteins on enzymes are listed below:

Changes in catalytic properties

- Increase in V_{max}
- Decrease in K_m
- Change in optimum pH
- Elimination of an inhibition site
- Alteration of specificity of reaction
- Elimination of a residue that confers instability

Changes in structural properties

- Improvement in thermostability
- Improvement in stability in organic solvents
- Changes in physiochemical properties
- Modification of ligand binding specificity

Creation of new systems

- Chimeric and multifunctional proteins
- Addition of tags for purification
- Improvement of effectiveness of therapeutic agents

There are two different approaches for doing this, rational design and random mutation. In rational design, close inspection of the enzyme structures and molecular dynamics calculations are used to suggest how to construct an improved enzyme. The changes are then made by site directed mutagenesis. It requires a through knowledge of three-dimensional structure for improving the enzyme properties. There are still a limited number of proteins that fulfill these requirements and examples of successful design of stabilized proteins are rare. The most widely used methods of random mutation are cassette mutagenesis and DNA shuffling. In casette mutagenesis, a limited region of amino acid sequence space is selected as being a likely region whereas in DNA shuffling the new properties might be encoded and all possible random variants of the amino acid sequence in this region are made using a combinatorial library. The library of clones is then grown on a Petri dish and a suitable screening reaction is used to reveal those clones which contain enzymes with the desired new properties[30, 31, 32, 33].

Previous studies showed that a complete switching of substrate specificity of *bs* LDH from pyruvate to malate was achieved by replacement of the glutamine residue at position 102 to an arginine residue found in the naturally occurring malate dehydrogenases by rational design. Substrate specificity of *bs* LDH has been very successfully altered by a semi rational approach which a *bs* LDH variant with Asn-101, Val-102 is as efficient with phenylpyruvate as is the wild type enzyme with pyruvate[34].

These studies offer that,

a) Efficient construction of *bs* LDH plasmid libraries,

b) Analysis of regions of the *bs* LDH enzyme able to tolerate variation in its primary sequence without affecting the protein fold, thermostability and expression,

c) Detection of new enzyme activity in the *bs* LDH library with a number of unnatural substrates on a Petri-dish screen.

More recently, Allen[35] used directed evolution approach (DNA shuffling) to produce a novel *bs* LDH that no longer requires the activator FBP which is expensive and representative of the sort of co-factor complications that are undesirable in industrial processes. An essential prerequisite of screening assay is that the *bs* LDH activity detected with a specific hydroxyl acid substrate must not be affected by the proteins, intracellular substrates and reduced coenzymes of the *E. coli* host. The screen was initially designed by A.S. El Hawrani[36] for use with a thermophilic protein and it is advantage that the LDH from *bs* is a thermophilic enzyme expressed in a bacterial strain (JM 105, *E. coli)*. Host proteins can be inactivated on the nitro cellulose filters using a heat step. The *bs* LDH from thermophilic bacteria is extremely stable to thermal denaturation. However, these thermophile enzymes have a very low diversity of substrate which they will transform

1. 5. Substrate Inhibition

LDH has been found to function as an allosteric enzyme that activated by FBP. The LDH is inhibited by pyruvate in lower at the low pH and in higher at the high pH[37]. Substrate inhibition is due to an abortive NAD^+-pyruvate complex decreasing the steady state concentration of functional LDH[38,39]. For reducing substrate inhibition

may focus on reducing the affinity of the LDH:NAD^+ complex for pyruvate or in this case, reducing the affinity of the enzyme for NAD^+. In the wild type enzyme, the side chain of Asp-52 forms a hydrogen-bonding interaction with the 2- and 3-hydroxyl groups of the adenine ribose ring. Substitution of this residue with the longer basic arginine side chain will perturb this interaction and consequently is expected to weaken NAD^+ binding, and therefore reduce the ability of the LDH:NAD^+ complex to form the abortive adduct. In addition to that, it is possible to investigate some molecular interactions which bind NAD^+ to a typical Rossmann fold which is the structure of the nucleotide binding domain and similar to that found in other dehydrogenases.

6. Background and aims of this project

NAD^+-dependent LDH from *Bacillus stearothermophilus* was isolated. Its terminal amino acid sequence was determined and it was cloned into pKK223-3 and transformed into *E. coli* and the enzyme was overproduced. The three-dimensional crystal structures of the enzyme were solved in the apo and holo forms. As explained earlier, LDH has an ability to produce chirally pure hydroxyl compounds. The goal of this project was to attempt to make LDH the best enzyme for producing enantiomerically pure α-hydroxy acids. We wish to combine the property of thermostability (which is an advantage for screening) with broad substrate of commercial relevants. Therefore, it is proposed to develop new routes to saturated and unsaturated α-ketoacids required to explore the specificity of L-lactate dehydrogenase and to provide chiral building blocks for more complex molecules. As a part of this project, we are interested to obtain *bs* LDH enzymes with altered substrate specificities by randomly shuffling the DNA sequence of the enzyme and screening the products against their ability to transform useful new substrates e.g. 4-phenyl-2-hydroxybutanoate, phenyllactate, S-ketoisocaproic. The work had three goals: The first is to reduce the substrate inhibition of LDH by using site directed mutagenesis, the second is to introduce new protein engineering methods such as DNA shuffling to the lab, the last, an attempt was made to obtain *bs* LDH enzymes with altered substrate specificities by randomly shuffling the DNA sequence of the

enzyme and screening the products against their ability to transform useful new substrates e.g. 4-phenyl-2-hydroxybutanoate, phenyllactate, S-ketoisocaproic.

---

2. THEORETICAL

2. 1. Growth of the bacterial culture

The word growth is defined as an increase in the number of cells in microbiology. Growth is an essential component of microbial function, as any given cell has a finite life span in nature and the species is maintained only as a result of continued growth of the population. Different organisms need different sets of nutrients and often need these nutrients in one or another specific form. And not all nutrients are required in the same amounts; some nutrients, called macronutrients, are required in large amounts, while others, called micronutrients, are required in lesser, sometimes even trace, amounts. Culture media are the nutrient solutions used to grow microorganisms in the laboratory. Two broad classes of culture media are used in microbiology: chemically defined and undefined (complex). Chemically defined media are prepared by adding precise amounts of highly purified inorganic or organic chemicals to distilled water. Therefore, the exact chemical composition of a defined medium is known. In many cases, however, knowledge of the exact composition of a medium is not critical. In these instances complex media may suffice or for various reasons may even be advantageous. Complex media often employ digests of casein (milk protein), beef, soybeans, yeast cells, or any of a number of other highly nutritious (yet chemically undefined) substances. Such digests are available commercially in powdered form and can be weighed out rapidly and dissolved in distilled water to give a medium. However, a major concession in using a complex medium is loss of control of its precise nutrient composition.

Once a culture medium has been prepared, it can be inoculated (that is, organisms added) and incubated under conditions that will support microbial growth. In most cases growth will be of a pure culture, a culture containing only a single kind of microorganism. To obtain or maintain a pure culture, it is essential that other organisms be prevented from entering it. A major method for obtaining pure cultures and for assessing the purity of a culture is the use of solid media, specifically, solid media in the Petri plate.

Culture media are frequently prepared in a semisolid form by the addition of a gelling agent to liquid media. Such solid culture media immobilize cells, allowing

them to grow and form visible, isolated masses called colonies. Bacterial colonies can be of various shapes and sizes depending on the organism, the culture conditions, the nutrient supply, and several other physiological parameters.

Solid media are prepared the same way as for liquid media except that before sterilizing the medium, agar is added as a gelling agent, usually at about 1.5%. The agar melts during the sterilization process, and the molten medium is then poured into sterile glass or plastic plates and allowed to harden before use.

Once a sterile culture medium has been prepared, it can receive an inoculum from previously grown pure culture to start the growth process again. This requires the practice of aseptic technique. Aseptic technique is the series of procedures used to prevent contamination during manipulations of cultures and sterile culture media. Aseptic transfer of a culture from one tube of medium to another is usually accomplished with an inoculating loop or needle that has been sterilized by incineration in a flame. Cultures in which growth has taken place can also be transferred to the surface of agar plates, where colonies develop from the growth and division of single cells.

As we mentioned, growth is described as an increase in the number of microbial cells in a population, also it can also be measured as an increase in microbial mass. Growth rate is defined as the change in cell number or cell mass per unit time. During the cell division cycle, all the structural components of the cell increase two times. Generation time is called the interval for the formation of two cells from one cell. The generation time is thus the time required for the cell population to double. Both the cell number and cell mass double during single generation. A generation time is different among organisms. Many bacteria have generation times of 1-3 h, but a few have generation times ten min or as long as several days. Also, generation time of any given organism is dependent to some extent on the growth medium used and the incubation condition employed. A typical growth curve for a population can be divided into some distinct phases called the lag, exponential, stationary and death phase. When a microbial population is inoculated into fresh medium, growth cannot begin immediately but only after a specific period of time that is called lag phase. The exponential phase is when number of microbial cells begins to increase. In stationary phase, there is increase in the number of the cell. If incubation continues

after a population reaches the stationary phase, the cells may remain alive and continue to metabolize, and even they may also die. The last phase is the death phase. In some cases death is accompanied by actual cell lysis[40, 41].

2. 2. Extraction and purification of plasmid DNA

Bacterial plasmids are double-stranded closed circular DNA molecules that range in size from 1 kb to more than 200 kb. They are found in a variety of bacterial species, where they behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosome. They have some roles in evolution period for adaptation to new environmental condition. Nevertheless, they rely on enzymes and proteins encoded by the host for their replication and transcription. Frequently, plasmids contain genes coding for enzymes that under certain circumstances in nature are advantageous to the bacterial host. Among the phenotypes conferred by plasmids are resistance to antibiotics; production of antibiotics; degradation of complex organic compounds; and production of colicins, enterotoxins, and restriction and modification enzymes. The most commonly selectable markers are genes that confer resistance to antibiotics such as ampicilin, tetracycline, chloramphenicol, and kanamycin. Each of these antibiotics operates through a different mechanism. Such as, ampicillin binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the cell wall. The ampicilin resistance (amp^R) gene carried on the plasmid codes for an enzyme that is secreted into the periplasmic space of the bacterium, where it catalyzes hydrolysis of the β-lactam ring, with concomitant detoxification of the drug.

Many methods have been developed to purify plasmid DNA from bacteria. These methods invariably involve three steps:

- Growth of the bacterial culture,
- Harvesting and lysis of bacteria,
- Purification of plasmid DNA.

Plasmids are almost always purified from cultures (grown in liquid medium containing the appropriate antibiotic) that have been inoculated with a single bacterial colony picked from an agar plate.

Bacteria are recovered by centrifugation and lysed by any one of a large method, like treatment with nonionic or ionic detergents, organic solvents, alkali, or heat. The choice among these methods is depends on three factors: the size of the plasmid, the strain of *E. coli*, and the technique that is to be used subsequently to purify the plasmid DNA[41].

Bacterial strain *Escherichia coli* JM105 {F'traD36proA+proB+laclq lacZΔM15/Δ(lac-pro) X111thi rpsL (Strr) endA sbcB supE hsdR9} was used as a host to prepare all dsDNA for mutagenesis and expression in plasmid pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The same *E. coli* strain was also used as a host for transformation and expression of *bs* LDH proteins in pKK223-3 (figure of pKK223-3 in appendix section). It contains 4584 bases and the strong tac promoter which, in an appropriate host, is regulated by lac repressor and induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to the medium. Immediately downstream of the tac promoter is the pUC8 multiple cloning site and the strong *rrn*B ribosomal terminator, which stabilizes this vector-host system by inhibiting read-through transcription initiated from the tac promoter in the parent plasmid. Genes containing a ribosome binding-site and an ATG start codon may be expressed by insertion into any unique site in the multiple cloning sites (MCS). The ribosome-binding site on the plasmid can be utilized to express inserts if the start codon of the insert is within 13 base pairs from the *Eco*RI site.

2. 3. Amplification of DNA with PCR and cutting-ligation

The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase. These oligonucleotides typically have different sequences and are complementary to sequence that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. This is done on an automated

cycler, which can heat and cool the tubes with the reaction mixture in a very short time. The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. Then the cycle of denaturation, annealing, and DNA synthesis is repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product[42, 43, 44].

Components and their final concentration that is commonly used in PCR; autoclaved ultra-filtered water (pH 7.0), 10x PCR buffer (50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$, 1X), dNTPs mix (200 µM each nucleotide), primer mix (0.4 µM each primer), *Pfu* DNA polymerase-native enzyme (1 Unit/25 µL), genomic DNA template (100 ng/25 µL). In this study, we selected *Pfu* DNA polymerase because of its high proofreading ability than *Taq* DNA polymerase is (Figure2.1)[45].

Recombinant *Pfu* DNA polymerase is purified from an *E. coli* strain carrying a plasmid with the cloned gene encoding the hyperthermophilic archae *Pyrococcus furiosus* DNA polymerase 92kDa. *Pfu* DNA polymerase is referred from here on as *Pfu*. *Pfu* has been shown to exhibit superior thermostability and proofreading properties compared to other thermostable polymerase. Unlike *Pfu* DNA polymerase, highly thermostable *Pfu* possesses 3' to 5' exonuclease proofreading activity that enables the polymerase to correct nucleotide-misincorporation errors. This means that *Pfu* generated PCR fragments will have fewer errors than *Pfu*-generated PCR inserts. The error rate for *Pfu* is reported to be seven to ten fold lower than that of no proofreading *Pfu* DNA polymerase and two to 30 fold lower than other proofreading enzymes. Using *Pfu* in your PCR reactions results in blunt-ended PCR products, which are ideal for cloning into blunt-ended vectors, such as the PCR-Script™ vectors. *Pfu* is superior for techniques that require high-fidelity DNA synthesis. The enzyme catalyzes the incorporation of nucleotides into duplex DNA in the 5'=>3' direction in the presence of Mg^{2+} at 70°C-80°C. *Pfu* DNA Polymerase exhibits 3'=>5' exonuclease (proofreading) activity, but has no detectable 5'=>3' exonuclease activity[46].

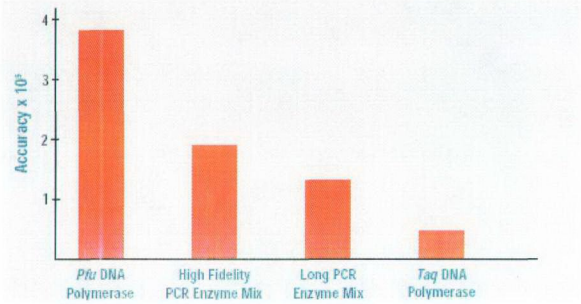


Figure 2. 1. Some accuracy of DNA polymerases and DNA polymerase mix[47].

Gene cloning requires that DNA molecules are cut in a very precise and reproducible fashion. Each vector is cut during construction of a recombinant DNA molecule. Each vector molecule must be cleaved at a single position, to open up the circle so that new DNA can be inserted; a molecule that is cut more than once will be broken into two or more separate fragments and will be of no use as a cloning vehicle. Restriction enzymes cut the covalent (phosphodiester) bonds between base pairs. If we are to study individual genes and individual sites on DNA, the large DNA molecules founding cells must be broken into manageable fragments. This is done using restriction endonucleases. These are nucleases that cleave DNA at particular sites by the recognition of specific sequences. Restriction enzymes used in molecular biology typically recognize short (4-8 bp) target sequences, usually palindromic, and cut at a defined position within those sequences. Restriction enzymes differ not only in the specificity and length of their recognition sequences, but also in the nature of the DNA ends they generate. Some enzymes generate staggered ends others generate flush ends[48].

The enzymes used are listed in the below with their cutting sides:

Restriction Enzymes	**Cutting sites**
*Pst*I	CTGCA^G
*Eco*RI	G^AATTC

The final step in construction of a recombinant DNA molecule is the ligation of the vector molecule and the DNA to be cloned. This process is referred to as ligation, and the enzyme that catalyses the reaction is called DNA ligase.

A target DNA is cleaved with a restriction enzyme to generate potential insert DNAs. Vector DNA that has been cut with the same enzyme mixed with these insert DNAs and DNA ligase is used to link the compatible ends of two DNAs. By adding an excess of the insert DNA relative to the plasmid DNA, the majority of vectors will reseal with insert DNA incorporated[49].

2. 4. Gel electrophoresis

Gel electrophoresis is a procedure for separating a mixture of molecules through a stationary material (gel) in an electrical field. DNA is negatively charged because the phosphates that form the sugar-phosphate backbone of a DNA molecule have a negative charge. A gel is prepared which will act as a support for separation of the fragments of DNA. The gel is a jello-like material, usually agarose, a substance derived from seaweed.

Holes are created in the gel. These will serve as a reservoir to hold the DNA solution. DNA solutions (mixtures of different sizes of DNA fragments) are loaded in a well in the gel. The gel matrix acts as a sieve for DNA molecules. Large molecules have difficulty getting through the holes in the matrix. Small molecules move easily through the holes.

Because of this, large fragments will lag behind small fragments as DNAs migrate through the gel. As the separation process continues the separation between the larger and smaller fragments increases. Molecular weight markers are often electrophoresed with DNAs.

Molecular weight markers are usually a mixture of DNAs with known molecular weights (figure of the marker that is used in the appendix section). Molecular weight markers are used to estimate the sizes of DNA fragments in your DNA sample. DNA samples are mixed with a "loading dye". The loading dye allows you to see the DNA as you load it and contains glycerol or sucrose to make the DNA sample heavy so that it will sink to the bottom of the well[48].

2. 5. Transformation-the uptake of DNA by bacterial cells

Most species of bacteria are able to take up DNA molecules from the medium in which they grow. Often a DNA molecule taken up in this way will be degraded, but occasionally it will be able to survive and replicate in the host cell. In particular this will happen if the DNA molecule is a plasmid with an origin of replication recognized by the host. Uptake and stable retention of a plasmid is detected by looking for expression of the genes carried by the plasmid. For example, *E. coli* cells are normally sensitive to the growth inhibitory effects of the antibiotics ampicilin tetracycline. However, cells that contain the plasmid are resistant to these antibiotics. Because of the plasmid that carries gene, this gene codes for enzyme that modifies antibiotic into a form that is non-toxic to the bacterium.

The term transformation has been extended to include uptake of any DNA molecule by any type of the cell. Most species of bacteria, including *E. coli,* take up only limited amounts of DNA under normal circumstances. In order to transform these species efficiently, the bacteria have to undergo some form of physical and/or chemical treatment that enhances their ability to take up DNA. Cells that have undergone this treatment are said to be component[48].

2. 5. 1. Preparation of chemical component cells

- Inoculate three ml LB medium with the appropriate *E. coli* strain and incubate the culture overnight at 37°C.
- Add the overnight culture to 500 ml SOB++ medium and incubate the culture at 25-30°C until the absorbance at 600 nm was approx. 0.5 (between 0.4 and 0.6).
- Chill the culture for at least ten min on ice. In the following steps, as much as possible the cell suspension should be kept on ice.
- Centrifuge the cell suspension for ten min at 4,500 rpm or 6,000 rpm at 4°C.
- Gently resuspend the pellet in 100 ml ice-cold TB buffer. Incubate the cell suspension on ice for ten min.

- Centrifuge for ten min at 250 rpm at 4°C.
- Gently resuspend the pellet in 18.6 ml ice-cold TB buffer and add 1.4 ml DMSO.
- Incubate the cell suspension on wet ice for at least ten min.
- Aliquot the cell suspension at 600 µl per tube. Shock-freeze the cell suspension in liquid nitrogen and store the tubes at -80° or in liquid nitrogen. At-80°C the cells will be competent for at least six months. In liquid nitrogen they will stay competent indefinitely[48].

2. 6. SDS-PAGE Electrophoresis

Proteins can be separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions. The mixture of proteins first dissolved in a solution of sodium dodecyl sulphate (SDS), an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone. It disrupts nearly all noncovalent interactions in native proteins. Anions of SDS bind to main chains at a ratio of about one SDS for every two amino acid residues. SDS confers a negative charge to the polypeptide in proportion to its length i.e. the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length.

During denaturation by boiling, it is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: This is done with 2-mercaptoethanol or dithiothreitol. Therefore, in denaturing SDS-PAGE separations, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

The negative charge acquired on binding SDS is usually much greater than the charge on the native protein; this native charge is thus rendered insignificant.

The SDS complexes with the denatured proteins are then electrophoresed on a polyacrylamide gel, typically in the form of a thin vertical slab. The direction of electrophoresis is from top to bottom.

Finally, the proteins in the gel can be visualized by staining them with silver or a dye such as Coomassie blue, which reveals a series of bands.

Typically, several samples are electrophoresed on one flat polyacrylamide gel. A μl syringe is used to place solutions of proteins in the wells of the slab. A cover is then placed over the gel chamber and 200 volts is applied. The negatively charged SDS-protein complexes migrate in the direction of the anode, at the bottom of the gel.

Small proteins move rapidly through the gel, whereas large ones stay at the top, near the point of application of the mixture. The mobility of most polypeptide chains under these conditions is linearly proportional to the logarithm of their mass. This empirical relationship is not obeyed by some proteins; for example, some carbohydrate-rich proteins and membrane proteins migrate anomalously. SDS-polyacrylamide gel electrophoresis is rapid, sensitive, and capable of a high degree of resolution. Electrophoresis and staining take several hours. As little as 0.1 μg of a protein gives a distinct band when stained with Coomassie blue, and even less (0.02 μg) can be detected with a silver stain. Proteins that differ in mass by about 2% can usually be distinguished[48].

2. 7. Sequencing DNA

DNA sequencing reactions all use a primer to initiate DNA synthesis. This primer will determine the starting point of the sequence being read, and the direction of the sequencing reaction. Most DNA sequencing reactions use dideoxy nucleotides (ddNTP) to stop DNA synthesis at specific nucleotides. For example, if the ddCTP to the right is incorporated into a growing strand of DNA, the lack of a free 3′ OH group would prevent the next nucleotide from being added, and the chain would terminate. In automated sequencing we use a different fluorescent label attached to each of the four dideoxy nucleotides (ddA, ddC, ddG and ddT). Thus we can determine the terminal base in each fragment of DNA. In two types, examples can be loaded to gels; automated sequencing and manual sequencing. Automated sequencing uses a different fluorescent dye attached to each ddNTP. Manual sequencing uses radio labeled dATP (35-S or 33-P) to label the DNA. The sample is then split into four tubes each with an individual ddNTP present. The samples are then subjected to acrylamide gel electrophoresis followed by autoradiography[48].

2. 8. Site-directed mutagenesis

Site-directed mutagenesis method was first developed in 70s and has become a popular technology in various fields of research. Using site-directed mutagenesis the information in the genetic material can be changed. A synthetic DNA fragment is used as a tool for changing one particular code word in the DNA molecule. This reprogrammed DNA molecule can direct the synthesis of a protein with an exchanged amino acid. Michael Smith's method has become one of biotechnology's most important instruments. Use of PCR in site-directed mutagenesis accomplishes strand separation by using a denaturing step to separate the complementing strands and allowing efficient polymerization of the PCR primers. PCR site-directed methods thus allow site-specific mutations to be incorporated in virtually any double-stranded plasmid; eliminating the need for M13-based vectors or single-stranded rescue[49].

In this method, the oligonucleotide is hybridized to the cloned DNA as template. In this way, a double-stranded molecule with one mismatch is made. The two strands are then separated and that with desired mismatch amplified further. We showed all of this as four steps (Figure2.2)[49, 50].

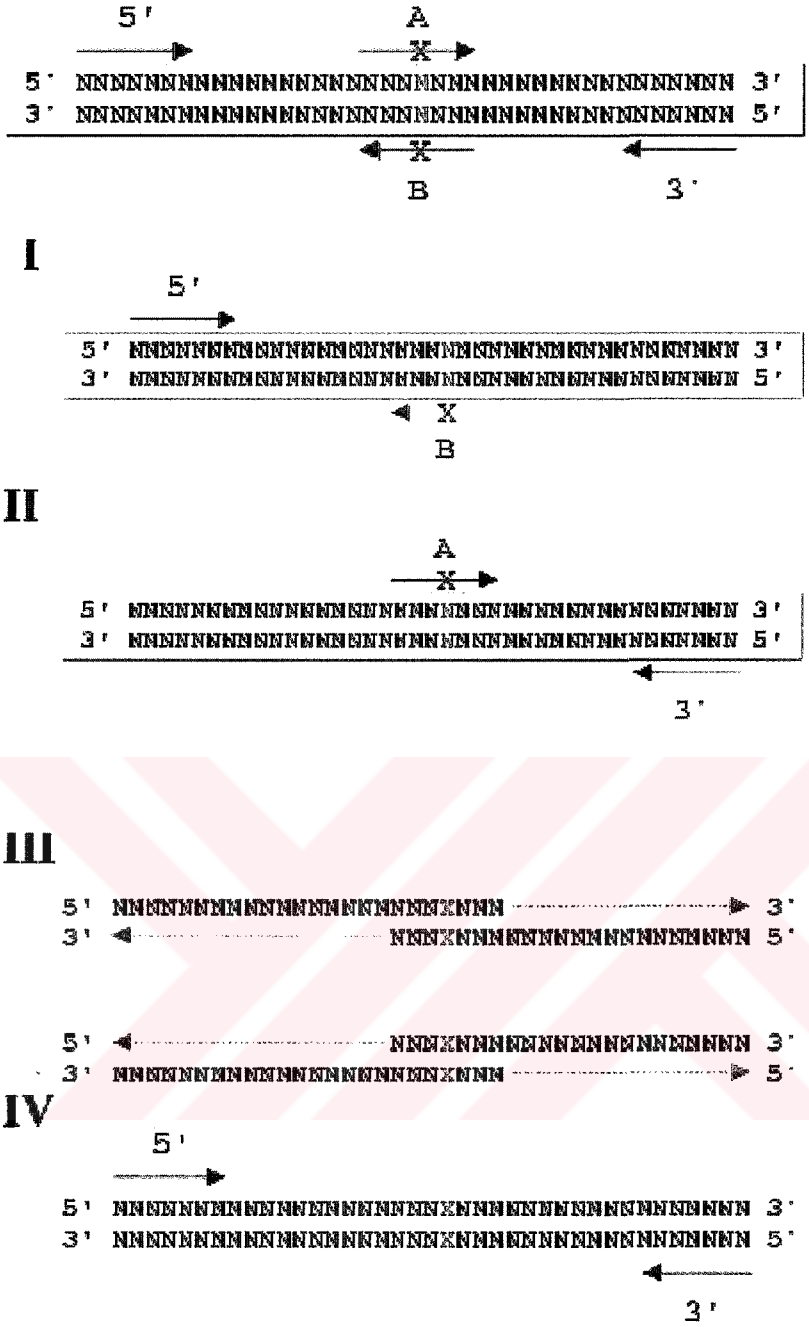


Figure 2. 2. Key steps of the PCR Based Overlap Extension Site-Directed mutagenesis. The primers A and B include desired mutation symbolized as the X[51].

By using site-directed mutagenesis, the researchers can study in detail how proteins function and how they interact with other biological molecules. Site-directed mutagenesis can be used, for example, to systematically change amino acids in enzymes, in order to better understand the function of these important biocatalysts. The researchers can also analyze how a protein is folded into its biologically active three-dimensional structure. The method can also be used to study the complex

cellular regulation of the genes and to increase our understanding of the mechanism behind genetic and infectious diseases, including cancer.

2. 9. DNA shuffling

The method of DNA shuffling, or "sexual PCR", is used to recombine homologous DNA sequences during *in vitro* molecular evolution. While randomly recombining the DNA sequences, the technique also introduces new point mutations at a relatively high rate. Though these point mutations may provide useful diversity for some in vitro evolution applications, they are problematic for others, especially when the mutation rate is high. Much lower mutagenesis rates are desired, for example, during the in vitro evolution of long genes or whole operons during recombination of beneficial mutations already identified previously, for DNA-based computing, or when the method is used to differentiate adaptive from neutral or deleterious mutations in evolutionarily-related sequences.

DNA shuffling consists of four steps: (i) preparation of genes to be shuffled, (ii) fragmentation with DNase I, (iii) reassembly by thermocycling in the presence of a DNA polymerase, and (iv) amplification of reassembled products by a conventional PCR (Figure2.3)[43].

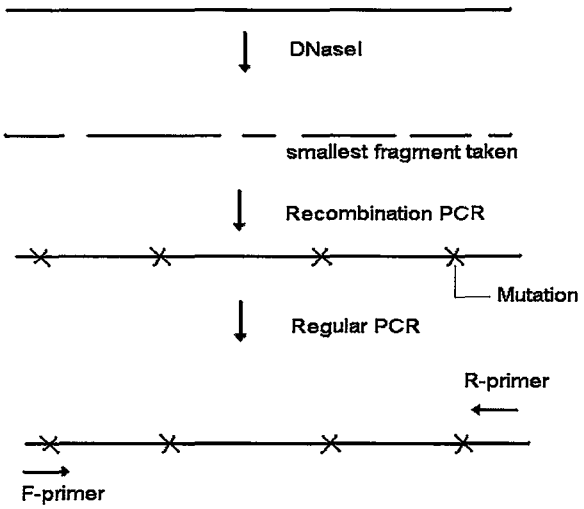


Figure 2. 3. DNA shuffling methodology.

The first step of this method is to randomly fragment a population of related genes using DNAse I. This produces fragments of various lengths that, after denaturation, hybridize to form an equal mixture of 5' and 3' overhangs. Using PCR techniques, the 5' overhang fragments can be extended by *Taq* DNA polymerase leaving the 3' overhang fragments unaffected. As a consequence of this extension, the average fragment length increases during each cycle. Recombination occurs, when a fragment derived from one template primes a template with a different sequence.

DNA shuffling technology offer:

- The ability to shuffle large or even multiple loci simultaneously
- The ability to shuffle mutant clusters as well as distant relatives
- Few bottlenecks-minimized genetic founder effects
- Large numbers of crossovers in a single round
- Low or controlled levels of mutagenesis
- Speed and few handling events
- All the above without exhaustive optimization.

3. EXPERIMENTAL

3. 1. Bacterial Growth of *Bacillus staerothermophilus* (*bs*) LDH

- *E. coli* bacterial strain JM 105 cells containing the *bs* LDH in pKK223-3 was streaked on a Luria-Bertani agar (LB) ampicillin plate and incubated at 37°C overnight.

- One single colony was removed with a sterile toothpick and inoculated into 5 ml of LB Broth ampicillin medium and incubated at 37°C overnight on an orbital shaker at 250 rpm.

- The plasmid was purified using the QIAGEN Plasmid Mini Kit (Catalog No. 12123).

- 1 µl of the sample from the previous step was run on a 1% agarose gel.

3. 2. Mutagenesis to construct *bs* LDH D52R

The first step was PCR-based overlapping extension of LDH gene. Fragments of 800bp and 150bp carrying desired mutation were produced using LDH gene in pKK223-3 vector as template. For 800bp fragment D52R, N-terminus was used as forward and *bs* C-terminus as reverse primer. For 150bp fragment *bs* N-terminus was used as forward and D52R as reverse primer.

Four oligonucleotides were used for PCRs: Following oligonucleotides were used as PCRs primers in order to amplify gene and construct mutant gene sequence;

5'CGGAATTCATGAAAAACAACGGTGGAGC-3' (forward primer),

5'AAAACTGCAGTCAT**CGC**GTAAAAGCACG-3' (with its complementary),

5`GTGCTCATCCGCGCGAATGAA-3` (reverse primer), M-13-F-40 (Sequencing primer, Qiagen PCR Cloning Kit, Cat. No. 231122).

Reaction mixtures:

800bp

Pfu buffer (10x)		
$MgCl_2$ [10mM]	-	2,5 µl
dNTPs mix (2mM each)	-	5 µl
D52R N-terminus primer [1mg/ml]	-	2,5 µl
bs LDH C-terminus primer [1mg/ml]	-	2,5 µl
LDH in pKK	-	1 µl
Distilled H_2O	-	34 µl
Pfu polymerase (Fermentas, EPO502)	-	0,5 µl

150bp

Pfu buffer (10x)	-	5 µl
$MgCl_2$ [10mM]	-	2,5 µl
dNTPs mix (2mM each)	-	5 µl
D52R C-terminus primer [1mg/ml]	-	2,5 µl
bs LDH N-terminus primer [1mg/ml]	-	2,5 µl
LDH in pKK	-	1 µl
Distilled H_2O	-	34 µl
Pfu polymerase	-	0,5 µl

Reaction conditions:

Initial denaturation:	94°C-5min	
Denaturation:	94°C-1min 30sec	20 cycles
Annealing:	55°C-2min	
Extension:	72°C-2min	
Final extension:	72°C-10min	

1% Agarose Gel Electrophoresis

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
PCR product (800bp) +10µl Loading Dye (6x)		PCR product (150bp) +10µl Loading Dye (6x)			Marker 4µl

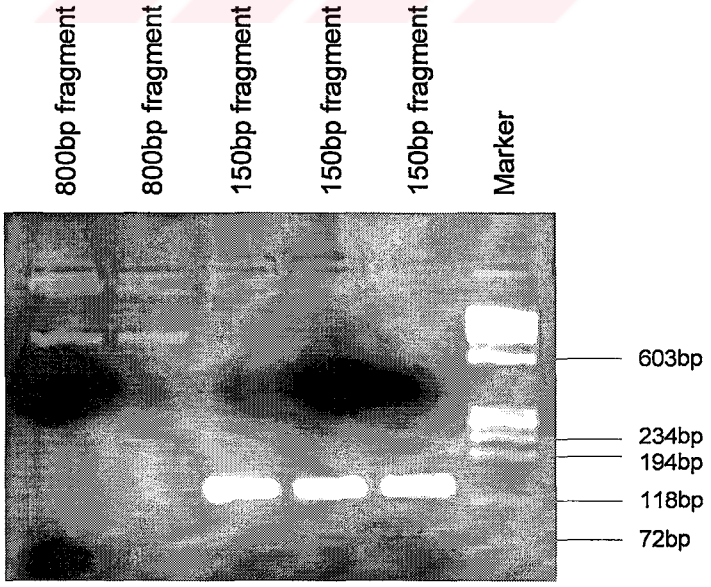


Figure 3. 1. 150 and 800 bp fragments after the PCR.

Bands were cut out of gel and DNA was extracted using Min Elute Gel Extraction Kits (Cat.No.28604).DNA was eluted in 25µl of EB buffer (10mM Tris-HCl, pH 8,5)

800bp and 150bp fragments concentration was estimated through gel electrophoresis:

800bp-about 200ng/µl

150bp-about 200ng/µl

Mutated LDH gene was constructed through 2-staged PCR reaction.

Stage 1

Reaction mixture:

Pfu buffer (10x)	-	2,5 µl
$MgCl_2$ [10mM]	-	2,5 µl
dNTPs mix [2mM each]	-	2,5 µl
800bp fragment [200ng/ul]	-	5 µl
150bp fragment [200ng/ul]	-	1 µl
Distilled H_2O	-	12,5 µl
Pfu polymerase	-	0,5 µl

Reaction conditions:

Initial denaturation:	94°C-5min	
Denaturation:	94°C-2min	7 cycles
Annealing:	50°C-1min	
Extension:	72°C-4min	
Final extension:	72°C-10min	

Stage 2

Reaction mixture:

Pfu buffer (10x)	-	2,5 µl
$MgCl_2$ [10mM]	-	2,5 µl
dNTPs mix [2mM each]	-	2,5 µl
bs N-terminus primer [1mg/ml]	-	2,5 µl
bs C-terminus primer [1mg/ml]	-	5 µl
Distilled H_2O	-	11,5 µl
Pfu polymerase	-	0,5 µl

Reaction conditions:

Initial denaturation:	94°C-5min	
Denaturation:	94°C-1min 30sec	20 cycles
Annealing:	55°C-1min	
Extension:	72°C-2min 30sec	
Final extension:	72°C-10min	

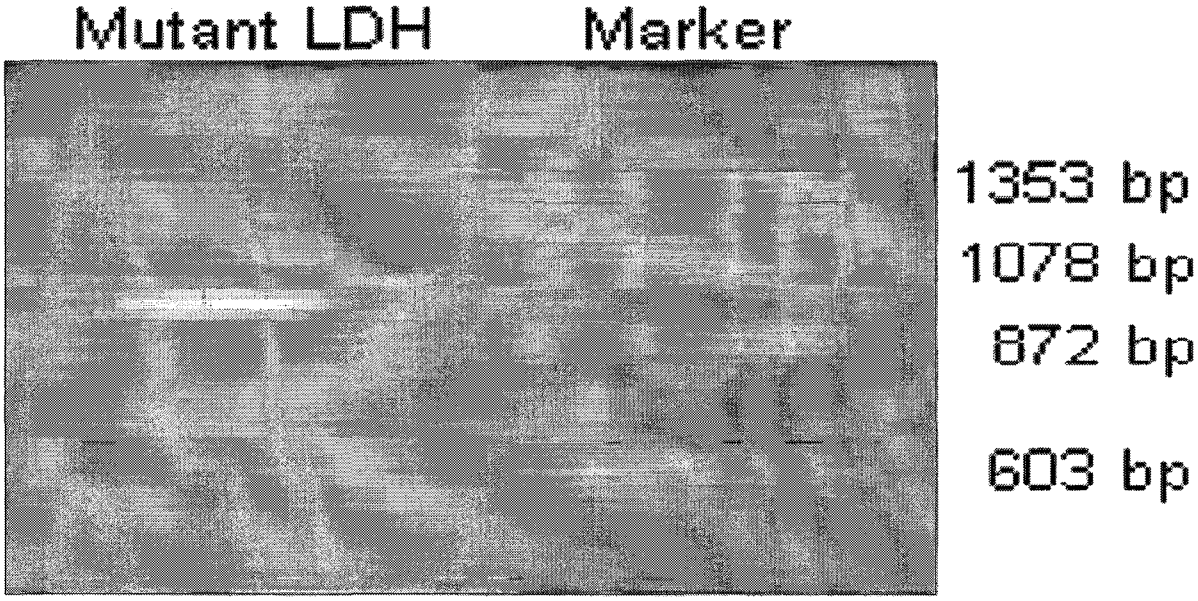


Figure 3. 2. The mutant LDH on the 1% agarose gel.

1% Agarose Gel Electrophoresis

Lane 1	Lane 2
PCR product (Mutant LDH)	Marker

Band of mutant LDH (about 950bp) was cut out of gel and DNA was extracted with the same kit.

Mutant LDH was cloned into pDrive cloning vector using QIAGEN PCR Cloning Kit. (Cat. No. 231122)

Ligation mixture:

Mutant LDH [40ng/µl]	-	4 µl
Ligation Master Mix (2x)	-	5 µl
pDrive Cloning Vector [50ng/µl]	-	1 µl

Ligation mixture was incubated for 45 minutes at 15°C and then for ten minutes at 70°C. 2 µl of ligation mixture was added to a tube JM 105 competent cells, mixed gently and incubated on ice for five minutes. Tube was heated at 42°C for 30 seconds

and incubated on ice for another two minutes. 250 µl of room temperature SOC medium was added to the tube and volumes of 100 µl were plated onto LB agar plates containing ampicillin. Plates were incubated at 37°C overnight.

6 colonies were selected and inoculated in 5 ml of LB Broth for 18 hours at 37°C. Plasmid DNA was purified using the same kit. Samples 2-6 were used for further analysis; sample 1 was discarded due to low DNA concentration.

DNA was digested using *Eco*RI and *Pst*I restriction enzymes.

Reaction mixtures :

		Tube 1	tube 2	tube 3
O^+ buffer (10x)	-	2 µl	2 µl	2 µl
Plasmid DNA	-	3 µl	3 µl	3 µl
Distilled H_2O	-	14 µl	14 µl	13 µl
*Eco*RI (Fermentas, ERO271)	-	1 µl	---	1 µl
*Pst*I (Fermentas, ERO611)	-	---	1 µl	1 µl

Digestion was carried out at 37°C for 1 hour.

1% Agarose Gel Electrophoresis

Row 1

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
Sample 1	sample 1	sample 1	sample 2	sample 2	sample 2	sample 3	sample 3	Marker
Tube 1	tube 2	tube 3	tube 1	tube 2	tube 3	tube 1	tube 2	

1 µl

+ 4 µl Loading Dye (6x)

Row 2

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
sample 3	sample 4	sample 4	sample 4	sample 5	sample 5	sample 5	---	Marker
tube 3	tube 1	tube 2	tube 3	tube 1	tube 2	tube 3		

1 µl

+ 4 µl Loading Dye (6x)

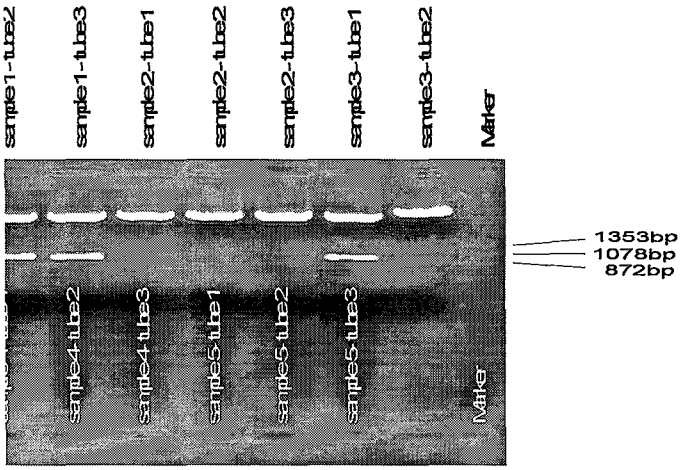


Figure 3. 3. After digestion the insert and the pDrive on the 1% agarose gel.

969bp and 3836bp of selected clones were expected in *Eco*RI-digested sample, band of 4805 bp was expected in *Pst*I-digested sample and bands of 23bp, 969bp and 3816bp were expected in sample digested with both enzymes.

Samples 3 and 4, which were selected clones carrying the expected mutant *bs* LDH gene, were used for sequencing.

PCR for sequencing was performed using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Reaction mixtures:

		Tube 1	tube 2
Sequencing RR-100	-	8 µl	8 µl
Sequencing Buffer (5x)	-	4 µl	4 µl
Template (mutant LDH in pDrive)	-	1 µl	1 µl
M-13-F-40 primer [1pmol/µl]	-	3,2 µl	3,2 µl
Distilled H_2O	-	3,8 µl	3,8 µl

Reaction conditions:

Initial denaturation: 95°C-5min

Denaturation:	95°C-1min	40 cycles
Annealing:	50°C-30 sec	
Extension:	60°C-4min	

2 µl of 3 M sodium acetate (pH 5, 2) and 50 µl of 95% ice-cold ethanol were added to each PCR sample. Tubes were kept on ice for 30 minutes and centrifuged at 14000 rpm for 30 minutes. Supernatant was discarded; each pellet was redissolved in 250 µl of 70% ethanol and centrifuged at 14000 rpm for 30 minutes. Supernatant was

discarded and pellet was air-dried at 95°C for 5 minutes. 20 µl of formamide was added to each tube and incubated for 3 minutes at 95°C and 3 minutes at -20°C.

Samples were stored at 4°C for further analysis. Sample 4 was chosen for further study and PCR for sequencing using *bs* N-terminus and *bs* C-terminus primers was performed.

Reaction mixtures:

		Tube 1	tube 2
Sequencing RR-100	-	8 µl	8 µl
Sequencing Buffer (5x)	-	4 µl	4 µl
Template (mutant LDH in pDrive)	-	1 µl	1 µl
bs N-terminus primer [1mg/ml]	-	3,2 µl	---
bs C-terminus primer [1mg/ml]	-	---	3,2 µl
Distilled H_2O	-	3,8 µl	3,8 µl

Reaction conditions and preparation of PCR product for sequencing were the same as described before.

LDH in pDrive cloning vector and pKK223-3 expression vector were digested using both *Eco*RI and *Pst*I restriction enzymes.

Reaction mixtures:

		Tube 1	tube 2
0^+ buffer (10x)	-	2 µl	2 µl
LDH in pDrive (sample 4)	-	3 µl	---
pKK223-3 vector [500µg/ml]	-	---	14 µl
*Eco*RI	-	1 µl	1 µl
*Pst*I	-	1 µl	1 µl

Digestion was carried at 37°C for 1 hour.

1% Agarose Gel Electrophoresis

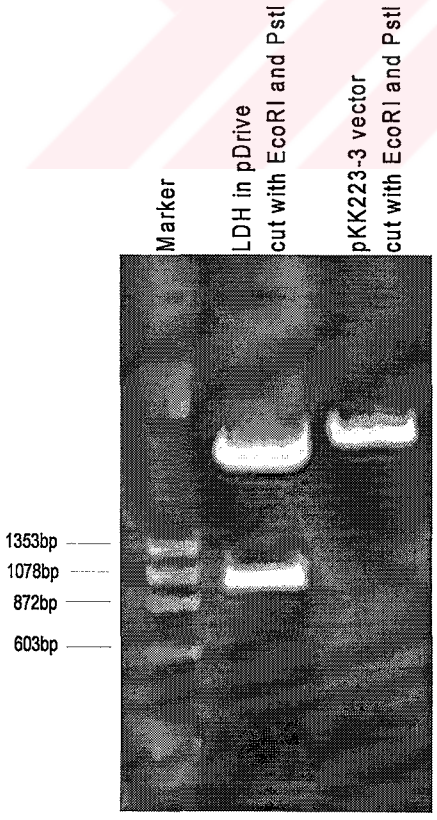


Figure 3. 4. After digestion the insert and the plasmid on the 1% agarose gel.

Bands of digested LDH gene and pKK223-3 vector were cut out of gel and purified using the kit.

For ligation, 10 µl of digested LDH and 2 µl of pKK223-3 vector were mixed, incubated for 5 minutes at 65°C and cooled on ice. 2 µl of ligase buffer (10x), 2 µl of 50% PEG, 2 µl of T4 DNA ligase and 4 µl of distilled water were added to the tube and incubated overnight at 16°C. Ligation product was purified using QIAGEN Min Elute Reaction Cleanup Kits 250 (Cat. No. 28206).

0, 8% Agarose Gel Electrophoresis

Lane 1	Lane 2
Marker	ligation product (1 µl + 1µl Loading Dye (6x))

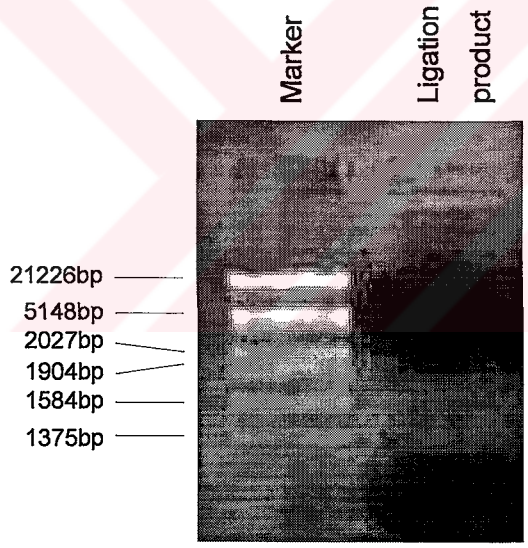


Figure 3. 5. After ligation the product on the 1% agarose gel.

The results of the sequence alignments in the appendix section.

Ligation mixture:

Mutant LDH [40ng/µl]	-	6 µl
pKK223-3 expression vector [50ng/µl]	-	2 µl

The reaction tube was kept at 65°C for 5 min. Then it is placed on ice. 1µl ligase (Fermentas, EL0011) 1µl ligation buffer was added. Finally, the reaction tube was kept at 16°C for overnight.

Ligation mixture was incubated for 10 minutes at 70°C. 2 µl of ligation mixture was added to tube JM105 *E. coli* competent cells, mixed gently and incubated on ice for 5 minutes. Tube was heated at 42°C for 30 seconds and incubated on ice for another 2 minutes. 250 µl of room temperature SOC medium were added to the tube and volumes of 100 µl were plated onto LB agar plates containing ampicillin. The plates were incubated at 37°C overnight.

2 colonies were selected and inoculated in 5ml of LB Broth for 18 hours at 37°C. Plasmid DNA was purified using the kit. Samples were used for further analysis.

LDH in pKK223-3 vector and pKK223-3 expression vector were digested using both *Eco*RI and *Pst*I restriction enzymes.

Reaction mixtures:

		Tube 1	tube 2
SH buffer (10x)	-	2 µl	2 µl
LDH in pKK223-3 vector	-	3 µl	---
pKK223-3 vector	-	---	14 µl
*Eco*RI	-	1 µl	1 µl
*Pst*I	-	1 µl	1 µl

Digestion was carried at 37°C for 1 hour.

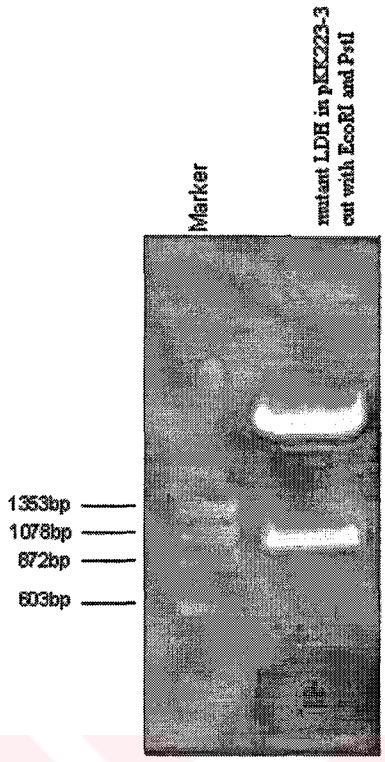


Figure 3. 6. After digestion the insert and the plasmid on the 1% agarose gel.

Reaction mixtures:

		tube 1	tube 2
Sequencing RR-100	-	8 µl	8 µl
Sequencing Buffer (5x)	-	4 µl	4 µl
Template (mutant LDH in pKK223-3)	-	1 µl	1 µl
bs C-terminus primer [1pmol/µl]	-	3, 2 µl	3, 2 µl
Distilled H_2O	-	3, 8 µl	3, 8 µl

Reaction conditions:

Initial denaturation:	95°C-5min	
Denaturation:	95°C-1min	40 cycles
Annealing:	50°C-30 sec	
Extension:	60°C-4min	

2 µl of 3 M sodium acetate (pH 5, 2) and 50 µl of 95% ice-cold ethanol were added to each PCR sample. Tubes were kept on ice for 30 minutes and centrifuged at 14000 rpm for 30 minutes. Supernatant was discarded; each pellet was redissolved in 250 µl of 70% ethanol and centrifuged at 14000 rpm for 30 minutes. Supernatant was discarded and pellet was air-dried at 95°C for 5 minutes. 20 µl of formamide was added to each tube and incubated for 3 minutes at 95°C and 3 minutes at -20°C. Samples were stored at 4°C for further analysis.

3. 3. Construct the shuffled *bs* LDH

Bacillus stearothermophilus LDH in pKK223-3 was isolated from *E. coli* JM 105 starins as mentioned in above. *bs* LDH was amplified by cutting the isolated plasmid.

Reaction mixtures:

	tube
O^+ buffer (10x)	1µl
Isolated *bs* LDH in pKK223-3	7µl
*Eco*RI	1 µl
*Pst*I	1 µl

Five reaction tubes were set up. Products of digestion were loaded agarose gel and purified from the gel by using the same kit.

Fragmentation

- 2 to 5 µg of parent DNA (7 µl) was incubated at 15°C.
- 0,000125U of DNase I solution was prepared and incubated at 15°C.
- Parent DNA was added the solution to the DNase I solution at 15°C.
- The reaction was stopped by adding the loading dye.
- The samples were electrophoresed on a 2% (w/v) Agarose gel.
- The region of the gel containing DNA of the desired molecular weight range between 100-300bp was excised.
- The DNA fragments from the Agarose gel with the same kit, was purified according to the manufacturer's instructions.

Assembly

- The following buffers are combined in a microcentrifuge tube:
  5 µl of 10X *Taq* Buffer,
  5 µl of dNTP mix(0.2 mM),
  10 µl of the purified fragments,
  2.5 Units of *Taq* Polymerase and *Pfu* polymerase,
  add ddH_2O to a total volume of 50 µl.
- The reaction was incubated for 3 min at 94°C.
- The DNA fragments were amplified in a thermocycler with the following program:
  30 sec at 94°C,
  1 min at 55°C,
  1 min at 72°C,
  lengthen each extension step by an additional 5 sec per cycle,
  repeated for 40 cycles.

Amplification

- The following components were mixed in a microcentrifuge tube:
  10 µl of 10 X *Taq* Buffer,
  10 µl of dNTP Mix,
  0.5 µM each Primer (*bs* N-terminus primer, *bs* C-terminus primer),
  1 µl of assembly Reaction,
  2.5 U *Taq/Pfu* (1:1) mixture and ddH_2O was added to a total volume of 100 µl.
- The DNA fragments were amplified in a thermocycler with the following program:
  2 min 96°C, 10 cycles of 30 s 94°C, 30 s 55°C, 45 s 72°C, followed by another 14 cycles of 30 s 94°C, 30 s 55°C, 45 s + 20 s/cycle 72°C, and finally 7 min 72°C.
- All of the reaction by electrophoresis was visualized on a 2% (w/v) agarose Gel
- The products of amplification were loaded agarose gel and purified from the gel by using the same method.

Reaction mixtures:

	Tube	tube
O^+ buffer (10X)	1µl	1µl
pKK223-3	7µl	---
Shuffled LDH	---	7µl
*Eco*RI	1 µl	1 µl
*Pst*I	1 µl	1 µl

Reaction was performed at 37°C for 1hour. Then the products of digestion were loaded to gel and cut. Finally the bands of the gels were purified with the same kit.

Ligation mixture:

Shuffled LDH	-	3 µl
pKK223-3 expression vector [50ng/µl]	-	5 µl

The reaction tube was kept at 65°C for 5 min. Then it is placed on ice. 1µl ligase, 1µl ligation buffer was added. Finally the reaction tube was kept at 16°C for overnight.

The same protocol was followed for the transformation that was mentioned in above.

Too many colonies were observed on plates.

4. PROTEIN PURIFICATION

Highly pure enzymes are essential for comprehending their structure, function, reaction mechanism and regulation. Reliable information concerning the kinetics, cofactors, active sites, structure and mechanism of action requires highly purified enzymes. In this part, we represent the essential methods that are used in order to purify enzymes[51, 52].

4. 1. Theoretical

4. 1. 1. Ultrasonic Extraction

The treatment of microbial cells in suspension with inaudible ultrasound (greater than about 18 kHz) results in their inactivation and disruption. Ultrasonication utilizes the rapid sinusoidal movement of a probe within the liquid. It is characterized by high frequency (18 kHz-1 MHz), small displacement, moderate velocities (a few m $s^{-1)}$, steep transverse velocity gradients (up to 4,000 s^{-1}) and very high acceleration (up to about 80,000 g). Ultrasonication produces cavitations phenomena when acoustic power inputs are sufficiently high to allow the multiple productions of micro bubbles at nucleation sites in the fluid. The bubbles grow during the rarefying phase of the sound wave, and then are collapsed during the compression phase. On collapse, a violent shock wave passes through the medium. The whole process of gas bubble nucleation, growth and collapse due to the action of intense sound waves is called cavitation. The collapse of the bubbles converts sonic energy into mechanical energy in the form of shock waves equivalent to several thousand atmospheres (300 MPa) pressure. This energy imparts motions to parts of cells which disintegrate when their kinetic energy content exceeds the wall strength. An additional factor which increases cell breakage is the microstreaming (very high velocity gradients causing shear stress) which occur near radially vibrating bubbles of gas caused by the ultrasound.

Much of the energy absorbed by cell suspensions is converted to heat so effective cooling is essential. Equipment for the large-scale continuous use of ultrasonic extraction has been available for many years and is widely used by the chemical industry but has not yet found extensive use in enzyme production. Reasons for this may be the conformational lability of some (perhaps most) enzymes to sonication

and the damage that they may realise though oxidation by the free radicals, singlet oxygen and hydrogen peroxide that may be concomitantly produced. Uses of radical scavengers (e.g. N_2O) have been shown to reduce this inactivation. As with most cell breakage methods, very fine cell debris particles may be produced which can hinder further processing. Sonication remains, however, a popular, useful and simple small-scale method for cell disruption[51,52].

Various intracellular enzymes are used in significant quantities and must be released from cells and purified. The amount of energy that must be put into the breakage of cells depends very much on the type of organism and to some extent on the physiology of the organism. Some types of cell are broken readily by gentle treatment such as osmotic shock (e.g. animal cells and some gram-negative bacteria such as *Azotobacter* species), whilst others are highly resistant to breakage. These include yeasts, green algae, fungal mycelia and some gram-positive bacteria which have cell wall and membrane structures capable of resisting internal osmotic pressures of around 20 atmospheres (2 MPa). Consequently, a variety of cell disruption techniques have been developed involving solid or liquid shear or cell lysis.

The rate of protein released by mechanical cell disruption is usually found to be proportional to the amount of releasable protein.

Hazards likely to damage enzymes during cell disruption;

- All mechanical methods require a large input of energy, generating heat. Cooling is essential for most enzymes. The presence of substrates, substrate analogues or polyols may also help stabilise the enzyme.

- Shear forces are needed to disrupt cells and may damage enzymes, particularly in the presence of heavy metal ions and/or an air interface.

- Disruption of cells will inevitably release degradative enzymes which may cause serious loss of enzyme activity. Such action may be minimised by increased speed of processing with as much cooling as possible. This may be improved by the presence of an excess of alternative substrates (e.g. inexpensive protein) or inhibitors in the extraction medium.

- Buffered solutions may be necessary. The presence of substrates, substrate analogues or polyols may also help stabilize the enzyme.

- Some enzymes may suffer conformational changes in the presence of detergent and/or solvents. Polyphenolics derived from plants are potent inhibitors of enzymes. This problem may be overcome by the use of adsorbents, such as polyvinylpyrrolidone, and by the use of ascorbic acid to reduce polyphenol oxidase action.

- Reducing agents (e.g. ascorbic acid, mercaptoethanol and dithiothreitol) may be necessary.

- The gas-liquid phase interfaces present in foams may disrupt enzyme conformation.

- Heavy metal ions (e.g. iron, copper and nickel) may be introduced by leaching from the homogenization apparatus. Enzymes may be protected from irreversible inactivation by the use of chelating reagents, such as EDTA[51,52].

4. 1. 2. By salt (ammonium sulfate) precipitation

The solubility of protein depends on, among other things, the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as salting-in. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as salting-out.

Many types of salts have been employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulfate has been the most widely used chemical because it has high solubility and is relatively inexpensive. Because

enzymes are proteins, enzyme purification can be carried out by following the same set of procedures as those for protein, except that some attention must be paid to the consideration of permanent loss of activity due to denaturation under adverse conditions.

There are two major salting-out procedures. In the first procedure, either a saturated salt solution or powdered salt crystals are slowly added to the protein mixture to bring up the salt concentration of the mixture. For example, the salt concentration reaches 25% saturation when 1 ml of the saturated salt solution is added to 3 ml of the salt-free protein solution; 50% for 3 ml added; 75% for 9 ml added; and so on. The precipitated protein is collected and categorized according to the concentration of the salt solution at which it is formed. This partial collection of the separated product is called fractionation. For example, the fraction of the precipitated protein collected between 20 and 21% of salt saturation is commonly referred to as the 20-21% fraction. The protein fractions collected during the earlier stages of salt addition are less soluble in the salt solution than the fractions collected later.

Whereas the first method just described uses increasing salt concentrations, the following alternative method uses decreasing salt concentrations. In this alternative method, as much protein as possible is first precipitated with a concentrated salt solution. Then a series of cold (near 0°C) ammonium sulfate solutions of decreasing concentrations are employed to extract selectively the protein components that are the most soluble at higher ammonium sulfate concentrations. The extracted protein is recrystallized and thus recovered by gradually warming the cold solution to room temperature. This method has the added advantages that the extraction media may be buffered or stabilizing agents be added to retain the maximum enzyme activity. The efficiency of recovery typically ranges from 30 to 90%, depending on the protein. The recrystallization of protein upon transferring the extract to room temperature may occur, immediately or may sometimes take many hours. Nevertheless, very rarely does recrystallization fail to occur. The presence of fine crystals in a solution can be visually detected from the turbidity[51,52].

4. 1. 3. Dialysis

Low molecular weight materials, such as salts, and some biological materials, such as amino acids, coenzymes, and low molecular weight carbohydrates, can be removed by dialysis from macromolecular weight carbohydrates, can be removed by dialysis from macromolecular materials such as proteins, nucleic acid, and polysaccharides. Dialysis is the process of separating smaller molecules from large ones in solutions by use of a semi permeable membrane that permits passage of the smaller, but not the larger molecules.

The method based on the availability of cellulose membranes that have well-defined molecular weight cutoffs (pore sizes) that allow small molecules to traverse the membrane, while restricting the movement of larger molecules .

To use dialysis tube one must first hydrate it to give flexibility, and then seal it on one end. The protein sample is then placed within the tubing, and the other end of the tube is sealed. The tube is than placed in a solution of buffer that has the final molecular composition desired for the protein. With time, molecules that are small enough to pass through the pores of the dialysis tubing will equilibrate between the internal and external solutions[51,52].

Dialysis tube is prepared according to the manufacturer of the dialysis tube (Sigma Cat. No. D9652)

4. 1. 4. Ion Exchange Chromatography (IEC)

Ion exchange chromatography is the most commonly practiced chromatographic method of protein purification (Figure4.1). It is relatively easy to scale-up, widely applicable and low in cost relative to other separation methods.

FPLC Setup:

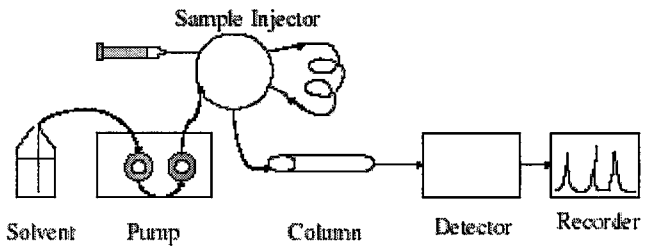


Figure 4. 1. Ion exchange chromatography.

- Proteins have charges due to amino acid side groups.
- Bind to charged column matrix depending on their charge at a particular pH.
- Anionic-negatively charged: phosphocellulose, heparin sepharose, S-sepharose.
- Cationic-positively charged: DEAE-sepharose, Q-sepharose. The functional group of Q sepharose is quaternary amine, which means that at all pH values these groups carry positive charges. This enables negatively charged proteins to stick.
- Elute bound proteins from column based on charge and displacement by salt or pH.

It is two types; cation exchange chromatography (CEC), positively charged ions bind to a negatively charged resin, another is anion exchange chromatography (AEC), the binding ions are negative, and the immobilized functional group is positive.

In anion exchange the surface charge of the solutes (proteins, nucleic acids, small molecules) which bind will be net negative, thus to get binding the buffer should be above the pI of solute. Commonly used anion exchange resins are Q-resin, a quaternary amine; and DEAE resin, DiEthylAminoEthane. AEC is often used as a primary chromatography.

In cation exchange the surface charge of the solutes will have a net positive charge, thus to get binding, the buffer should be below the pI of the solute. Commonly used cation exchange resins are Sresin, sulfate derivatives; and CM resins, carboxylate derived ions.

Retention and elution of solutes in IEC depend on the interactions of solutes with both the mobile and stationary phases. To describe how well solutes are retained on a column with different solvents, the terms weak mobile phase and strong mobile phase are used. Solutes can be eluted from a column by using either constant column conditions or gradient elution. The use of a constant mobile phase composition to elute is known as isocratic elution. Gradient elution in IEC is normally performed by changing the composition of the mobile phase with time. Known as solvent programming, this is done by going from a weak mobile phase to a strong one.

Eluants need to take into account the solubility of the chromatographed substances in the mobile phase and the polarity. For polar substances, solvents of non-polar character (saturated or halogenated hydrocarbons) are weak eluants compared with polar solvents (e.g. alcohols and acids).

Elution power is influenced by some factors; interaction between solvent molecules and chromatographed compound, interaction between adsorbed molecules of the mobile phase and the molecules of the sample in the adsorbed phase, interaction between the adsorbed molecules of the mobile phase and the adsorbent[51,52].

Detection is made with some methods;

• Fluorescence

-For impregnated plates, green fluorescence under UV light (254 Nm): some chromatographed substances appear as black spots.

• Chemical detection

-Iodine/ bromine/ammonia vapor.

-Ninhydrin.

-Bromocresol Green or Blue.

-Sulphuric acid.

• Radioactivity

-Plate can be divided up into sections, scraped and extracted with solvent prior to counting in liquid Scintillation Counter.

-Use radioactive counter.

-Use autoradiography[51,52].

4. 1. 5. Affinity chromatography

Affinity chromatography is a powerful technique that can be used to purify proteins that have affinity for some specific ligands, such as a substrate or inhibitor.

There are some types of affinity chromatography that can be very useful-but they are usually used late in the purification process after much of the garbage is removed. The ligand is covalently attached to a solid support such as agarose, and the protein mixture passed through a column (an affinity column) of this material. If your protein binds a specific carbohydrate or requires a specific co-factor, you may be able to get it to bind to a column on which that carbohydrate or co-factor is immobilized. Proteins that bind the ligand are retained by the column and can be eluted from it by washing with the free ligand, a high concentration of the carbohydrate or co-factor or by changing the conditions to reduce binding.

Mimics for binding sites can sometimes be used as affinity matrices; this imagination is the only limit for some proteins. Antibodies can be purified by the use of bacterial proteins that bind to antibodies-Protein A and Protein G.

One type of affinity chromatography is dye affinity chromatography using immobilized reactive triazine dyes. The dyes act as pseudo-affinity ligands which partially mimic the NAD-cofactor and can bind a wide range of proteins often with a surprising degree of specifity. Any proteins that do not have this particular structure will not bind to the column and will be eluted, with a low salt wash. Bound proteins can be biospecifically by using a cofactor. The Blue Sepharose has Cibacron Blue dye covalently bound to the agarose beads. The dye binds to the Rossman fold (NADH binding) sites of the protein. The protein would preferently bind the soluble NADH in this eluting buffer rather than the blue dye bound on the Sepharose beads. Therefore the protein would be eluted from the column. The NADH wash is not used because NADH has an absorbance at 280 nm and this make it difficult to monitor elution[51,52].

4. 1. 6. 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 useful to the cell.

4. 1. 6. 1. Product accumulation is often linear with time

In most cases, an enzyme converts one chemical (the substrate), into another (the product). A graph of product concentration vs. time follows three phases as shown in the following graph (Figure 4.2).

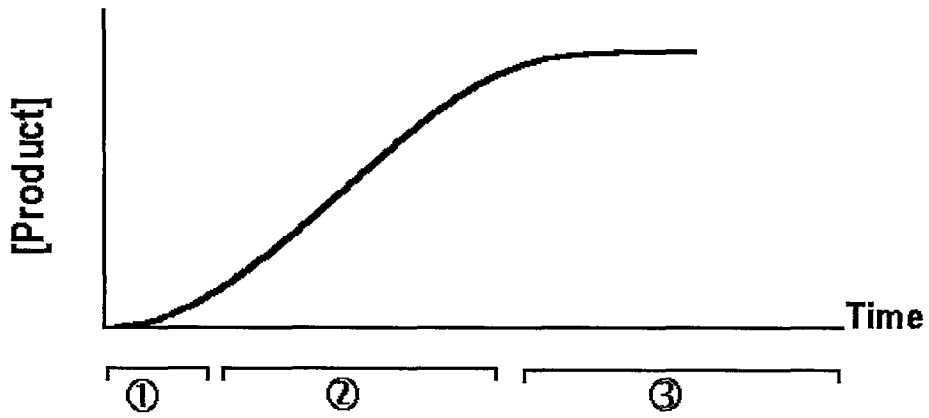


Figure 4. 2. The phases of the enzyme kinetics.

At very early time points, the rate of product accumulation increases over time. Special techniques are needed to study the early kinetics of enzyme action; since this transient phase usually lasts less than a second (the figure greatly exaggerates the first phase). For an extended period of time, the product concentration increases linearly with time.

At later times, the substrate is depleted, so the curve starts to level off. Eventually the concentration of product reaches a plateau and does not change with time.

It is difficult to fit a curve to a graph of product as a function of time, even if you use a simplified model that ignores the transient phase and assumes that the reaction is irreversible. The model simply cannot be solved to an equation that expresses product concentration as a function of time. To fit these kinds of data (called an enzyme progress curve) you need to use a program that can fit data to a model defined by differential equations or by an implicit equation[53].

Rather than fitting the enzyme progress curve, most analyses of enzyme kinetics fit the initial velocity of the enzyme reaction as a function of substrate concentration. The velocity of the enzyme reaction is the slope of the linear phase, expressed as amount of product formed per time. If the initial transient phase is very short, you can simply measure product formed at a single time, and define the velocity to be the concentration divided by the time interval.

We consider the data collected only in the second phase. The terminology describing these phases can be confusing. The second phase is often called the "initial rate", ignoring the short transient phase that proceeds. It is also called "steady state", because the concentration of enzyme-substrate complex does not change. However, the concentration of product accumulates, so the system is not truly at steady state until, much later, the concentration of product truly does not change over time[51,52].

4. 1. 6. 2. Enzyme velocity as a function of substrate concentration

When enzyme velocity is measured at many different concentrations of substrate, the graph is obtained as given in figure 4.3.

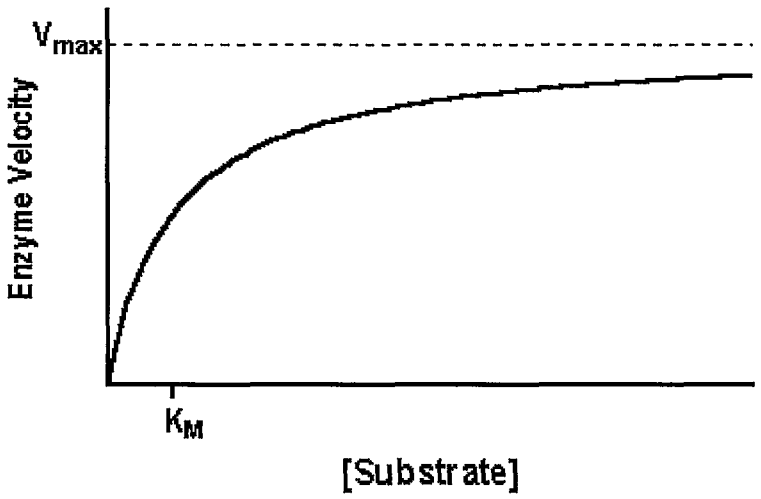


Figure 4. 3. Enzyme velocity plotted against substarte concentration obeying Michaelis-Menten kinetics.

Enzyme velocity as a function of substrate concentration often follows the Michaelis-Menten equation:

$$\text{Velocity} = V = \frac{V_{max}[S]}{[S] + K_M}$$

V_{max} is the limiting velocity as substrate concentrations get very large. V_{max} (and V) are expressed in units of product formed per time. If the molar concentration of enzyme is known, the observed velocity by the concentration of enzyme sites in the assay, and express V_{max} as units of moles of product formed per second per mole of enzyme sites can be divided. This is the turnover number, the number of molecules of substrate converted to product by one enzyme site per second. In defining enzyme concentration, distinguish the concentration of enzyme molecules and concentration of enzyme sites (if the enzyme is a dimer with two active sites, the molar concentration of sites is twice the molar concentration of enzyme).

K_M is expressed in units of concentration, usually in Molar units. K_M is the concentration of substrate that leads to half-maximal velocity. To prove this, set [S] equal to K_M in the equation above and $V = V_{max/2}$.

To understand the meaning of K_M, you need to have a model of enzyme action. The simplest model is the classic model of Michaelis and Menten, which has proven useful with many kinds of enzymes[51,52].

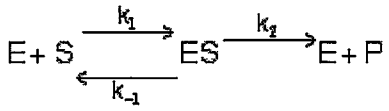


Figure 4. 4. The equation of enzyme-substrate reaction.

The substrate (S) binds reversibly to the enzyme (E) in the first reaction. In most cases, this step can't be measured. Production of product (P) is created by the second reaction.

From the model, Derived an equation that describes the rate of enzyme activity (amount of product formed per time interval) as a function of substrate concentration. The rate of product formation equals the rate at which ES turns into E + P, which equals k_2 times [ES]. This equation isn't helpful, because we don't know ES. We need to solve for ES in terms of the other quantities. This calculation can be greatly simplified by making two reasonable assumptions. First, we assume that the concentration of ES is steady during the time intervals used for enzyme kinetic work. That means that the rate of ES formation equals the rate of ES dissociation (either back to E + S or forward to E + P). Second, we assume that the reverse reaction (formation of ES from E + P) is negligible, because we are working at early time points where the concentration of product is very low. V_{max} (the velocity at maximal concentrations of substrate) as k_2 times E_{total}, and K_M, the Michaelis-Menten constant, as $(k_2 + k_{-1}/k_1$.

Substituting:

$$\text{Velocity} = V = \frac{V_{max}[S]}{[S] + K_M}$$

K_M is not a binding constant that measures the strength of binding between the enzyme and substrate. Its value includes the affinity of substrate for enzyme, but also

the rate at which the substrate bound to the enzyme is converted to product. Only if k_2 is much smaller than k_{-1} will K_M equal a binding affinity[51,52].

4. 1. 7. Enzyme protein assays

All steady-state kinetic parameters are measured in 100 mM triethanolamine buffer, pH 6, containing various amounts of substrate and coenzyme at 25°C. Initial rates is determined at 340 nm by measuring the NADH consumption and fitted using the GRAFIT (GRAFIT Data Analysis and Graphics program Version 3,01. CJ Smith. Erithacus Software Ltd., Staines, UK).

4. 1. 7. 1. Assumptions of enzyme kinetic analysis

Standard analyses of enzyme kinetics assume:

- The production of product is linear with time during the time interval used.
- The concentration of substrate vastly exceeds the concentration of enzyme. This means that the free concentration of substrate is very close to the concentration you added, and that substrate concentration is constant throughout the assay.
- The concentration of substrate vastly exceeds the concentration of enzyme. This means that the free concentration of substrate is very close to the concentration you added, and that substrate concentration is constant throughout the assay.
- A single enzyme forms the product.

Steady-state kinetics were preformed with 0, 2 nM NADH 5 nM LDH enzyme and various concentrations of pyruvate in the presence or absence FBP (5mM), enzyme activator. Protein concentration of *bs* LDH preparations were determined spectrophotometrically using an absorption coefficient (A_{280} in 0.1%) of 0,91 calculated from the content of trytophan and tyrosine.

4. 2. Experimental

- Bacterial Growth of *Bacillus staerothermophilus* LDH
- *E. coli* bacterial strain JM105 cells containing the *bs* LDH gene was streaked on a 2xYT Agar ampicillin plate and incubated at 37°C overnight.
- Three-single colony were removed with a sterile toothpick and inoculated into three 5 ml of 2xYT Broth ampicillin medium and incubated at 37°C overnight on an orbital shaker at 250 rpm.
- These starter cultures were added to three100 ml of 2xYT Broth ampicillin medium in 500 ml flask and incubated at 37°C overnight on an orbital shaker at 250 rpm.
- These starter cultures were added to three 1L of 2xYT Broth ampicillin medium in 3 L flask and incubated at 37°C overnight on an orbital shaker at 250 rpm.

4. 2. 1. Ultrasonic Extraction

- Cells grown in 3L of 2xYT medium at 37°C were transferred to centrifuge tubes.
- The cells were harvested by centrifugation at 4000 rpm in a 2x500 ml rotor for 20 minutes.
- The cells were suspended in minimum 50 mM TEA pH 7.5 buffers.
- The cells were broken open by sonication on ice (B.Braun Biotech International B.BRAUN LABSONIC® U).
- After sonication, the solution was centrifuged at 15000 rpm in a 2x20 ml rotor for 30 minutes. The supernatant was used for further purification.

4. 2. 2. Ammonium Sulphate Precipitation

The LDH enzyme was precipitated by adding 430g/L ammonium sulphate (Aldrich Cat. No.21 558-9) to supernatant and recovered by centrifugation at 15000 rpm for 30 minutes.

4. 2. 3. Dialysis

4. 2. 3. 1. Preparation of dialysis tube:

- Tubes were boiled in 1 mM EDTA and 10 mM $NaHC0_3$ solution for 30 minutes.
- Tubes were boiled in distilled H_20 for 10 minutes twice.
    - Tubes were tied with two tight knots on one end of the tubing and after centrifugation pellets solved in 5 ml of 0.05 M pH 7.5 TEA buffer were pipetted into the tubes.
    - The other ends of the tubings were closed off with two tight knots.
    - Tubes were taken into flasks containing 500 ml of same buffer. Buffers were stirred slowly with a stir bar and magnetic stir plate for 2 hours at 37°C.
    - Dialysis buffer were discarded and replaced with the same volume of fresh dialysis buffer. Dialysis process kept on for an additional 2 hours at the same temperature.
    - The tubes were removed from the beaker containing dialysis buffer and carefully cut one end of the tubing. Dialyzed protein solutions were pipetted into tubes.

4. 2. 4. SDS-PAGE

Gel casette assembly

- The glass plates, combs, and any other pertinent materials were cleaned and completely dried.
- Short plates were placed on top of a spacer plate. Both plates were inserted into the casting frame on a flat surface. The casting frames were clamped and checked that the plates are level on the bottom. Casting frames were put into the casting stand.

Preparation of the gel

- All reagents were combined except the TEMED for the separating gel.
- Seperating gel composition

Acryl/Bis mixture	2.5 ml
Buffer with Gylcerol	4.7 ml
Water	2.8 ml
APS	70 µl
TEMED	16 µl
Total	10.086 ml

- TEMED was quickly added when gel was ready to pour. Solution was mixed using a pipette and separating gel solution poured between the glass plates in the casting chamber to about ¾ below the short plate.
- Both the separating and stacking gels should polymerize within 15 minutes.
- All reagents were combined except the TEMED for the stacking gel.
- Stacking gel composition

Acrylamide Bisacrylamide mixture	600 µl
Buffer without Glycerol	1.2 ml
Water	4.2 ml
Ammonium Persulphate (APS)	70 µl
TEMED	16 µl
Total	6.086 ml

- A small layer of butanol was added on top of the gel prior to polymerization of separating gel. The top layer of the gels was rinsed with deionized water prior to pouring the stacking gel. The well forming combs were inserted into the opening between the glass plates.

- Once the stacking gel had been polymerized, the combs were gently removed.

Sample Preparation

- Some water in a 600 ml beaker was placed and leaved on a hot plate to boil.

- Samples were prepared by mixing the 500 µl volume of protein solution and 500 µl of sample application buffer. Samples and prestained molecular weight marker (Fermentas, SM0431, its figure on the appendix) were boiled for about 5 min.

Electrophoresis

- The gel cassettes were removed from the casting stand and placed in the electrode assembly with the short plate on the inside.
- Some tank electrophoresis buffer was poured into the opening of the casting frame between the gel cassettes. The wells of the gels were filled with buffer.
- 15 µl of denaturized samples were slowly pipetted with a Hamilton syringe.
- The region outside of the frame was filled with tank buffer.
- The electrophoresis tank was connected to the power supply. Separation was run at 110 V, until the dye front has reached to the bottom of the gel.
- When electrophoresis was complete, the power supply was turned off and the apparatus was disassembled.

4. 2. 5. Ion Exchange Chromatography

- The column (3 cm x 12 cm) was filled with Q Sepharose (Amersham Bioscience) and put FPLC (Bio-Rad Bio Logic Duo Flow).
- The column was washed with two column volumes of 50mM TEA, pH 7, 5.
- The sample from the end of the dialysis was loaded to the column and eluted with a gradient from 0 M to 0, 5 M NaCl in 50 mM TEA pH 7, 5.
- We saw only one pick in the display chromatogram of the computer so we think time our protein was eluted at the same.

4. 2. 6. Affinity Chromatography

- The column (3 cm x 12 cm) was filled with Blue Sepharose and put FPLC.
- The column was washed with two column volumes of 50 mM TEA, pH 6.
- The sample from the end of the dialysis was loaded to the column.
- The sample from the end of the dialysis was loaded to the column and eluted with a gradient from 0 M to 1 M NaCl in 10mM TEA pH 6.
- We saw only one pick that we think time our protein was eluted at the same, in the display chromatogram of the computer.
- Fractions containing LDH were loaded to the SDS-PAGE.
- SDS-PAGE and brilliant blue Coomassie staining showed the proteins to be >98% pure after described purification.

4. 2. 7. Enzyme Kinetics

The steady-state catalytic properties of both the wild type and mutant were measured at 25°C by following the decrease in A_{340} in the NADH/NAD^+ conversion by using spectrophotometer (Perkin Elmer Instruments Lambda 25 UV/VIS spectrometer) in the presence (+FBP 5mM) and absence of FBP (-FBP) (Sigma, F-4757).

All assays were carried in 10mM TEA. Determinations of K_{cat} and K_M for the substrates were made at saturating NADH concentrations (0,2 mM). Enzyme concentration is 5nM and pyruvate concentration is between 0, 25 μM and 40 mM. Kinetic data were analyzed using the GRAFIT.

5. RESULTS AND DISCUSSION

5. 1. Site-directed mutagenesis

5. 1. 1. Molecular Biology Studies

In the *bs* LDH gene, the conserved aspartic acid residue at position 38 was replaced by the longer basic arginine by using PCR based overlap extension method. Mutant double stranded DNA was sequenced to check for the correct amino acid change using the ABI Prism 3100 Avant automated sequencer at Molecular Biology and Genetics Dept., ITU. The sequencing result confirmed that the expected sequence was obtained for D38R mutation (Figure5.1).

Figure 5. 1. Sequencing result of the mutant LDH.

5. 1. 2. Expression of cloned DNA molecules in *E.coli*

Further experiments showed the gene was overproducing protein (Figure5.2) and enzymatic activity indicated that the protein was correctly folded.

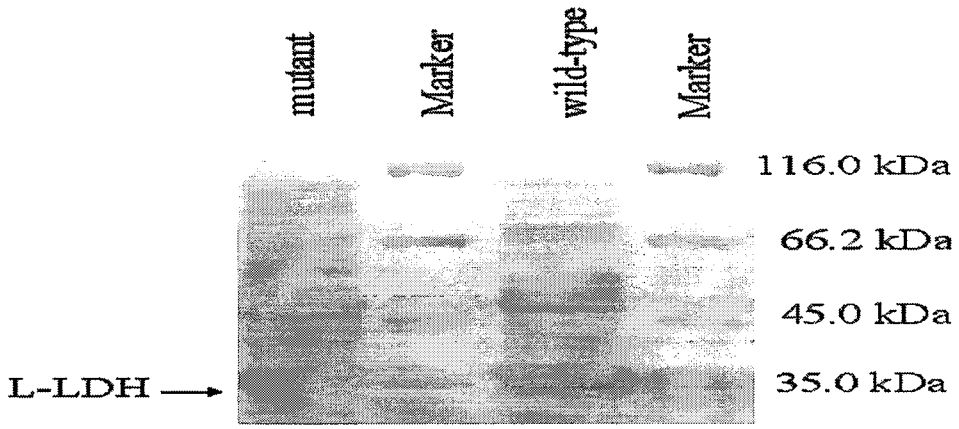


Figure 5. 2. The SDS-PAGE gel of the over-expressed D38R *bs* LDH by Coomassie Blue.

5. 1. 3. Purification

Both wild type and mutant LDH proteins have been purified. The result of Coomassie Blue-stained SDS Polyacrylamide gel showed that both proteins to be ≥ 98 % pure after the purification. As it can be seen in Figure5.3, the proteins are sufficiently pure to use for steady-state kinetics.

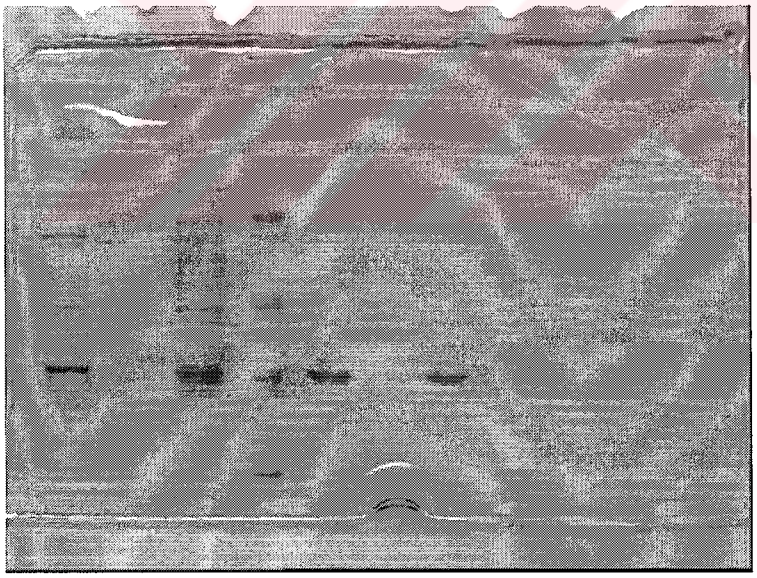


Figure 5. 3. The result of SDS-PAGE from the Q-Sepharose and Blue-Sepharose. (The first line is total cell, the second line is empty, the third line is after dialysis for wt, and the fourth line is marker the fifth is wt LDH, the sixth line is empty and the seventh line is mutant LDH).

5. 1. 4. Steady-state kinetics

Steady-state kinetic results for wild type and mutant proteins are shown in Table5.1. The inhibition constant (K_i) for pyruvate in the wild type is 10.9 mM but in the mutant is 31.1 mM. Therefore, the substrate inhibition was reduced three fold by exchange of aspartate for arginine (Table5. 2).

Table 5. 1. Showing the absorbance of NADH for both wt and the mutant LDH.

mutant LDH –FBP		**mutant LDH +FBP**	
Substrate	Absorbance A_{340}	Substrate	Absorbance A_{340}
0,25µM	0,01990	0,25µM	0,00420
0,5µM	0,01530	0,5µM	0,02310
0,75µM	0,02720	0,75µM	0,04720
1 mM	0,03310	1mM	0,08375
2mM	0,05560	2 mM	0,11500
4 mM	0,06110	4 mM	0,16310
8 mM	0,08990	8 mM	0,18850
10 mM	0,12120	10 mM	0,19830
20 mM	0,18800	20 mM	0,17980
40 mM	0,24710	40 mM	0,14160

wild type LDH –FBP		**wild type LDH +FBP**	
Substrate	Absorbance A_{340}	Substrate	Absorbance A_{340}
0,25µM	0,00410	0,25 µM	0,0011
0,5µM	0,00510	0,5µM	0,0023
0,7µM	0,00750	0,75µM	0,0044
1 mM	0,01080	1mM	0,0083
2 mM	0,01570	2 mM	0,0108
4 mM	0,01920	4 mM	0,0153
8 mM	0,01840	8 mM	0,0198
10 mM	0,01790	10 mM	0,2434
20 mM	0,01370	20 mM	0,2827
40mM	0,00810	40 mM	0,3101

Table 5. 2. Kinetic results for both wild type and mutant LDH.

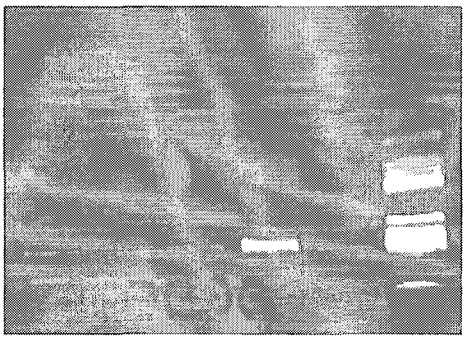
	WILD TYPE LDH		MUTANT LDH	
	+FBP	–FBP	+FBP	–FBP
V_{max} (abs / min)	0,0393	0,6106	0,3328	0,36
K_m (mM)	2,7617	32	3,7	19,3
K_i (mM)	10,9	-	31,1	-
K_{cat} (sec-1)	21,06	327,22	178,35	194,85

5. 2. DNA Shuffling Experiments

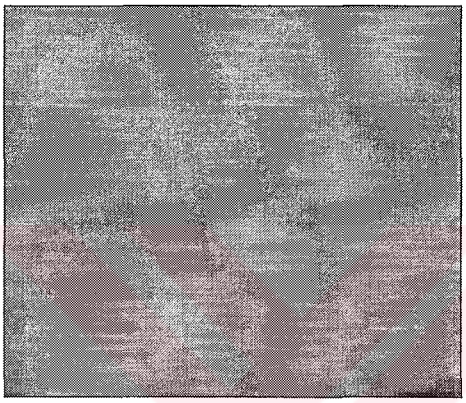
When DNA shuffling method was applied to LDH it first involved isolating the coding DNA (1 kb fragment) with a 5' region containing an *Eco*RI site and a 3' region with a *Pst* I site. The product is of the correct size (Figure 5. 4). The gene is then randomly cleaved with a non-specific endo DNAse I. This should give a smear of randomly sized oligonucleotides smaller than 1 kb. These are visible in figure 5. 4 together with a small amount of undigested gene.Fragments with sizes 100-300 bp were purified from the corresponding region of a 2 % low melting point agarose gel. These fragments were then amplified by PCR 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 (Figure 5.4). The full-length gene is finally isolated by a short PCR step using the correct primers for the 5' *Eco*RI (N-terminus) and 3' *Pst* I (C-terminus) sites. The gel (Figure 5. 4) shows a high yield of a randomly, shuffled library of 1 kb genes probably having being randomised by DNA shuffling. This preliminary experiment showed that the method could be applied to LDH.

The 1 kb gene product obtained was then cut with restriction enzymes *Eco*RI and *Pst*I and ligated into similarly digested pKK223-3 following purification from a 0.8% agarose gel and transformed into JM 105 *E. coli* cells.

a. 954 bp PCR product was digested with DNase I.



b. The oligonucleotides after primerless PCR.



c. 954 bp product containing library after a final PCR step using primers.

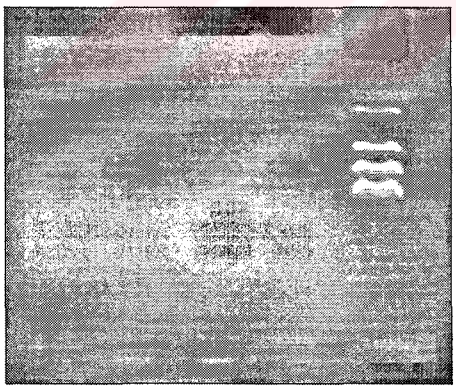


Figure 5. 4. Agarose gels of the steps used randomly shuffle the *bs* LDH.

6. CONCLUSION

The studies described in this thesis are directed towards an understanding of the relationship between the structure and the function of LDH from *Bacillus stearothermophilus* with the overall aim of developing a novel protein, which has been engineered to be a commercially useful enzyme for industrial purposes. Protein engineering is a promising technique, which can be used to create enzymes with desired new features such as, reducing of the substrate inhibition for biotechnological applications. These thesis details two strategies used to produce novel LDH proteins.

In the first strategy, the role of the conserved aspartic acid residue at position 38 was investigated. This aspartate has been replaced by arginine and the effect of this change on the kinetic behavior of enzyme determined. The desired mutated protein was achieved using site directed mutagenesis and kinetically characterized. The steady state catalytic properties of both the wild type (aspartate 38) and mutant (arginine38) with pyruvate were compared in the presence and absence of FBP. The results showed that, substrate inhibition was reduced threefold by exchange of aspartate for arginine.

In the second strategy, we tried to obtain *bs* LDH enzymes with altered substrate specificities by randomly shuffling the DNA sequence of the enzyme and screening the products against their ability to transform useful new substrates (e.g. 4- phenyl-2 -hydroxybutanoate). The results of DNA shuffling experiments showed that a high yield of a random library of the LDH gene was produced by using the DNA shuffling method and transformed into JM 105 *E. coli* cells. As a future work, all the colonies will be screened to obtain the desired mutant of LDH from random library.

7. REFERENCES

1. **Holbrook, JJ., Liljas, A., Steindel, SJ., Rossmann MG.,** 1975. Lactate dehydrogenase. In: Anonymous Enzymes, 3rd Ed., volume 11, 191-292. Edited by: Boyer, Paul D. Academic: New York, N. Y.

2. **Fine, I., Kaplan, N.O., and Kufinec, D.**, 1963. *Biochemistry* **2**, 116.

3. **Clarke, AR., Waldman, ADB., Munro, I., Holbrook, JJ,.** 1985. Changes in the state of subunit association of LDH from *Bacillus stearothermophilus*. *Biochim Biophys Acta*; **828**: 375-379.

4. **Slender, R. K., Yang, S. Y.**, 1970. *Science* **169**, 179

5. **Wirz, B., Sulter, F., Zuber, H.,** 1983. Structure and function of L-LDHs from thermophilic and mesophilic bacteria. III - The primary structure of thermophilic lactate dehydrogenase from Bacillus stearothermophilus. Hydroxylamine -, o - iodosobenzoic acid - and tryptic - fragments. The complete amino - acid sequence. *Hoppe - Seyler 's Z Physiol Chem Ed*; **364**: 893 – 909.

6. **Adams, M. J., Buehner, M., Chandrasekhar, Ford, K. G. C., Hackert, M. L., Liljas, A., Rossman, M. G., Smiley, I.E., Allison, W.S., Kaplan, N. O., and Taylor, S. S,.** 1968. *Proc. Natl. Acad. Sci.* **70**.

7. **Piontek, K., Chakrabarti, P., Schar, H. P., Rossmann, M. G., Zuber, H.,** 1990. Structure determination and refinement of Bacillus stearothermophilus lactate dehydrogenase. *Proteins* 7 pp. 74.

8. http://www.ncbi.nlm.nih.gov/

9. **Wigley DB, Gamblin SJ, Turkenburg JP.,** 1992. Structure of a ternary complex of an allosteric lactate dehydrogenase from *Bacillus stearothermophilus* at 2.5 Å resolution. *J Mol Biol.* **223**: 317-335.

10. **S. Arnott, D. W. L. Hukins.**, 1972. *BJ* **13**, 453.

11. **M. Sundaralingam.**, 1969. *Biopolymers* 7, 821.

12. **Wigley, D. B., Clarke, A.R., Holbrook, J. J.,** 1987. Hydrogem bonding of the carboxyamide of NADH is not important for catalysis in lactate deydrogenase. *Prot. Eng.* 1, 260.

13. **Fine, I., Kaplan, N. O., and Kuftinec.,** 1963. *Biochemistry* **2**, 116.

14. **Wilks, HM., Hart, KW., Feeney, R.,** 1988. A specific, highly active malate dehydrogenase by redesing of a lactate dehydrogenase framework. *Science*, 1541 - 1544.

15. **Bur, D., Clarke, AR., Friesen, JD.,** 1989. On the effect on specificity of Thr 246-Gly mutation in L - lactate dehydrogenase of *Bacillus stearothermophilus. Bichem Biophys Res commun;* **161**: 59-63.

16. **Adams, MJ., Buehner, M., Chandrasekher, K.,** 1973. Structure function relationships in LDH. *Proc Nat Acad Of Sci* **70**: 1968 – 1972.

17. **Dunn, CR.,** 1983. Acrystallographic study of structural changes in L - Lactate dehydrogenase induced by the binding of substrate. 1989; *Nature* **302**:842-844.

18. **Coulson, CJ., Rabin, BR.,** 1969. Inhibition of lactate deydrogenase by high concentrations of pyruvate: the nature and removal of the inhibitor. *FEBS Lett.***3**, 333.

19. **Clarke, AR., Evington, JRN., Dunn, CR., Atkinson, T., Holbrook, JJ.,** 1986. The molecular pathway by which fructose 1,6 bisphosphate induces the assembly of a bacterial lactate dehydrogenase. *BBA*; **870**:112 - 126.

20. **Clarke, A. R., Waldman, A. D. B., Munro, I., Holbrook, J. J.,** 1985. *BBA* **828**, 375 -379.

21. **Clarke, A. R., and Daffron, T. R.,** 1998. In comprehensive biological catalysis, vol 3, m. sinnott, ed., Academic Press. USA.

22. **Adams, M. J.,** 1968. *PNAS* **70.**

23. **White, J.L.,** 1976. *J. Molec. Biol.***102**, 759.

24. **Holbrook, J.J.,** 1973. *FEBS letter* **31**, 57.

25. **Holbrook, J.J.,** 1974. *Biochem J.* **139**, 677.

26. **Clarke, A. R., Atkinson, T., Holbroook J. J.,** 1989. *Trends. Biochem. Sci.* **14**, 101.

27. **Holbrook J. J., Ingram V. A.,** 1973. *Biochem. J.* **131**,729.

28. **Bizzari, S., Kishi, A.** CEH Report. Lactic Acid, Its Salts and Esters. Published April 2003.

29. **Furlenmeier, A., Quitt, P., Vogler, K., Lanz, P.,** 1976. inventors Hoffmann La Roche Inc., assignee, USA, 3957758.

31. **Wu, KC., Chan, K., Lee, CUG, Lau, YF.,** 1987. *Biochem. Biophys. Res. Commun.* **146**, 964-970.

30. **Casy, G., Lee, T.V., and Lovell, H.,** 1992. *Tetrahedron Letters,* **33**, 817.

31. **Clarke, A.R., Smith, C.J., Hart, K.W., Wilks, Chia, HM., Lee, WN., Birktoft, TV., Banaszak, JJ., Barstow, LJ., Atkinson, DA.,& Holbrook,**

JJ.,1987. *Biochemical and Biophysical Research Communications* **148**, 15-23.

32. **El Hawrani, A.S., Sessions, RB., Moreton, K.M., and Holbrook, JJ.,** 1996. Guided evolution of enzymes with new substrate specificities, *J. Mol. Biol.*, 0-14.

33. **Allen, SJ., Holbrook, JJ.,** 2000. Production of an activated form of *Bacillus stearothermophilus* L-2- hydroxyacid dehydrogenase by directed evolution, *Protein engineering* Vol. **13**, No:1 P:5 - 7.

34. **El Hawrani, A.S., Sessions, RB., Moreton K.M., and Holbrook, JJ.,** 1996. Guided evolution of enzymes with new substrate specificities, *J. Mol. Biol.*, 0 - 14.

35. **Winer, A. D., Schwert, G. W.** 1958. *JBC* **231**, 1065.

36. **Karagüler Gül, N., Sessions, R. B., and Holbrook, John J.,** 2001. Role of glutamate - 52 in the mechanism of L - lactate dehydrogenase from Bacillus stearothermophilus. *Biotechnology Letters* . **23**: 395 - 399.

37. **Hewitt, CO., Eszes, CM., Sessions, RB., Moreton, KM., Dafforn, TR., Takei, J., Dempsey, CE., Clarke, AR., Holbrook, JJ.,** 1999. A general method for relieving substarte inhibition in lacatate dehydrogenase. *Prot. Eng.* Jun;**12** (6):491-6.

38. **Wilson, K., Goulding, KH.,** 1986. Principles and techniques of practical biochemistry. Third ed., Edward Arnold,. London

39. **Madigan M. T., Martinko J., Parker J.,** 2003. 10th ed. Brock Biology of Microorganisms. USA.

40. **Brown, T.A.,** 2001. Gene cloning and DNA analysis : an introduction.. 4th ed. Blackwell Science, Oxford.

41. **Old RW, Primrose SB.** 1994. Principles of gene manipulations. Fifth edition. Blackwell Scientific Publications, Oxford.

42. **Nicholl, DST.,** 1994. An introduction to genetic engineering. Cambridge University Pres, Cambridge.

43. **Lundberg, K.S.,** 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosus, *Gene,* **108**, 1-6.

44. **Cline, J, Braman, JC., Hogrefe, HH.,** 1996.. Sep 15. *Nucleic Acids Res.* **24**(18):3546-51.PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases.

45. http://www.fermentas.com

46. **Sambrook, J., Fritsch, EF., & Maniatis, T.,** 2001. Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

47. **Griffiths, AJF., Miller, JH., Suzuki, DT., Lewontin, RC., Gelbart, WM.,** 1993. An introduction to genetic analysis. Fifth edition. W. H. Freeman and Company. New York

48. **Fisher, C. L., and Pei, G. K.,** 1997. Modification of a PCR-based site-directed mutagenesis method. *Biotechniques* **23**, 570-574

49. **Griffin, A.M., and Griffin, H.G.,** 1995. Molecular Biology: Current Innovations and Future Trends. Eds. Horizon Scientific Press, Norfolk.

50. **Jakoby, W.B.,** 1971. Methods in Enzymology: Enzyme purification and related techniques. Vol. XXII. Academic Press, USA, pp: 273-315.

51. **Duggleby, RG.,** 1995. Analysis of Enzyme Reaction Progress Curves by Nonlinear Regression. Methods in Enzymology, 249.

52. **Murray, RK., Granner, DK., Mayes, PA., Rodwell VW.,** 1993. Harper's Biochemistry. 23rd edition. Connecticut: Publishing Division of Prentice Hall, USA.

8. APPENDIX

Ths seconder structure of the *bs* LDH

```
M  K  N  N  G  G  A  R  V  V  V  I  G  A  G  F  V  G  A  S
  1 atgaaaaacaacggtggagcccgagtagtggtcatcggcgccgggtttgtcggcgccagt 60

     Y  V  F  A  L  M  N  Q  G  I  A  D  E  I  V  L  I  D  A  N
 61 tatgtgtttgccttaatgaatcaagggattgccgatgagatcgtgctcatcgatgcgaat 120

     E  S  K  A  I  G  D  A  M  D  F  N  H  G  K  V  F  A  P  K
121 gaaagcaaggccataggcgatgcgatggacttcaaccatgggaaagtatttgcgccgaag 180

     P  V  D  I  W  H  G  D  Y  D  D  C  R  D  A  D  L  V  V  I
181 ccggttgacatttggcacggcgattacgatgattgccgcgatgccgatttggttgtcatt 240

     C  A  G  A  N  Q  K  P  G  E  T  R  L  D  L  V  D  K  N  I
241 tgcgccggcgccaaccaaaaaccgggcgagacgcggcttgatcttgtggacaaaaacatt 300

     A  I  F  R  S  I  V  E  S  V  M  A  S  G  F  Q  G  L  F  L
301 gccattttccgctcgatcgttgagtcggtcatggcatccggatttcaaggactgtttctc 360

     V  A  T  N  P  V  D  I  L  T  Y  A  T  W  K  F  S  G  L  P
361 gtcgccaccaatccggtcgacattttaacgtacgcgacgtggaaattcagcggcctgccg 420

     H  E  R  V  I  G  S  G  T  I  L  D  T  A  R  F  R  F  L  L
421 catgagcgggtgatcggttcggggacgattttagatacggcgcggttccgcttttgttg 480

     G  E  Y  F  S  V  A  P  Q  N  V  H  A  Y  [redacted]
481 ggcgagtatttctctgtcgctccgcaaaatgttcatgcctatattattggggaacacggc 540

     [redacted]  L  P  V  W  S  Q  A  Y  I  G  V  M  P  I  R  K  L
541 gacactgaactcccggtctggagccaggcttatatcggcgtcatgccgatccgcaagctg 600

     V  E  S  K  G  E  E  A  Q  K  D  L  E  R  I  F  V  N  V  R
601 gtcgagtccaaaggggaagaagcgcaaaaagatctcgagcgcatttttgtcaatgtgcgc 660

     D  A  A  Y  Q  I  I  E  K  K  G  A  T  Y  Y  G  I  A  M  G
```

```
661 gatgccgcctaccaaattattgagaaaaaaggagcgacgtactacgggattgcgatgggg 720

    L  A  R  V  T  R  A  I  L  H  N  E  N  A  I  L  T  V  S  A
721 cttgcccgcgtgacgcgcgccattttgcataacgaaaacgctattttgaccgtatcagcc 780

    Y  L  D  G  L  Y  G  E  R  D  V  Y  I  G  V  P  A  V  I  N
781 tacctcgatggcctatatggggagcgcgacgtctacatcggagtgccggctgtcattaac 840

    R  N  G  I  R  E  V  I  E  I  E  L  N  D  D  E  K  N  R  F
841 cgcaatggcatccgcgaggtgatcgaaattgaattgaatgatgacgaaaaaaatcgattc 900

    H  H  S  A  A  T  L  K  S  V  L  A  R  A  F  T  R  *
901 catcatagcgcagctacattaaaaagcgtgctagcccgtgcttttacgcgatga       954
```

RVVVIGAGFVGASYVFALM Nucleotide(NADH,FAD,ADP) Binding Site,

Ref.: WIERENGA, R.K. & HOL, W.G.J. 1983.NATURE 302:842-844.

Motif: *!E*[AIVLM]!PG*G**[GA]**[LIVMCYF]***[AILVM]*

AGANQKPGETRL Active Site Loop

Ref.: LERCH H.P. et al., 1989.GENE 83:263-279.

Motif: *[LIVAM]G[EQ]HG[DN][ST]*

[redacted] LDH Active Site

Ref.: D.B.Wigley et.al.,1992, Structure of a ternary complex of an allosteric lactate dehydrogenase from Bacillus stearothermophilus at 2.5 A resolution, Mol. Biol.223, 317-335.

Results of the aligments

Sequence alignment for sample 3

(All sequences were aligned by using BLAST -

http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)

'query' is wild type LDH gene

'sbjct' is sequence of sample 3

Identities = 691 / 709 (97,5%), Gaps = 7 / 709 (0,9%)

```
Query: 1   atgaaaaacaacggtggagcccgagtagtggtcatcggcgccgggtttgtcggcgccagt 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 140 atgaaaaacaacggtggagcccgagtagtggtcatcggcgccgggtttgtcggcgccagt 199
Query: 61  tatgtgtttgccttaatgaatcaagggattgccgatgagatcgtgctcatcgatgcgaat 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 200 tatgtgtttgccttaatgaatcaagggattgccgatgagatcgtgctcatccgcgcgaat 259
                                                    desired mutation
Query: 121 gaaagcaaggccataggcgatgcgatggacttcaaccatgggaaagtatttgcgccgaag 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 260 aaaagcaaggccataggcgatgcgatggacttcaaccatgggaaagtatttgcgccgaag 319
Query: 181 ccggttgacatttggcacggcgattacgatgattgccgcgatgccgatttggttgtcatt 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 320 ccggttgacatttggcacggcgattacgatgattgccgcgatgccgatttggttgtcatt 379
Query: 241 tgcgccggcgccaaccaaaaaccgggcgagacgcggcttgatcttgtggacaaaaacatt 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 380 tgcgccggcgccaaccaaaaaccgggcgagacgcggcttgatcttgtggacaaaaacatt 439
Query: 301 gccattttccgctcgatcgttgagtcggtcatggcatccggatttcaaggactgtttctc 360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 440 gccattttccgctcgatcgttgagtcggtcatggcatccggatttcaaggactgtttctc 499
Query: 361 gtcgccaccaatccggtcgacattttaacgtacgcgacgtggaaattcagcggcctgccg 420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 500 gtcgccaccaatccggtcgacattttaacgtacgcgacgtggaaattcagcggcctgccg 559
Query: 421 catgagcgggtgatcggttcggggacgattttagatacggcgcggttccgcttttgttg 480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 560 catgagcgggtgatcggttcggggacgattttagatacggcgcggttccgcttttgttg 619
Query: 481 ggcgagtatttctctgtcgctccgcaaaatgttcatgcctatattattggggaacacggc 540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 620 ggcgagtatttctctgtcgctccgcaaaatgttcatgcctatattattggggaacacggc 679
Query: 541 gacactg-aactcccggtctggag-ccaggcttatatcggcgtcatgccgatccgcaagc 598
           ||||||| |||||||||||||||| ||||||||||||||||||||||||||| |||||
Sbjct: 680 gacactgaaactcccggtctggagnccaggcttatatcggcgtcatgccgatccccaagc 739
Query: 599 tggtcgagtccaaaggggaagaagcgcaaaaagatctcgagcgcatttttgtcaatgtgc 658
           |||||||||||||||||| | || ||| ||||||||||||||||||||||||||||||
Sbjct: 740 tggtcgagtccaaaggggg-anaancgc-aaaagatctcgagcgcatttttgtcaatgtgc 797
Query: 659 gcgatgccgcctaccaaattattgagaaaaaaggagcgacgtactacgg 707
           |||||||| |||||| |||| | || ||||||||| |||| ||||||||
Sbjct: 798 gcgatgccncctacc-aattntnga-aaaaaaggaccgac-tactacgg 843
```

Sequence alignment for sample 4

'query' is wild type LDH gene

'sbjct' is sequence of sample 4

Identities = 664 / 676 (98, 2%), Gaps = 4 / 676 (0, 6%)

```
Query: 1   atgaaaaacaacggtggagcccgagtagtggtcatcggcgccgggtttgtcggcgccagt 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 133 atgaaaaacaacggtggagcccgagtagtggtcatcggcgccgggtttgtcggcgccagt 192
Query: 61  tatgtgtttgccttaatgaatcaagggattgccgatgagatcgtgctcatcgatgcgaat 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||   ||||||
Sbjct: 193 tatgtgtttgccttaatgaatcaagggattgccgatgagatcgtgctcatccgcgcgaat 252
Query: 121 gaaagcaaggccataggcgatgcgatggacttcaaccatgggaaagtatttgcgccgaag 180
           ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 253 gaacgcaaggccataggcgatgcgatggacttcaaccatgggaaagtatttgcgccgaag 312
Query: 181 ccggttgacatttggcacggcgattacgatgattgccgcgatgccgatttggttgtcatt 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 313 ccggttgacatttggcacggcgattacgatgattgccgcgatgccgatttggttgtcatt 372
Query: 241 tgcgccggcgccaaccaaaaaccgggcgagacgcggcttgatcttgtggacaaaaacatt 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 373 tgcgccggcgccaaccaaaaaccgggcgagacgcggcttgatcttgtggacaaaaacatt 432
Query: 301 gccattttccgctcgatcgttgagtcggtcatggcatccggatttcaaggactgtttctc 360
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| .
Sbjct: 433 gccattttccgctcgatcgttgagtcggtcatggcatccggatttcaaggactgtttctc 492
Query: 361 gtcgccaccaatccggtcgacattttaacgtacgcgacgtggaaattcagcggcctgccg 420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 493 gtcgccaccaatccggtcgacattttaacgtacgcgacgtggaaattcagcggcctgccg 552
Query: 421 catgagcgggtgatcggttcggggacgattttagatacggcgcggttccgctttttgttg 480
           |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 553 catgagcgggtgatcggttcagggacgattttagatacggcgcggttccgctttttgttg 612
Query: 481 ggcgagtatttctctgtcgctccgcaaaatgttcatgcctatattattggggaacacggc 540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 613 ggcgagtatttctctgtcgctccgcaaaatgttcatgcctatattattggggaacacggc 672
Query: 541 gacactgaactcccggtct-ggagccaggcttatatcggcgtcatgccgatccgcaagct 599
           |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct: 673 gacactgaactcccggtctgggagccaggcttatatcggcgtcatgccgatccgcaagct 732
Query: 600 ggtcgagtccaaaggggaagaagcgcaaaaagatctcgagcgcatttttgtcaatgtgcg 659
           |||||||||||||||||| || | | |||| ||||||||||||||||||||||||||||
Sbjct: 733 ggtcgagtccaaaggggaa-aaacnc-aaaanatctcgagcgcatttttgtcaatgtgcg 790
```

Sequence alignment sample 4 using *bs* N - terminus primer

'query' is wild type LDH gene

'sbjct' is sequence of sample 4

Identities = 786 / 802 (98,0%), Gaps = 3 / 802 (0,4%)

```
Query: 50  cgggtttgtcggcgccagttatgtgtttgccttaatgaatcaagggattgccgatgagat 109
           ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 25  cgggtttctcggcgccagttatgtgtttgccttaatgaatcaagggattgccgatgagat 84
Query: 110 cgtgctcatcgatgcgaatgaaagcaaggccataggcgatgcgatggacttcaaccatgg 169
           |||||||||||   |||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 85  cgtgctcatccgcgcgaataaaagcaaggccataggcgatgcgatggacttcaaccatgg 144
Query: 170 gaaagtatttgcgccgaagccggttgacatttggcacggcgattacgatgattgccgcga 229
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 145 gaaagtatttgcgccgaagccggttgacatttggcacggcgattacgatgattgccgcga 204
Query: 230 tgccgatttggttgtcatttgcgccggcgccaaccaaaaaccgggcgagacgcggcttga 289
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 205 tgccgatttggttgtcatttgcgccggcgccaaccaaaaaccgggcgagacgcggcttga 264
Query: 290 tcttgtggacaaaaacattgccattttccgctcgatcgttgagtcggtcatggcatccgg 349
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 265 tcttgtggacaaaaacattgccattttccgctcgatcgttgagtcggtcatggcatccgg 324
Query: 350 atttcaaggactgtttctcgtcgccaccaatccggtcgacattttaacgtacgcgacgtg 409
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 325 atttcaaggactgtttctcgtcgccaccaatccggtcgacattttaacgtacgcgacgtg 384
Query: 410 gaaattcagcggcctgccgcatgagcgggtgatcggttcggggacgattttagatacggc 469
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 385 gaaattcagcggcctgccgcatgagcgggtgatcggttcggggacgattttagatacggc 444
Query: 470 gcggttccgctttttgttgggcgagtatttctctgtcgctccgcaaaatgttcatgccta 529
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 445 gcggttccgctttttgttgggcgagtatttctctgtcgctccgcaaaatgttcatgccta 504
Query: 530 tattattggggaacacggcgacactgaactcccggtctggagccaggcttatatcggcgt 589
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 505 tattattggggaacacggcgacactgaactcccggtctggagccaggcttatatcggcgt 564
Query: 590 catgccgatccgcaagctggtcgagtccaaaggggaagaagcgcaaaaagatctcgagcg 649
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 565 catgccgatccgcaagctggtcgagtccaaaggggaagaagcgcaaaaagatctcgagcg 624
Query: 650 cattttgtcaatgtgcgcgatgccgcctaccaaattattgagaaaaaaggagcgacgta 709
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 625 cattttgtcaatgtgcgcgatgccgcctaccaaattattgagaaaaaaggagcgacgta 684
Query: 710 ctacggg-attgcgatggggc-ttgcccgcgtgacgcgcgccattttgcataacgaaaac 767
           |||| || |||||||||||| |||||||||||| |||||||||||||| |||||||
Sbjct: 685 ctacnggaattgcgatggggctttgcccgcgtgacccgcgccattttgcatancgaaaac 744
```

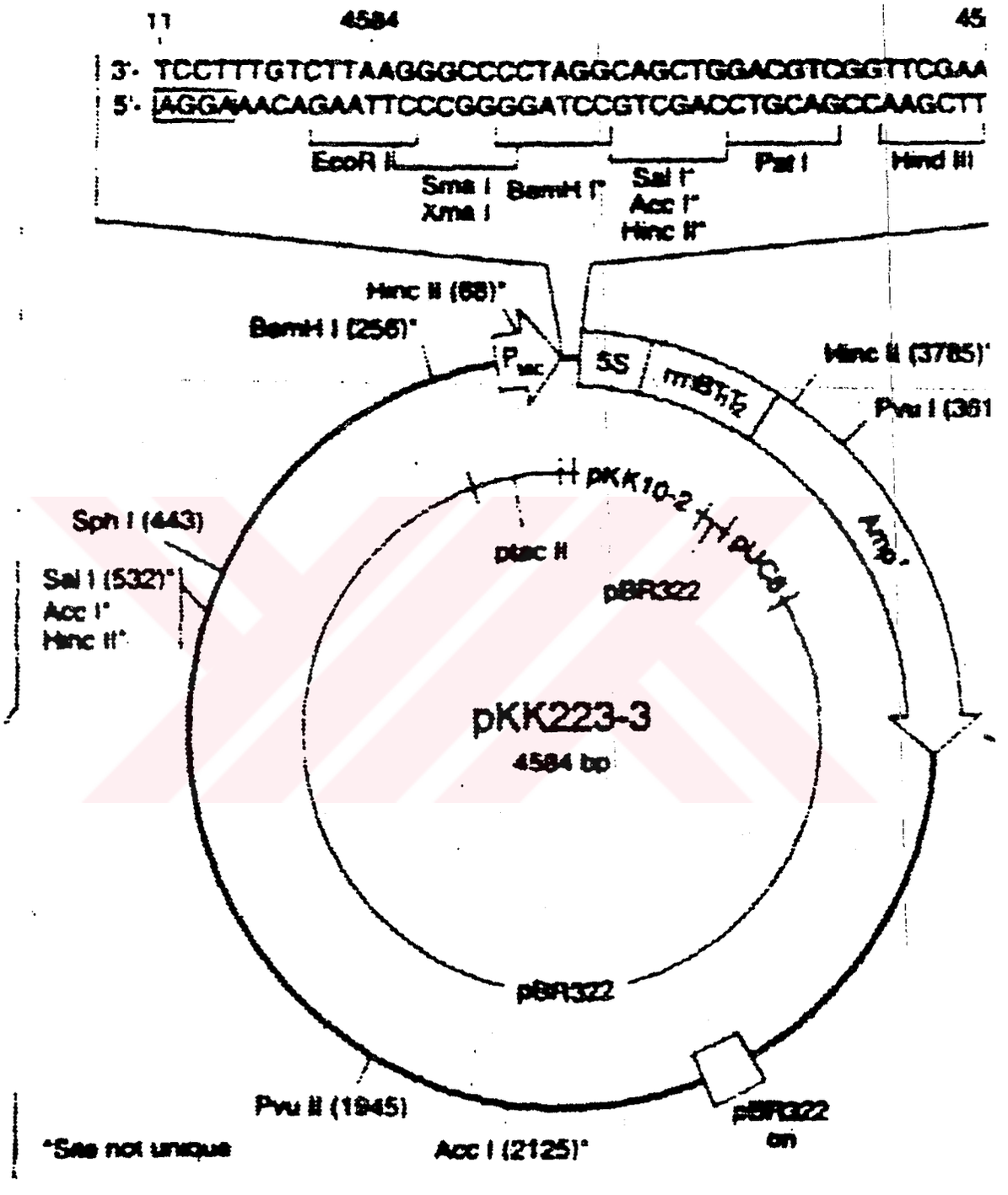
Sequence alignment sample 4 using *bs* C - terminus primer

'query' is wild type LDH gene

'sbjct' is sequence of sample 4

identities = 830 / 852 (97, 4%), Gaps = 3 / 852 (0,4%)

```
Query: 94   ggattgccgatgagatcgtgctcatcgatgcgaatgaaagcaaggccataggcgatgcga 153
            ||| |||||||||||||||||||||    ||| |  |||||||| ||||||||||||||
Sbjct: 172  ggantgccgatgagatcgtgctcatccgcgcgnaaaaaagcaag-ccataggcgatgcga 230
Query: 154  tggacttcaaccatgggaaagtatttgcgccgaagccggttgacatttggcacggcgatt 213
            | ||| |||||||| ||||||||||||||||||||||| |||||||||||||||||| |
Sbjct: 231  tngacntcaaccatnggaaagtatttgcgccgaagccggntgacatttggcacggcgant 290
Query: 214  acgatgattgccgcgatgccgatttggttgtcatttgcgccggcgccaaccaaaaaccgg 273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 291  acgatgattgccgcgatgccgatttggttgtcatttgcgccggcgccaaccaaaaaccgg 350
Query: 274  gcgagacgcggcttgatcttgtggacaaaaacattgccattttccgctcgatcgttgagt 333
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 351  gcgagacgcggcttgatcttgtggacaaaaacattgccattttccgctcgatcgttgagt 410
Query: 334  cggtcatggcatccggatttcaaggactgtttctcgtcgccaccaatccggtcgacattt 393
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 411  cggtcatggcatccggatttcaaggactgtttctcgtcgccaccaatccggtcgacattt 470
Query: 394  taacgtacgcgacgtggaaattcagcggcctgccgcatgagcgggtgatcggttcgggga 453
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 471  taacgtacgcgacgtggaaattcagcggcctgccgcatgagcgggtgatcggttcgggga 530
Query: 454  cgattttagatacggcgcggttccgcttttgttgggcgagtatttctctgtcgctccgc 513
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 531  cgattttagatacggcgcggttccgcttttgttgggcgagtatttctctgtcgctccgc 590
Query: 514  aaaatgttcatgcctatattattggggaacacggcgacactgaactcccggtctggagcc 573
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 591  aaaatgttcatgcctatattattggggaacacggcgacactgaactcccggtctggagcc 650
Query: 574  aggcttatatcggcgtcatgccgatccgcaagctggtcgagtccaaaggggaagaagcgc 633
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 651  aggcttatatcggcgtcatgccgatccgcaagctggtcgagtccaaaggggaagaagcgc 710
Query: 634  aaaaagatctcgagcgcatttttgtcaatgtgcgcgatgccgcctaccaaattattgaga 693
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 711  aaaaagatctcgagcgcatttttgtcaatgtgcgcgatgccgcctaccaaattattgaga 770
Query: 694  aaaaaggagcgacgtactacgggattgcgatggggcttgcccgcgtgacgcgcgccattt 753
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 771  aaaaaggagcgacgtactacgggattgcgatggggcttgcccgcgtgacgcgcgccattt 830
Query: 754  tgcataacgaaaacgctattttgaccgtatcagcctacctcgatggcctatatggggagc 813
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 831  tgcataacgaaaacgctattttgaccgtatcagcctacctcgatggcctatatggggagc 890
```



Map and Properties of pKK 223-3 Vector

Protein Molecular Weight Marker