Expression, Purification and Kinetic Characterization of *Lactate Dehydrogenase* of Lactic Acid Bacteria and ProB of *Bacillus subtilis*

Project submitted

For the award of

Master of Science

In

Life science (Biochemistry)

By

GUNJAN

Supervisor

Dr Malkhey Verma

Department of Biochemistry and Microbial Sciences

School of Basic and Applied Sciences

Central University of Punjab

Bathinda 151001

May, 2018

DECLARATION

I declare that the project report entitled **"Expression, Purification and Kinetic Characterization of *Lactate Dehydrogenase* of Lactic Acid Bacteria and ProB of *Bacillus subtilis* "** has been prepared by me under the guidance of Dr Malkhey Verma, Associate Professor, Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

GUNJAN

Regd. No: 16mslsbc06

Department of Biochemistry and Microbial Sciences,

School of Basic and Applied Sciences,

Central University of Punjab, Bathinda-151001

Date:

CERTIFICATE

I certify that Gunjan has prepared her project report entitled **"Expression, Purification and Kinetic Characterization of *Lactate Dehydrogenase* of Lactic Acid Bacteria and ProB of *Bacillus subtilis*"** for the award of MSc. degree from the Central University of Punjab, under my guidance. She has carried out this work at the Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

Dr MALKHEY VERMA

Associate Professor,

Department of Biochemistry and Microbial Sciences,

School of Basic and Applied Sciences,

Central University of Punjab, Bathinda-151001

Date:

ACKNOWLEDGEMENTS

Although only one name appeared on the cover of this M.Sc. Project but there are many hidden names that helped me to make contents in between the covers. I take this opportunity to express my thankfulness to all of them.

Although I did not find words that could express my thanks to a comparable extent for the help and support that he provided me but still trying to thank my guide Dr Malkhey Verma, who guided my M.Sc. and provided all facility to complete my Project. I have no words to express my gratitude for him. He has motivated me very much and this M.Sc. work would not have been completed without his help. I thank you, sir, for all the help and advice (both personal as well as professional) you have given me at right time and in the right direction.

It's my privilege to convey my heartfelt acknowledgement to Dr Ramakrishna Wusirika, HOD, Department of Biochemistry and Microbiology Sciences, School of Basic and Applied Sciences for his valuable guidance, Prof. (Dr.) R.K. Kohli present Vice Chancellor, Central University of Punjab, Bathinda for providing me research facilities to carry out my work.

I am extremely thankful to my seniors Priyanka Singh, my classmates Nitin Dogra, Debasis, Arti Negi and all my friends in CUP, Bathinda, for their cooperation, guidance, support and help during M.Sc. and I am also thankful to my juniors for their support and help during M.Sc. degree.

Lastly, I pay my deepest regards to my parents (Mr Ritesh Kumar and Mrs Anju Devi) for their endless support and encouragement.

Gunjan

Department of Biochemistry and Microbial Sciences

ABSTRACT

"Expression, Purification, Kinetic Characterization of *Lactate Dehydrogenase* of Lactic Acid Bacteria"

Name of student: Gunjan
Registration number: 16mslsbc06
Degree for which submitted: Master of Science
Name of supervisor: Dr Malkhey Verma
Name of Centre: Department of Biochemistry and Microbial Sciences
Name of school: School of Basic and Applied Sciences

Key Words: *L. lactis*, ProB*, B. subtilis,* Lactate Dehydrogenase, Lactic Acid Bacteria, Proline.

Lactate dehydrogenase is very well known for its role in glucose metabolism in Lactic Acid Bacteria (LAB). These bacteria are widely used in the industrial and research areas. LAB use NADH as a cofactor to produce lactate or lactic acid from glucose through fermentation, whereas ProB is an enzymatic protein which catalyzes the conversion of L-glutamate to ɣ-glutamyl phosphate at the expense of ATP in proline biosynthesis. Both the recombinant proteins are expressed in the *E. coli* BL 21 strain using pET plasmids. Proteins are purified using affinity chromatography and purity is established by a single band in SDS Page. Proteins were quantified by Bradford assay. In this study, the proteins selected are Lactate Dehydrogenase and ProB. In the absence of FBP, Pi is an activator of *L. lactis* LDH at pH 6. This effect can be interpreted by considering the computed binding affinities of Pi to the catalytic and allosteric binding sites of the enzymes modelled in protonation states corresponding to pH 6 and pH 7. In this study, we can find out the delicate interplay among the effects of Pi, FBP, and pH that results in different regulatory effects on the LDH of LAB and ProB of *B. subtilis.*

Gunjan **Dr Malkhey Verma**

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviations
1.	2-(N-Morpholino) Ethane Sulfonic Acid	MES
2.	Adenosine Diphosphate	ADP
3.	Adenosine Triphosphate	ATP
4.	Ammonium Persulphate	APS
5.	Carbon Dioxide	CO_2
6.	Dihydroxyacetone Phosphate	DHAP
7.	Embden-Meyerhof	EM
8.	Fructose 1,6-Bisphosphate	FBP
9.	Fructose1,6-Diphosphate	FDP
10.	ɣ -Glutamyl Phosphate Reductase	GPR
11.	ɣ-Glutamyl Kinase	GK
12.	Glyceraldehyde 3-Phosphate	GAP
13.	Hydrochloric Acid	HCL
14.	Isopropyl-1-Thiogalactopyranoside	IPTG
15.	Lactate Dehydrogenase	LDH
16.	Lactic Acid Bacteria	LAB
17.	Luria Bertani	LB
18.	Nicotinamide Adenine Dinucleotide	NAD
19.	Oxygen	O_2
20.	Phosphate Buffer Saline	PBS
21.	Phosphoenolpyruvate	PEP
22.	Phosphoketolase	PK
23.	Phosphotransferase System	PTS
24.	Polyacrylamide Gel Electrophoresis	PAGE
25.	Sodium Dodecyl Sulphate	SDS

LIST OF TABLES

LIST OF APPENDICES

Chapter 1

Introduction

1. Introduction

A typical lactic acid bacterium (LAB) is gram-positive, aerotolerant, acid tolerant, and fermentative rod or coccus in shape with a DNA base composition of not more than 53mol% G+C and producing lactic acid as a major product (Unden *et al.,* 2009). They require for complex growth factors such as vitamins and amino acids. Lactic acid bacteria are characterized by the manufacture of lactic acid as a main catabolic product from glucose. They ferment glucose mainly to lactic acid, CO_2, and ethanol. All LAB grows anaerobically, but distinct most anaerobes, they grow in the presence of O_2 as "aerotolerant anaerobes"(Liong.,2015).

Although many genera of bacteria produce lactic acid as a major or minor end-product of fermentation, the term **Lactic Acid Bacteria** is typically reserved for genera in the order *Lactobacillales*, which include *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Streptococcus,* in addition to *Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus,* and *Weisella* (Kenneth., 2011).

Due to the fact they gain energy most effective from the metabolism of sugars, lactic acid bacteria are limited to environments in which sugars are present. They have restrained their biosynthetic capabilities, as they are evolved in environments that are rich in amino acids, nutrients, purines and pyrimidines, so that they ought to be cultivated in complex media that fulfil all their nutritional requirements. The majority are free-living or live in favourable or harmless relations with animals, while some are opportunistic pathogens. They're found in milk and milk products and in decomposing plant resources. They're normal parts of human beings inside the oral cavity, the intestinal tract and the vagina, where they play an important function (Kenneth ., 2011).

Lactic acid bacteria are some of the most important of microorganisms utilized in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage microorganism by using producing growth-inhibiting substances and large quantities of lactic acid. Fermentation LAB is concerned in making yoghurt, cheese, cultured butter, bitter cream, sausage,

cucumber pickles, olives and sauerkraut, but some species may also destroy beer, wine, and processed meats (Buckenhüskes *et al.,*1993).

1.2 Brief knowledge about the targeted lactic acid bacteria (LAB) in the study

Lactobacillus Plantarum, Streptococcus pyogenes, Enterococcus faecalis, and Lactococcus lactis are all Gram-positive microorganisms which belong to the phylogenetic order Lactobacillales. These bacteria are mainly found in a specific environment (*L. lactis*, milk; *L. Plantarum*, plants; *E. faecalis*, faeces; *S. pyogenes*, skin and mucosal membranes) and interact with humans also. *L. lactis* and *L. plantarum* are bacteria of important significance to be used in the food industry (Siezen *et al.,* 2011) *E. faecalis* is a vital commensal of the human intestine, a food contaminant, and a facultative pathogen. *S. pyogenes* is a completely human pathogen inflicting illnesses like tonsillitis, pharyngitis, scarlet fever, and necrotizing fasciitis. Though they differ in the form of their living, all four species predominantly advantage energy through homolactic acid fermentation ((Fisher, and Phillips, 2009).The free energy generated for the duration of homolactic acid fermentation is 2 mol of ATP/1 mol of glucose. The vital enzyme in this pathway is LDH, which is responsible for catalyzing the reversible reduction of pyruvate to lactate. This reaction serves solely to balance the redox potential by oxidation of $NADH + H^+$ to NAD^+ (Feldman-Salit *et al.,* 2013).

The LAB genomes vary in the quantity and kind of LDH enzymes that they encode. The *L. lactis* strain MG1363 genome possesses three L-LDH genes (llmg_1120, L-LDH1; llmg_0392, L-LDH2; and llmg_1429, L-LDH). The Biological assembly of bacterial LDH is a homotetramer. Each monomer has one active site, and the tetramer has two allosteric sites, each situated at the interface between two monomers (Iwata *et al.,* 1994). Fructose1, 6bisphosphate (FBP) has been shown to allosterically regulate some LDHs, including *L. lactis* LDHs, and to bind at these allosteric sites. The mechanism of LDH regulation by FBP has been defined to be allosteric because of a sequential intersubunit rearrangement of the LDH tetramer accompanied by local intra-subunit conformational change (Cameron *et al.,* 1994).

Lactococcus lactis is a Gram-positive bacterium and spherical in shape. It is widely used for commercial production of fermented dairy products such as milk, cheese, and yoghurt. They are non-motile and do not form spores. *Lactococci* are found associated with plant fabric, especially grasses, from which they are easily inoculated into milk. Hence, they are found usually in milk and may be a natural cause of souring. *Lactococcus lactis* has two subspecies, *lactis* and *cremoris*, both of which are important in the manufacture of many forms of cheese and different varieties of fermented milk products. FBP dependent LDH (a key enzyme in homolactic fermentation) is responsible for the

reduction of lactate by oxidation of NADH. Sugar is broken down as phosphate and disaccharides in mono by hydrolytic reduction and mono by fermentative process glycolysis (Kenneth *et al.,* 2011).

1.3 About Proline

The structure of Proline is unique because of its side chain which connects the molecule to the protein backbone at two ends, which results in the formation of a ring and this ring is not a part of the structure of other amino acids. With this, we can say that proline is a secondary amino acid, also known as an imino acid. Since it is the only proteinogenic imino acid of its type because of which people often refer to it as an amino acid.

L-proline comes under the category of non-essential amino acids and is synthesized mainly in the liver by ornithine, glutamine, and glutamate. Proline is among one of the principal amino acids which our body uses to build collagen (Cox *et al.,* 2008).

1.4 Benefits of proline

- ✓ **Healthy skin**

Skin is the first line of defence of our body protecting it from infection. The body uses hydroxyproline to make collagen protein which aid in skin elasticity and thickness. In old age, the skin loses its thickness and became thinner and less fibrous due to less production of collagen synthesis. Because of UV exposure and free radical damage, the skin loses its smoothness and gets wrinkled. It has been found that Proline increases collagen synthesis in the fibroblast cells from its precursors, glutamate and pyrroline-5-carboxylate. Thus, we can say that proline can slow down ageing and enhance the health of skin (Murakami *et al.,* 2012).

- ✓ **Tissue repair**

In case of injury whenever there is soft tissue damage, the body increases the production of proline so that the wound can be healed fast. It has been seen in animal studies that current administration of l-proline at the wounded site can accelerate the process of healing much faster than the oral administration. This amino acid is also present in the medical dressings that use collagen fragments to initiate wound healing (Ponrasu *et al.,* 2013).

- ✓ **Cardiovascular health**

In Arteriosclerosis the arteries become thick and hard which is the leading cause of heart disease. This condition arises as the arteries become stiff due to fat accumulating on the walls of the arteries. The accumulated fat results in the restriction of the transport of nutrients and oxygen throughout the body. Which reduces blood flow and build up the pressure increasing the risks of heart attacks (Ivanov *et al.,* 2007).

1.5 Biosynthesis of Proline in *Bacillus subtlis* and action in osmoregulation.

Proline biosynthesis in *Bacillus subtlis* occurs through a series of three enzymatic reactions catalyzed by the gene products of the *proB, proA, and proC* loci through feedback inhibition of -y-glutamyl kinase by proline (Baich *et al.,*1969).

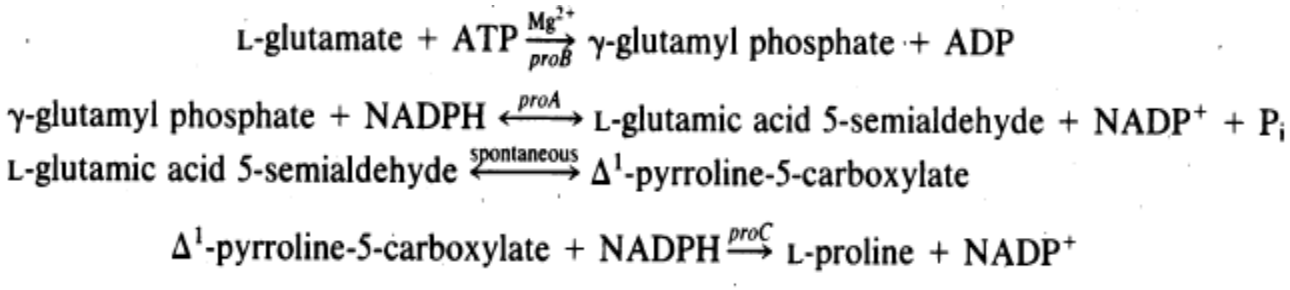

Figure 1.5 Reaction Involved in Proline Biosynthesis (Jeffrey Smith *et al.,* 1983)

Proline is a highly water-soluble imino acid and its accumulation not only serves as a countermeasure for water exit from osmotically stressed cells but its function-preserving and protein anti-aggregating physio-chemical properties also helps to optimize the composition of the cytoplasm for biochemical activities. Both prokaryotic and eukaryotic cells to fend off the detrimental effects of high osmolarity on cell physiology. It also exhibits protein-stabilizing properties and promotes protein folding under unfavourable conditions (Brill *et al.,* 2011)

1.6 Knowledge Gap

The information regarding the allosteric regulation of the isoenzyme of LDH isolated from *L. lactis* is not entirely known till now. The procedure of LDH regulation by FBP has been defined as allosteric because of subsequent intersubunit rearrangement of the LDH tetramer accompanied by local intra-subunit conformational changes. Kinetic characterization of proB has not done before and is

important to understand the growth under osmotic conditions. Further, this study could be extended to understand plants growth under salt stress.

1.7 Hypothesis

Kinetic characterization of glycolytic enzymes of the LAB will help to understand how differently glycolysis is regulated in the pathogenic and non-pathogenic LAB. Further, this information can be used for metabolic engineering of industrial strains of the LAB and for the industrial production of lactic acid and also drug development for the pathogenic LAB. Characterization of proB of B. subtilis will help to understand how proline biosynthesis is regulated under osmotic conditions.

1.8 Objectives

Protein expression and purification of LDH and proB using affinity chromatography

Kinetic characterization of LDH of *L. lactis* and proB of *Bacillus subtilis*

Chapter-2

Review of literature

2.1 Metabolism of lactic acid bacteria

According to sugar fermentation patterns, lactic acid bacteria are classified into two broad metabolic categories: homofermentative and heterofermentative. The primary class, homofermentative LAB, includes some lactobacilli and most species of *enterococci, lactococci, pediococci, streptococci, tetragenococci,* and *vagococci,* that ferment hexoses through the Embden-Meyerhof (EM) pathway. Heterofermentative the second category, LAB, includes some of the *lactobacilli, leuconostocs, oenococci,* and *weissella* species. These two categories are differentiated based on apparent distinct enzyme level and the presence or absence of the necessary cleavage enzymes of the EM pathway (fructose 1,6-diphosphate) and the PK pathway (phosphoketolase) (Plumed-Ferrer *et al.,* 2007).

2.2 Homolactic Fermentation

Under conditions of limited oxygen and excess glucose, homolactic LAB catabolizes one mole of glucose to yield two moles of pyruvate in the Embden-Meyerhof pathway. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. The homolactic LAB genera hold *Lactococcus, Lactobacillus, Streptococcus, Enterococcus,* and *Pediococcus* species. The transport and phosphorylation of sugars occur by transport of free glucose and phosphorylation by an ATP-dependent hexose kinase (while the other sugars namely, mannose and fructose, enter into the major pathways at the level of glucose-6-phosphate or they can also enter at the level of fructose-6-phosphate after isomerization or phosphorylation or both); or the phosphoenolpyruvate (PEP) sugar phosphotransferase system (PTS), in which PEP is the phosphoryl donor for the uptake of sugar. Some species of LAB use the PTS for transport of galactose only; others utilize the PTS for all sugars (Goffin *et al.,* 2004).

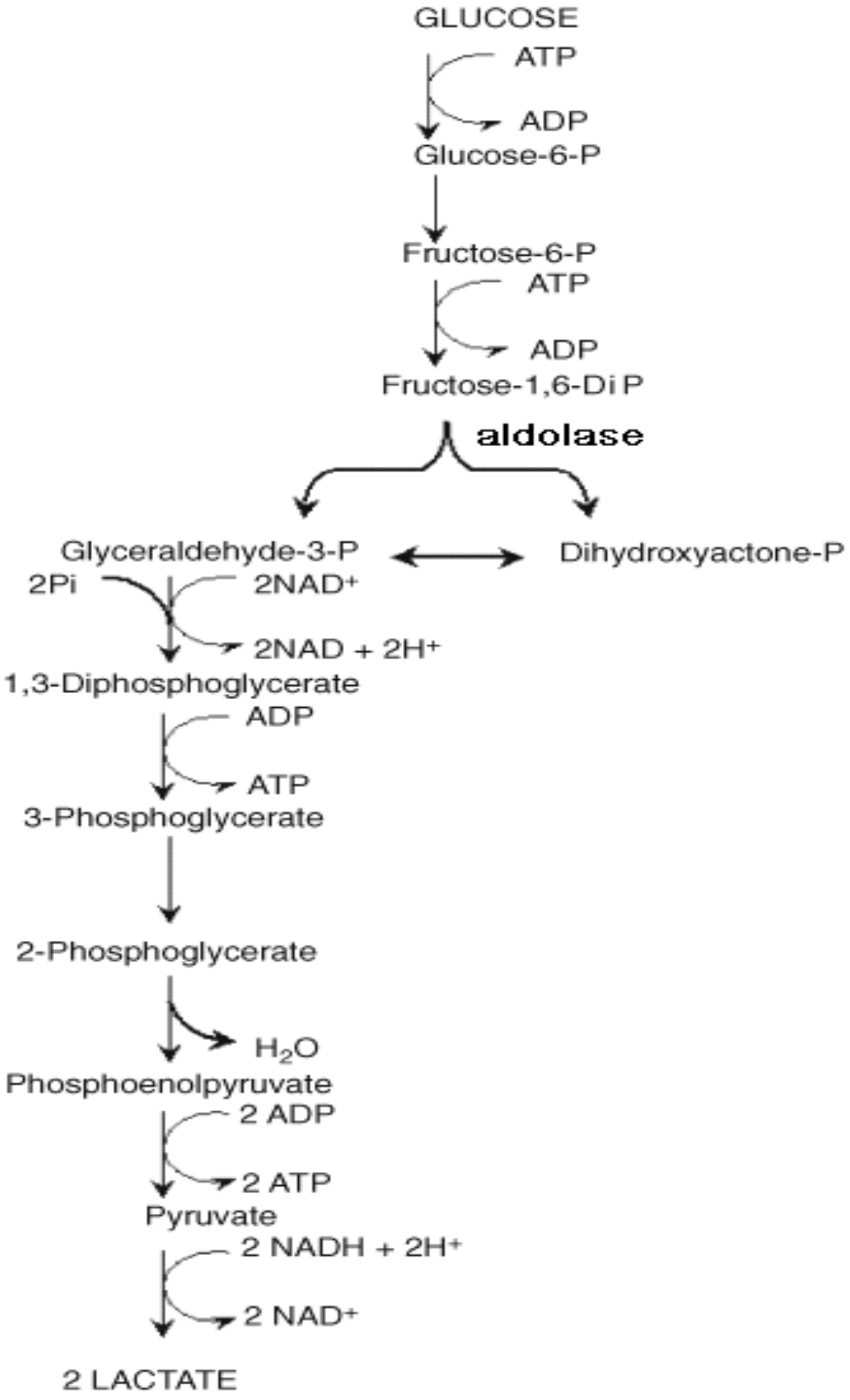

Figure 2.2 The pathway of homolactic acid fermentation in Lactic Acid Bacteria (Axelsson *et al.,* 2004)

2.3 Heterolactic Fermentation

Phosphoketolase pathway (pentose phosphate pathway) is employed by the heterofermentative LAB to dissimilate sugars. One mole of glucose-6-phosphate is primarily dehydrogenated to 6-phosphogluconate and then decarboxylated to yield one mole of CO_2 and pentose-5-phosphate obtained is cleaved into one mole of glyceraldehyde phosphate (GAP) and one mole of acetyl phosphate. Just like homofermentation GAP is further metabolized to lactate, with the acetyl phosphate reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. The end- products (CO_2, lactate and ethanol) are formed in equimolar quantities from the catabolism of one mole of glucose. *Leuconostoc, Oenococcus, Weissella*, and certain lactobacilli are all obligate heterofermentative LAB (Ganzle *et al.,* 2015).

Lactic acid bacteria have a very limited capacity to synthesize amino acids using inorganic nitrogen sources. Which means they depend on preformed amino acids being present in the growth medium as a source of nitrogen. The requirement for amino acids is different for different species and strains within the species. Some strains are prototrophic for most amino acids, whereas others may require 13–15 amino acids. There is a requirement of a proteolytic system for hydrolyzing the peptides and proteins to obtain essential amino acids because the quantities of free amino acids present in their environment are not sufficient to result into high cell density growth of bacteria. Proteolytic activity is found in all dairy lactococci used for acidification of milk. In the lactococcal proteolytic system, the enzymes are found outside the cytoplasmic membrane, transport systems, and intracellular peptidases. The proteolytic activity of LAB adds the flavour, aroma and texture of the fermented products. Many known varieties of cheeses, such as Swiss and Cheddar, proteolysis adds the required flavour tones to them (Goffin *et al.,* 2004).

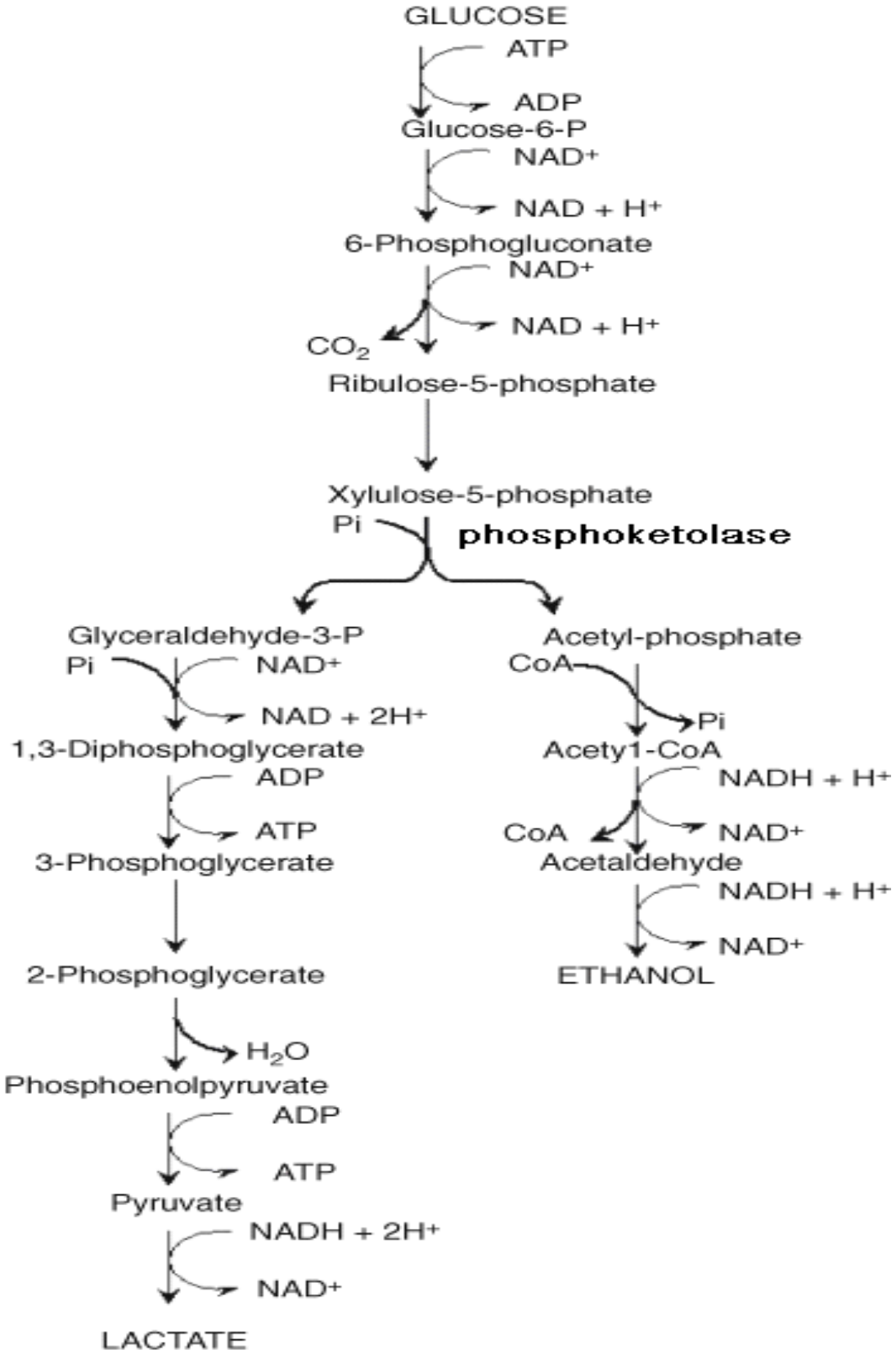

Figure 2.3 The pathway of heterolactic acid fermentation in Lactic Acid Bacteria (Axelsson *et al.,* 2004).

2.4 Glycolysis (Embden-Meyerhof pathway)

In glycolysis (Embden-Meyerhof pathway), under typical conditions where sugars are not limiting, and oxygen is restricted, utilization of one glucose molecule is theoretically fermented to two lactic acid molecules resulting in a net gain of two molecules of ATP. The primary steps of glycolysis are the phosphorylation of glucose to fructose1,6-bisphosphate (FBP) and its breaking into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), (DHAP formed is also converted to GAP). GAP is then converted to pyruvate through a route that involves two substrate-level phosphorylation steps. Finally, pyruvate is reduced to lactic acid by lactate dehydrogenase (LDH) using NADH as the cofactor. In glycolysis, the reduced cofactors NADH are reoxidized to NAD+ and thus a redox balance is obtained (Kandler *et al.,* 1983).

2.5 Fermentation of Hexoses

There are three main routes of hexose fermentation, hexose is most frequently substrate for lactic acid bacteria. The end-product of fermentation of glucose under typical conditions (excess sugar and limited access to oxygen) is lactic acid. This means to facilitate homofermentative lactic acid bacteria theoretically produce two molecules of lactic acid from consumption of one molecule of glucose. Hexoses other than glucose, such as mannose, galactose, and fructose are also fermented by lots of LAB. These sugars enter the major pathways of glycolysis after isomerization and phosphorylation. In further steps of the fermentation reaction, the phosphorylated hexose is broken into triose phosphates and then through oxidation and dephosphorylation to pyruvic acid. The final process of the fermentation process is the reduction of pyruvic acid to lactic acid. In the heterofermentative LAB, another fermentation pathway is characteristic resulting in two main end-products—lactic acid and ethanol, and carbon dioxide (Axelsson *et al.,* 2004).

2.6 Importance of lactic acid bacteria

The formation of acetoin in lactic acid bacteria is helpful in providing flavours and quality to the foods and help them from spoiling.

The process malolactic fermentation coverts malic acid to much milder lactic acid. It is useful in reducing the acid level and enhance the taste of wine especially white wine and changes the flavour concentration through carbohydrate and citrate metabolism.

Fermented milk and cheese are dairy products preserved partly by acid produced by bacterial activity (Ganzle *et al.,* 2015).

2.7 Proline

Proline is a non-essential, cyclic amino acid but mainly itis an imino acid in humans which is synthesized from glutamic acid and different amino acids, proline is a constituent of many proteins. It is found in high concentrations in collagen, proline constitutes nearly a third of the residues. Collagen is a structural protein which forms the elastic fibers in the skin, bones, ligaments and tendons. Together with lysine and vitamin C, proline is converted into hydroxy lysine and hydroxyproline to help form collagen. D-proline is an isomer of the naturally occurring amino acid, L-proline. D-amino acids have been found in moderately high abundance in human plasma and saliva. These amino acids can be of bacterial origin, but there is also proof that they are endogenously produced through amino acid racemase activity (Cox *et al.,* 2008).

2.8 Biosynthesis of Proline

A Schiff base is formed with the main amine group on the α carbon of glutamate semialdehyde and the aldehyde which is then reduced resulting in the production of proline. When proline is in a peptide bond, it does not have a hydrogen at the α amino group position, so it cannot donate a hydrogen bond to stabilize an α helix or a β sheet. Proline cannot have found in an α helix but when proline is found in an α helix, the helix will have a slight bend because of the need of the hydrogen bond.

Proline is frequently found at the ending of α helix or in turns or loops. Proline can exist in the *cis*-configuration in peptides unlike other amino acids which only exist entirely in the *trans*- form in polypeptides. The *cis* and *trans* forms are almost isoenergetic. The *cis/trans* isomerization can play an essential role in the folding of proteins.

Glutamic acid can be converted into proline. Firstly, the γ carboxyl group is reduced to the aldehyde, resulting in the production of glutamate semialdehyde. Then this aldehyde formed reacts with the α-amino group and eliminate water as it forms the Schiff base. The second step is the reduction step, in which the Schiff base is reduced, and formation of proline takes place (Smith *et al.,* 1984).

2.9 *Bacillus subtilis*

Bacillus subtilis are rod-shaped and gram-positive bacteria that are naturally found in the soil. As such it is appreciably more like Archaea than is *E. coli*. The common environment of *B.subtilis* is stress and starvation, therefore, *Bacillus subtilis* has evolved a place of strategies that allow survival under these harsh conditions. One approach, for example, the formation of stress-resistant endospores. *Bacillus subtilis* can also gain extra protection more quickly against many stress conditions which is comprises of acidic, alkaline, osmotic, or oxidative conditions, and heat or ethanol (Balitsky *et al.,* 2001).

2.10 Pathway of Proline Biosynthesis in *Bacillus subtilis*

In plants and in animal's proline can be synthesized from ornithine, the primary precursor for proline biosynthesis in bacteria and in osmotically stressed plant cells is glutamate the pathway of proline synthesis from glutamate, comprises three enzymatic steps. Bacterial proline synthesis from glutamate occurs through three enzymatic reactions, catalyzed by γ-glutamyl kinase (GK) (*proB* product, EC 2.7.2.11), γ-glutamyl phosphate reductase (GPR) (*proA* product, EC 1.2.1.41), and Δ^1-pyrroline-5-carboxylate reductase (P5C) (*proC* product, EC 1.5.1.2). For many bacteria, the *proB* and *proA* genes compose an operon, which is distant from *proC* on the chromosome. The first enzyme of the proline biosynthesis pathway encoded by the *proB* gene. Although *B. subtilis* has an only *proA* like gene, a second *proB*-like gene, *proJ* of the *proHJ* locus has been found. In a way exceptional to this bacterium, either *proB*-like enzyme can provide enough g-glutamyl kinase activity to support growth in the absence of exogenous proline. The first enzyme of the proline biosynthesis pathway encoded by the proB gene. So according to the first reaction of proline biosynthesis, the enzymatic reaction is dependent on proB gene (Brill *et al.,* 2011).

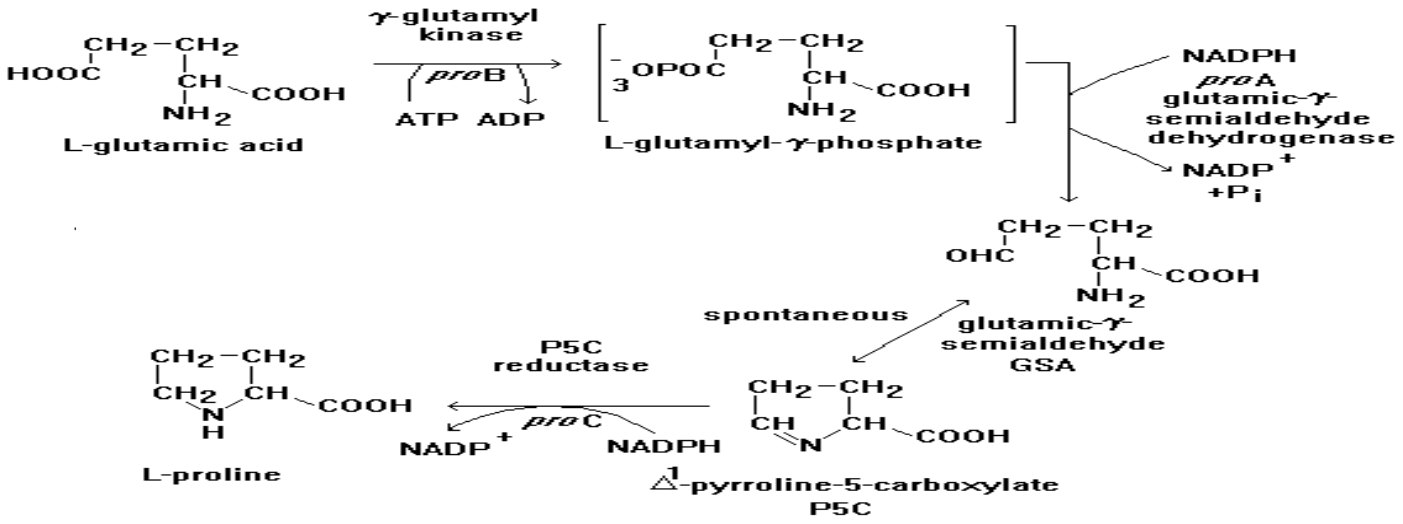

Figure 2.10 Pathway of Proline Biosynthesis in Bacteria (Krishna *et al.,* 1979)

2.11 Proline Acts as an Osmoprotectactant

One of the well-suited solutes most extensively employed by members of the *Bacteria* is the amino acid L-proline. In addition to its characteristic as a water-attracting osmolyte, proline additionally serves as a “chemical chaperone” by aiding the folding of proteins and by way of stopping their aggregation in osmotically challenged cells. The soil-dwelling bacterium *Bacillus subtilis* makes use of proline as an osmoprotectant via both *de novo* synthesis and uptake. It lives in a taxing habitat where desiccation strategies create microniches with low water availability and high salinity, thereby necessitating suitable osmostress- relieving cellular responses. To fend off the detrimental effects *B. subtilis* uses many preformed compatible solutes like glycine betaine of high osmolarity on cellular physiology and for the growth with the aid of importing them through several osmotically controlled uptake systems.

In the absence of an exogenous supply of osmoprotectants, *B. subtilis* must rely on its own devices to deal with sustained high osmolarity environment. It does so by primarily importing significant amounts of potassium ions and by the subsequent synthesis of very large quantities of L-proline. For osmoadaptive proline synthesis, *B. subtilis* depends on the concerted actions of the *proJ-proA-proH* enzymes, whereas in case of anabolic proline manufacturing, enzymes *proB-proA-proI* are involved. Predictably, the genetic disruption of the osmostress-responsive *proJ-proA-proH* proline biosynthetic pathway causes osmotic sensitivity. In addition, proline also serves as a nutrient for *B. subtilis*. Glutamate is an essential metabolite positioned at the intersection of carbon and nitrogen metabolism and serves as the precursor for proline biosynthesis in *B. subtilis* (Zaprasis *et al.,* 2013).

Chapter-3
Material and Methods

3.1 Material Required

Luria bertani agar, isopropyl-1-thiogalactopyranoside(IPTG), Petri plates, Antibiotics (Ampicillin and kanamycin), Conical flask, Distilled water, 70% ethanol, Tris-HCL (pH7), β—Mercaptoethanol (1mM), NaCl(100mM), Falcon tubes (15, 50 ml), Pipette and tips (1000µl, 100µl, 20 µl), Shaker incubator, Autoclave, Weighing balance, pH meter, refrigerator and deep freezers (-80ºC, -20ºC), Laminar air flow, Centrifuge machine, Column tubes, Column Stand, Eppendrof tubes (2ml, 1.5ml), Beaker, Reagent bottle, Metal ion resins, Tissue paper, Parafilm, Ice bucket, ice flakes.

3.2 Procedure

3.2.1 Preparation of LB agar plates:

Two sets of 50mL of LB broth (20g broth powder per 1L distilled water) in 250mL conical flasks were prepared, 2 grams of agar was added to these conical flasks. Swirled to mix. Added cotton plugs on the top of the flasks and, a fresh piece of aluminium foil to cover the top. After that, these flasks were autoclaved (at 121 ºC for 15 minutes) and cooled up to ~40 °C. Then antibiotics were added (ampicillin and kanamycin) 50μL into the flasks containing media one in each, in the laminar air flow cabinet and swirled vigorously for several seconds to mix. The plates in the laminar air flow were set and labelled at the bottom with the appropriate antibiotic: A=ampicillin, K=kanamycin, initial of the name and the date.

Media was poured into the petri plates as follows:

Removed the lid of the petri plates and removed the foil cover to the flask. Around 25mL LB agar was poured into each petri plates to completely cover the bottom of the dish and then keep the lid back. When plates got solidified, then streaking was done with recombinant *E. coli* strains carrying pET plasmid containing LDH of *L. lactis* and proB of *B. subtilis* on the petri plates and kept for incubation for 24 hours at 37ºC.

NOTES-

You should finish pouring dishes within 5 minutes of adding the antibiotic. Always stored the petri plates upside down in the refrigerator.

Work quickly because the agar will harden soon once it is cool enough it does not pour properly in the plates and formation of solidifying lumps takes place.

3.2.2 Seed Culture Preparation

E. coli growth was seen on petri plates containing antibiotic. With the help of inoculating loop, a single isolated colony from the petri plate was taken and inoculated in the flask. This flask was later kept in the incubator shaker for 24 hours at 37℃ and 180 rpm.

3.2.3 Cells Cultivation for protein expression

When the media in the flask became turbid. Four sets of 100mL of LB media in the 500mL conical flask were prepared autoclaved and kept inside the LAF. Then 100 μL of antibiotic (in which bacterial growth was observed) was added to each flask after that 10mL of seed was added into each flask if the seed would have been less dense then 15mL of the seed was added per 100 ml of cultivation media. These four flasks were later placed in the incubator shaker for 24 hours at 37℃ and 180 rpm.

The recombinant *E. coli* cell was taken after one hour at 600 nm when O.D. was reached in the range of 0.5- 0.6, 100μL of IPTG was added in each of the four flasks to induce the recombinant protein expression and later cells were grown for 3-5 hrs.

3.2.4 Cell Harvesting

The cultivated broth was centrifuged in 50mL falcon tube at 6000rpm for 10 mins at 4℃, till all the broth was used, using the same falcon tube. The pellet was collected, and the supernatant was discarded after autoclaving it. Then cells were washed by vortexing in washing buffer. After the pellet was suspended in the buffer it was centrifuged at 6000rpm for 5 min at 4℃. The above step was repeated twice after that pellet was suspended in the suspension buffer by vortexing and stored at -80^{o}C for further use.

3.2.5 Protein Purification by Affinity Chromatography

The pellet was sonicated at one-minute interval for 15 times at a temperature of 10ºC. Cell debris was removed by centrifugation at 7500rpm for 30min at 4℃ and supernatant was collected. The metal ion resins (Talon resins) was washed with suspension buffer for 3 to 4 times. After this, the supernatant collected was mixed with the washed metal ion resins. For optimum binding of the protein with the resins, it was placed on rocker shaker for 1 hour. Later resins mixed with the supernatant was transferred into the column. The supernatant was collected in another falcon tube and it was stored at 4℃ with proper labelling. Resins were washed with the suspension buffer (3-4mL) for 3- 4 times. The final wash was given by 20mM imidazole buffer and recombinant protein was eluted with 150mM imidazole buffer and collected in three fractions in the labelled Eppendorf tube. Used resins were recharged back by using 10mL of 20mM MES buffer. The resins were washed with 20mL of distilled water after which they were kept in the refrigerator for further use.

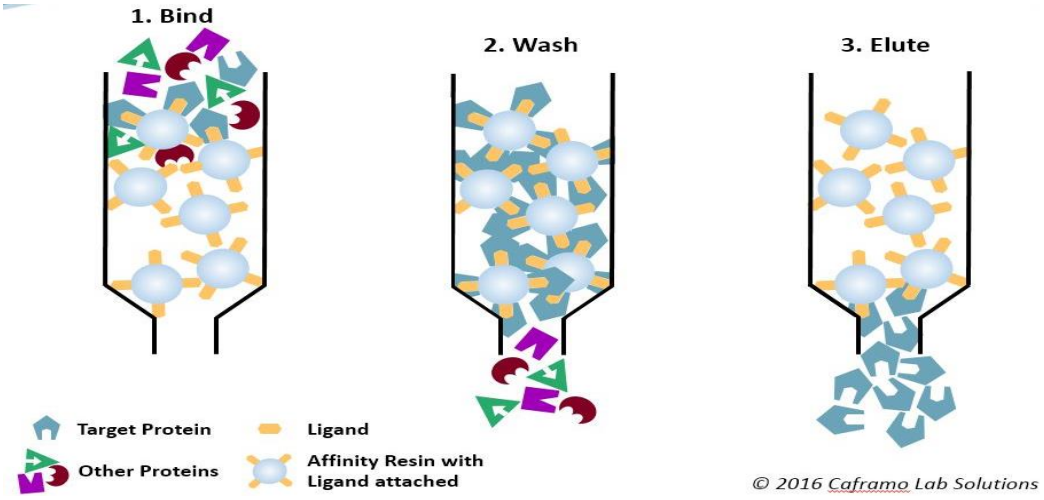

Figure 3.2.5 Protein Purification by Affinity Chromatography

NOTE-

Never let metal ion resins dry. Always keep it in the buffer.

3.2.6 Bradford Assay

Bradford assay is used for protein quantification. The Bradford assay involves the binding of proteins to Coomassie brilliant blue, to form a protein-dye complex that shifts the absorption maximum of the dye from 465 to 595 nm, turning the colour of the solution from red-brown to blue. This technique is simpler, faster, and more sensitive than the Lowry method. The Bradford and Lowry assays have various strengths and weakness in terms of substance interference and protein size.

Total protein concentration was quantified by Bradford method, using bovine serum albumin(BSA) (0.1μg/μl) as standard. Bradford assay is preferable than Lowry assay due to its rapidity and suitability in assessing protein concentration for gel electrophoresis. The protocol for preparing the Bradford reagent: “100mg Coomassie brilliant blue G-250 was dissolved in 50mL 95% ethanol. To this solution, 100mL 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre and filtered through Whatman filter paper”. Final concentration in the Bradford reagent was 0.01% (w/v) Coomassie brilliant blue G-250, 4.7%(w/v) phosphoric acid.

The standard curve was prepared using serial dilution of BSA. The protein sample was added to 1X PBS to make final volume 80 µl. To this solution, 20 µl of Bradford reagent was added so that the final volume reaches to 100 µl. An incubation of 10-15 min was given at 37^{o}C, to obtain a blue colour solution which was measured at 595 nm wavelength with a microplate reader (Bradford *et al.,* 1976).

NOTE-

You Bradford reagent is light sensitive, perform the experiment in dark.

Keep the 96 well plate wrapped in Aluminium foil for incubation.

3.2.7 SDS- PAGE Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) is used to separate biological macromolecules, which include proteins and nucleic acids based on their molecular weight, shape and size of the molecule and charge. A series of proteins of known molecular mass known as molecular weight markers are run on a gel in a fixed separated track adjacent to the protein of unknown molecular mass under the effect of electric field. In case of proteins, sodium dodecyl sulfate, act as an anionic detergent which linearizes and imparts a negative charge on the protein. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass which makes the separation much easier.

Preparation of gel:

The glass plates were prepared for the experiment by first washing in warm detergent solution, rinsing subsequently in tap water, deionized water and ethanol and drying them. Spacer strips were arranged at the sides and bottom of the plates. The inner plates were laid in position, resting on the spacer strips and the complete arrangement was mounted vertically. After mixing the solution it was poured into the space between the two plates leaving the upper space unfilled. Isopropanol was carefully laid over the surface of the poured gel mixture to avoid air contact, which reduces the polymerization reaction. The gel mixture could polymerize if left undisturbed for 30 minutes. In the meantime, gel mixture for stacking gel was prepared. After the separating gel was polymerized the over laid water was removed carefully with filter paper. Stacking gel was added over the separating gel and an appropriate comb was inserted between the plates

Reagents	**Volume**
30% acrylamide (bisacrylamide and acrylamide)	3.34 ml
1.5 M Tris-Cl (pH 8.8)	2.25 ml
10% APS	100 µl
10% SDS	100 µl
TEMED	10 µl
Distilled Water	4 ml

Table 3.2.7a. List of reagents used in 10% mini SDS preparation

Reagents	**Volume**
30% acrylamide (bisacrylamide and acrylamide)	850 µl
0.5 M Tris-Cl (pH 8.8)	625 µl
10% APS	50 µl
10% SDS	50 µl
TEMED	5 µl
Distilled Water	3.4 ml

Table 3.2.7b List of chemicals used for 4% stacking gel of 5ml.

Preparation of protein samples:

The eluted protein samples were added and mixed with the loading dye. The samples were incubated or 2min in a boiling water bath prior to loading. When the polymerization was completed the comb was removed and the lower spacer strip was carefully removed. The gel was attached to the electrophoresis unit using appropriate clips/clamps and unit was placed in the tank. The lower reservoir was filled with 1x running buffer. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with 1x running buffer. The upper reservoir was also carefully filled with 1x running buffer. The electrodes were connected to a power pack providing a constant voltage of 70 V. The gel was run at a constant voltage for 4-6 hrs. at room temperature. Electrophoretic mobility of the samples was determined by bromophenol blue front. At the end of the run, the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted and the gel was carefully removed.

Staining of the gel:

The gel was fixed with 20% trichloroacetic acid for 5minutes in the oven then washed with water and stained with staining dye. The gel was stained overnight.

Reagents	**Quantity for 100ml Staining dye**
Acetic acid	10 ml
Methanol	20 ml
Distilled water	70 ml
Coomassie brilliant blue G-250	0.025g

Table 3.2.7c List of chemicals used to make staining dye.

Destaining of the gel:

The destaining of the stained gel was done by the destaining dye.

Reagents	**Quantity for 100ml Destaining dye**
Acetic acid	10 ml
Methanol	20 ml
Distilled water	70 ml

Table 3.2.7d List of chemicals used to make Destaining dye.

NOTE-

Never touch any of the reagents with bare hands.

Always add APS and TEMED just prior to the pouring of gel.

Chapter 4

Result

Recombinant *E. coli* strains' (carrying LDH gene of *L. lactis* and proB gene of *B. subtilis*) growth were observed on LB agar plate having antibiotic ampicillin and kanamycin which shows that these bacterial strains were resistant to these antibiotics shown in figure 4.1.

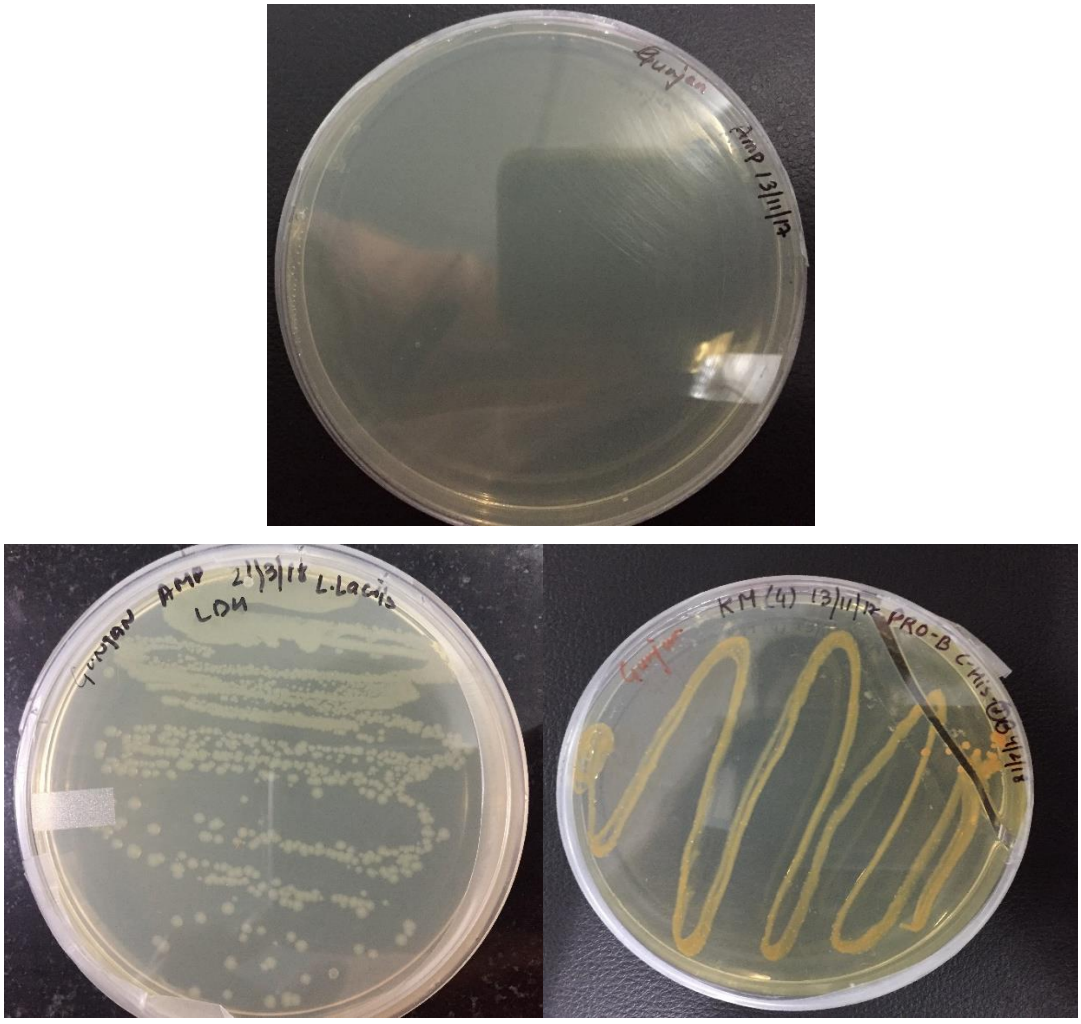

Figure 4.1 Recombinant *E. coli* growth for LDH and proB.

The flasks containing LB media with antibiotic ampicillin and kanamycin for LDH and ProB respectively after incubation showed a dense growth (as shown in Figure 4.2).

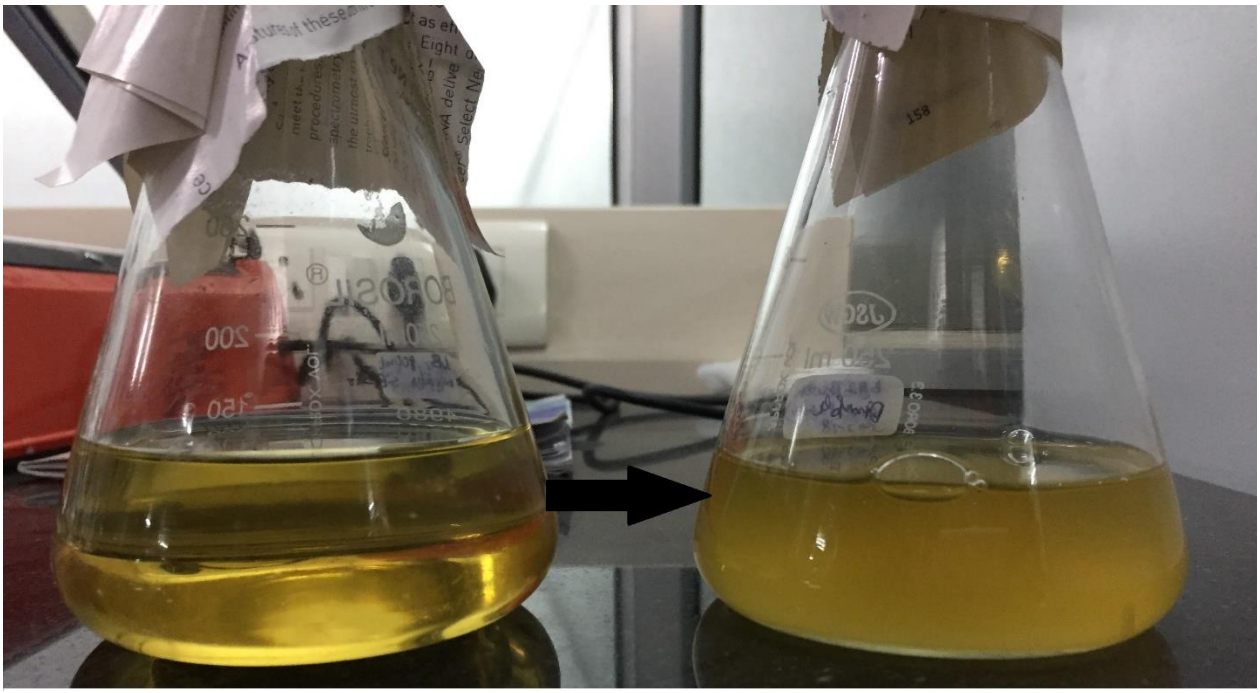

Figure 4.2 Turbidity shows the dense growth of both the bacterial strains after keeping the flask in a shaker for 24 hours

The protein LDH and proB were extracted from the recombinant *E. coli* strains by employing affinity chromatography. For determining the quality as well as the quantity of the extracted proteins further assays were performed. Results are shown in figure 4.3.

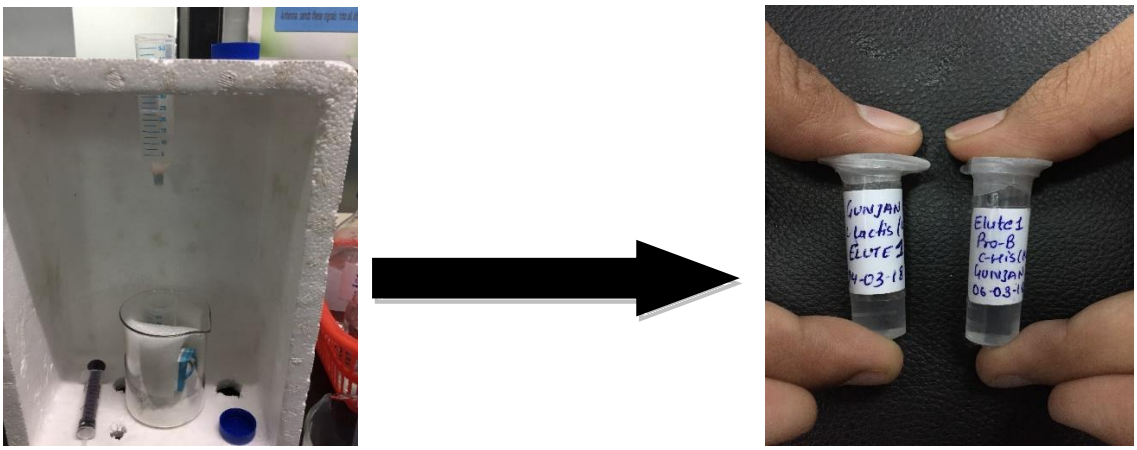

Figure 4.3 Fractions of protein collected from affinity chromatography.

4.4 Bradford Assay

A standard curve of Bovine Serum Albumin (BSA) (0.1 µg/µl), estimated by Bradford assay.

S. No.	Concentration of BSA (µg/µl)	O.D. at 595nm
1	2µg/µl	0.047
2	4µg/µl	0.091
3	6µg/µl	0.136
4	10µg/µl	0.155
5	15µg/µl	0.187
6	20µg/µl	0.219

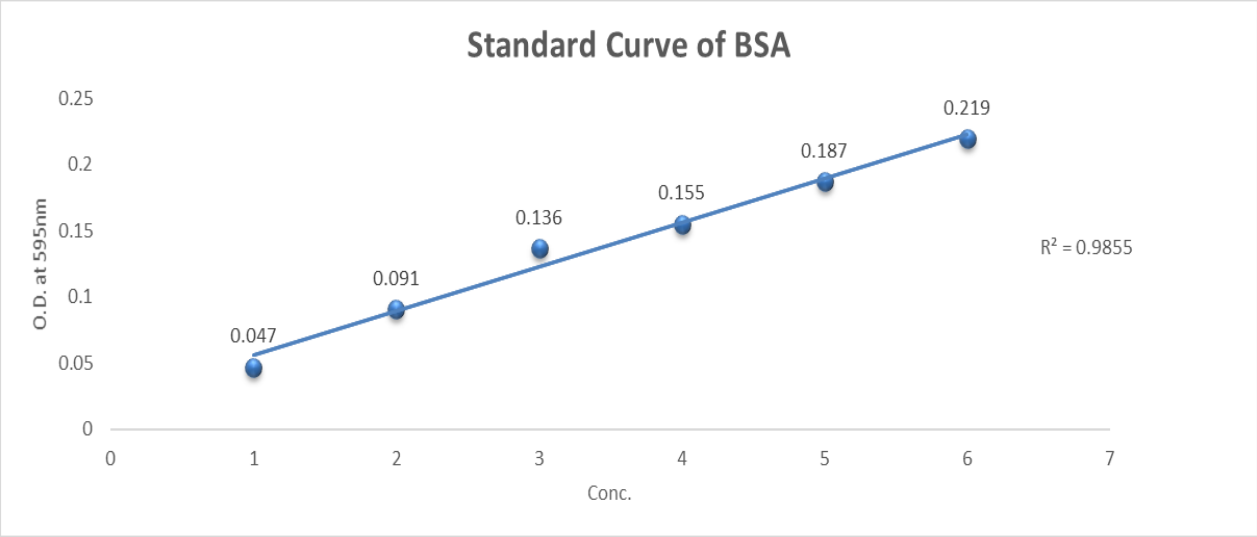

Figure 4.4 Standard curve for protein quantification using Bovine Serum Albumin (BSA).

Proteins quantification were performed by Bradford assay and proteins concentrations were observed 2.66 µg/µl for LDH and 4.19 µg/µl for proB.

4.5 SDS-PAGE Gel Electrophoresis

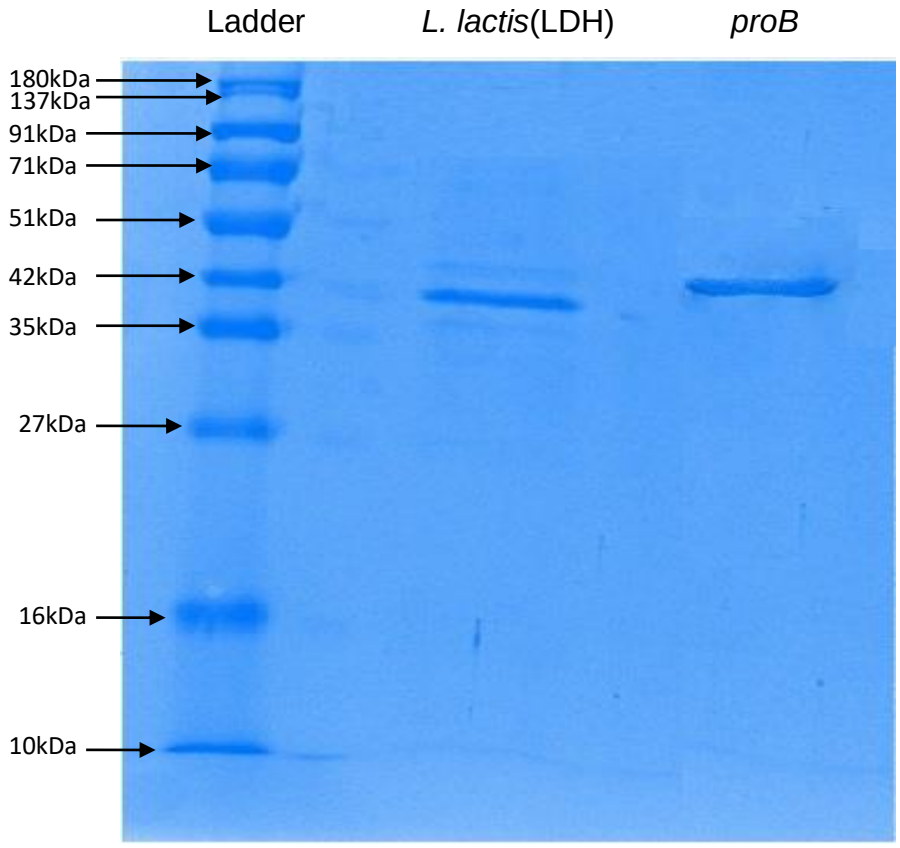

Figure 4.5 Electrophorogram Showing the Protein Expression.

Single bands on SDS PAGE gel were observed (as shown in Figure 4.5). According to the ladder which was used as reference molecular weight showed that protein LDH having molecular weight approximately 39kDa and *ProB* having molecular weight approximately 42kDa were expressed.

Chapter 5
Discussion

The recombinant *E. coli* strains were obtained by incorporating LDH gene of L. lactis and ProB gene of B. subtilis in the pET 30a plasmid and transformed into *E.coli BL21* DE3. These recombinant strains of *E. coli* were grown on LB media to produce proteins LDH and *ProB*. These proteins were purified and expressed and purity was established by SDS-PAGE obtaining a single desired band. Later we were supposed to determine the amount of enzyme obtained for which these proteins were made to metabolize their respective substrate. For LDH pyruvate was used, while for *proB* L-glutamate was used. Based on the amount of product formed in each reaction the amount of protein was determined.

Further, kinetic studies were supposed to performed using protocols developed by our research group including pH and temperature profiling at various pH and temperatures, respectively. But we couldn't perform these experiments for the above discussed enzymes because of unavailability of required chemicals. In the future depending on the availability of chemical these experiments will be performed and data can be analyzed. These studies can be used to improve the industrial strains of the LAB to improve the production in larger scale.

APPENDIX A

Sr. No.	Stock Solution	Methods of Preparation
1	Luria Bertani (LB) Broth	20g of Luria Bertani broth was dissolved in 1L of Distilled Water.
2	LB Agar	2.5g of agar was dissolved in 100ml of LB broth for plating.
3	Antibiotics (Ampicillin and kanamycin 50mg/ml)	250mg of kanamycin and ampicillin were dissolved in 5 mL of distilled water separately and stored in -20° C.
4	IPTG (Isopropyl β-D-1-thiogalactopyranoside)	2.4g of IPTG was dissolved in 10mL of distilled water and stored at -20° C.
5	Tris-Cl Buffer (0.5M)	60.5g of Tris-CL, 90g of NaCl and 36 µl of β—Mercaptoethanol was dissolved in 900ml of distilled water and make up the final volume 1000mL, adjust the pH=7 using concentrated HCl.
6	Washing/suspension buffer (50Mm)	100mL of tris-HCl buffer was mixed in 900mL of distilled water.
7	150mM imidazole	5.1g imidazole was dissolved in 500ml of washing buffer.
8	20mM imidazole	27mL of 150mM imidazole was mixed with 200mL of washing buffer.
9	MES (2-(*N*-morpholino) ethane sulfonic acid) buffer	2.2 gm MES was dissolved in 500mL of distilled water and the pH-5 was adjusted using 10N NaOH.
10	30% acrylamide (bisacrylamide and acrylamide)	Dissolve 29.2g Acrylamide and 0.8g bisacrylamide in distilled water to make the final volume of 100ml. Note: Acrylamide solution is light sensitive. Store it in dark at 40°C. It is toxic. Wear gloves.
11	1.5 M Tris-Cl (pH 8.8)	Dissolve 9.085g of Tris base in 20ml distilled water. Adjust the pH using concentrated. HCl. Make final volume 50ml using distilled water

12	10% APS	1.5g in 15ml distilled water
13	10% SDS	1.5g in 15ml distilled water
14	0.5M Tris-Cl (pH6.8)	Dissolve 3.02g of Tris base in 20ml distilled water. Adjust the pH using conc. HCl. Make final volume 50ml using distilled water.
15	1X PBS	Prepare 800 mL of distilled water in a suitable container. Add 8 g of NaCl to the solution. Add 200 mg of KCl to the solution. Add 1.44 g of Na_2HPO_4 to the solution. Add 240 mg of KH_2PO_4 to the solution. Add distilled water until the volume is 1 L.

APPENDIX B

SDS-PAGE (Polyacrylamide gel electrophoresis) buffers and gel composition

1. SDS-PAGE:

a. 10X Running buffer: 10X Running buffer for SDS-PAGE comprises:

- 250mM Tris Base (pH 8.3)
- 2M Glycine
- 1% SDS

To prepare running buffer dissolve 30.2g Tris base, 150g glycine and 10g SDS in distilled water to make final volume 1litre. Dilute the running buffer to 1X before use.

Note: SDS is a respiratory irritant in solid form. Mask should be worn while weighing.

b. 4X SDS sample loading buffer: It is used for preparation and loading protein samples onto a gel for SDS-PAGE analysis. 1X SDS sample buffer comprises:

- 50mM Tris-Cl (pH 6.8)
- 2% SDS
- 10% Glycerol
- 1% β-mercaptoethanol
- 12mM EDTA
- 0.02% Bromophenol blue

To prepare 4X SDS sample buffer, mix 4ml of 0.5M Tris-Cl pH 6.8, 0.8g of SDS, 4ml of 100% glycerol, 0.4ml of β-mercaptoethanol, 1ml of 0.5M EDTA, 8mg bromophenol blue. Make up the volume with distilled water to 10ml. Dilute protein sample and sample loading buffer in ratio 3:1 before loading.

Note: β-mercaptoethanol is a severe irritant, and it gets absorbed through the skin.

c. Gel composition for separating gel: The components for 10% Separating gel (10ml):

- 30% acrylamide = 3.34ml
- 1.5M Tris-Cl (pH 8.8) = 2.5ml
- 10% APS = 0.1ml
- 10% SDS = 0.1ml
- TEMED = 0.010ml
- Distilled water = 4ml

d. Gel composition for stacking gel: The components for 4.5% Stacking gel (5ml):

- 30% acrylamide = 0.850ml
- 0.5M Tris-Cl (pH 6.8) = 0.625ml
- 10% APS = 0.05ml
- 10% SDS = 0.05ml
- TEMED = 0.005ml
- Distilled water = 3.4ml

References

Belitsky, B. R., Brill, J., Bremer, E., & Sonenshein, A. L. (2001). Multiple genes for the last step of proline biosynthesis in Bacillus subtilis. *Journal of Bacteriology*, **183**(14), 4389-4392.

Blättel, V., Wirth, K., Claus, H., Schlott, B., Pfeiffer, P., & König, H. (2009). A lytic enzyme cocktail from Streptomyces sp. B578 for the control of lactic and acetic acid bacteria in wine. *Applied Microbiology and Biotechnology*, **83**(5), 839-848.

Brill, J., Hoffmann, T., Bleisteiner, M., & Bremer, E. (2011). Osmotically controlled synthesis of the compatible solute proline is critical for cellular defense of Bacillus subtilis against high osmolarity. *Journal of Bacteriology*, **193**(19), 5335-5346.

Buckenhüskes, H. J. (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. *FEMS Microbiology Reviews*, **12**(1-3), 253-271.

Cameron, A. D., Roper, D. I., Moreton, K. M., Muirhead, H., Holbrook, J. J., & Wigley, D. B. (1994). Allosteric activation in Bacillus stearothermophilus lactate dehydrogenase investigated by an X-ray crystallographic analysis of a mutant designed to prevent tetramerization of the enzyme. *Journal of Molecular Biology*, ***238***(4), 615-625.

Feldman-Salit, A., Hering, S., Messiha, H. L., Veith, N., Cojocaru, V., Sieg, A., ... & Fiedler, T. (2013). Regulation of the activity of lactate dehydrogenases from four lactic acid bacteria. *Journal of Biological Chemistry*, ***288***(29), 21295-21306.

Fiocco, D., Capozzi, V., Goffin, P., Hols, P., & Spano, G. (2007). Improved adaptation to heat, cold, and solvent tolerance in Lactobacillus plantarum. Applied Microbiology and Biotechnology, **77**(4), 909-915.

Fisher, K., & Phillips, C. (2009). In vitro inhibition of vancomycin-susceptible and vancomycin-resistant Enterococcus faecium and E. faecalis in the presence of citrus essential oils. *British Journal of Biomedical Science*, ***66(***4), 180-185.

Ivanov, V., Roomi, M. W., Kalinovsky, T., Niedzwiecki, A., & Rath, M. (2007). Anti-atherogenic effects of a mixture of ascorbic acid, lysine, proline, arginine, cysteine, and green tea phenolics in human aortic smooth muscle cells. *Journal of Cardiovascular Pharmacology*, ***49(***3), 140-145.

Iwata, S., Kamata, K., Yoshida, S., Minowa, T., & Ohta, T. (1994). T and R states in the crystals of bacterial L–lactate dehydrogenase reveal the mechanism for allosteric control. *Nature Structural and Molecular Biology*, **1**(3), 176.

Kenneth, T. (2011). Todar's online textbook of bacteriology. *Bacterial Protein Toxins*.

Koistinen, K. M., Plumed-Ferrer, C., Lehesranta, S. J., Kärenlampi, S. O., & Von Wright, A. (2007). Comparison of growth-phase-dependent cytosolic proteomes of two Lactobacillus plantarum strains used in food and feed fermentations. FEMS Microbiology Letters, **273**(1), 12-21.

Krishna, R. V., Beilstein, P., & Leisinger, T. (1979). Biosynthesis of proline *in Pseudomonas aeruginosa*. Properties of γ-glutamyl phosphate reductase and 1-pyrroline-5-carboxylate reductase. *Biochemical Journal*, **181**(1), 223.

Liong, M. T. (Ed.). (2015). *Beneficial Microorganisms in Agriculture, Aquaculture and Other Areas* (Vol. **29**). Springer.

Murakami, H., Shimbo, K., Inoue, Y., Takino, Y., & Kobayashi, H. (2012). Importance of amino acid composition to improve skin collagen protein synthesis rates in UV-irradiated mice. *Amino Acids*, **42**(6), 2481-2489.

Ponrasu, T., Jamuna, S., Mathew, A., Madhukumar, K. N., Ganeshkumar, M., Iyappan, K., & Suguna, L. (2013). Efficacy of L-proline administration on the early responses during cutaneous wound healing in rats. *Amino acids*, **45**(1), 179-189.

Siezen, R. J., & van Hylckama Vlieg, J. E. (2011). Genomic diversity and versatility of Lactobacillus plantarum, a natural metabolic engineer. *Microbial Cell Factories*, **10**(1),

Smith, C. J., Deutch, A. H., & Rushlow, K. E. (1984). Purification and characteristics of a gamma-glutamyl kinase involved in Escherichia coli proline biosynthesis. *Journal of Bacteriology*, **157**(2), 545-551.

Taskila, S., & Ojamo, H. (2013). The current status and future expectations in industrial production of lactic acid by lactic acid bacteria. In *Lactic acid bacteria-R & D for food, health and livestock purposes*. InTech. Incomplete Ref

Unden, G. (2009). *Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 3-30). H. König, & J. Fröhlich (Eds.). Springer.

Wessels, S., Axelsson, L., Hansen, E. B., De Vuyst, L., Laulund, S., Lähteenmäki, L., ... & von Wright, A. (2004). The lactic acid bacteria, the food chain, and their regulation. *Trends in food science & technology*, **15**(10), 498-505.

Zaprasis, A., Brill, J., Thüring, M., Wünsche, G., Heun, M., Barzantny, H., ... & Bremer, E. (2013). Osmoprotection of Bacillus subtilis through import and proteolysis of proline-containing peptides. *Applied and eEnvironmental Mmicrobiology*, **79**(2), 576-587.

Urkund Analysis Result

Analysed Document:	Gunjan URkund_20 May 2018.docx (D38914289)
Submitted:	5/20/2018 2:15:00 PM
Submitted By:	malkhey.verma@cup.edu.in
Significance:	7 %

Sources included in the report:

Gunjan URkund.docx (D38904461)
http://jb.asm.org/content/183/14/4389.full
http://aminoacidstudies.org/l-proline/
http://slideplayer.com/slide/10617049/
https://www.ncbi.nlm.nih.gov/pubmed/11571156

Instances where selected sources appear:

18