Comparison of Pyruvate Kinase of different species of Lactic Acid Bacteria

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In
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By

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May 2018

DECLARATION

I Debasis Sahoo declare that the project report entitled “COMPARISON OF PYRUVATE KINASE OF DIFFERENT SPECIES OF LACTIC ACID BACTERIA" 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.

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CERTIFICATE

I certify that Debasis Sahoo has prepared his project report entitled “COMPARISON OF PYRUVATE KINASE OF DIFFERENT SPECIES OF LACTIC ACID BACTERIA” for the award of M.Sc. degree from the Central University of Punjab, under my guidance. He has carried out this work at the Department of Biochemistry and Microbial Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ABSTRACT

Comparison of Pyruvate kinase of different species of Lactic Acid Bacteria

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Key Words: Pyruvate kinase, Lactic acid bacteria, cellular metabolism, recombinant protein expression.

Pyruvate kinase (PK) is an important allosterically regulated enzyme that connects Glycolysis from the primary energy to cellular metabolism. It catalyzes the last step of Glycolysis by producing one mole of energy in the form of ATP. On the other hand, Lactic acid bacteria (LAB) under anaerobic condition produce energy by Glycolysis process that strengthens the vital role of PK in their cellular metabolism. The recombinant proteins of PKs expression were performed by inserting the PKs genes of LAB strains in the pET 30 plasmid and transformed into the *E. coli* BL21 (DE3) cells. After cell cultivation, recombinant protein expression was induced. The targeted protein was purified by Affinity chromatography and quantified by Bradford quantitative assay. Protein expression was compared by the SDS-PAGE. Here in this study, the main targeted protein is Pyruvate kinase and its activity is compared among *Enterococcus faecalis, Lactococcus lactis, Lactococcus plantarum and Streptococcus pyogenes.*

Debasis Sahoo Dr. Malkhey Verma

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Needless to say, omissions and errors if any is mine.

Debasis Sahoo

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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.	Glyceraldehyde 3-Phosphate	GAP
11.	Hydrochloric Acid	HCL
12.	Isopropyl-1-Thiogalactopyranoside	IPTG
13.	Lactate Dehydrogenase	LDH
14.	Lactic Acid Bacteria	LAB
15.	Luria Bertani	LB
16.	Nicotinamide Adenine Dinucleotide	NAD
17.	Oxygen	O_2
18.	Phosphate Buffer Saline	PBS
19.	Phosphoenolpyruvate	PEP
20.	Polyacrylamide Gel Electrophoresis	PAGE
21.	Pyruvate kinase	PK
22.	Sodium Dodecyl Sulphate	SDS

CHAPTER 1

INTRODUCTION

The role of Lactic Acid Bacteria (LAB) in fermentation is known for thousands of years. Many different cultures experienced this method of food processing to improve the storage quality, palatability, and nutritive value of perishable foods such as milk, meat, fish and some vegetables. The micro-organisms that produce lactic acid are known as lactic acid bacteria. Nowadays, lactic acid bacteria are mainly linked with a group of dairy products such as cheese, yoghurt, kefir and buttermilk. Lactic acid bacteria have been associated with positive health effects. The LAB is being used as probiotics to accomplish intestinal disorders such as lactose intolerance, acute gastroenteritis, constipation, and inflammatory bowel disease. (Buckenhuskes*,* 1993).

Lactic acid bacteria (LAB) constitute a group of Gram-positive, non-spore forming, anaerobic bacteria which excrete lactic acid as the chief fermentation product into the medium if supplied with appropriate carbohydrates (Kandler, 1983). It also synthesizes some antimicrobial substances like bacteriocins that possess the capability to inhibit the growth of pathogenic and food spoilage bacteria (Salit *et al*., 2013; Veith *et al*., 2013).

Since LABs mostly produce energy by the metabolism of sugars, they are restricted to such environments in which sugars are abundant. They have got the self-possessed biosynthetic capability, having evolved in environments that are rich in purines and pyrimidines, amino acids and nutrients so they must be grown in complex media to fulfill all their nutritional requirements (Wessels *et al*., 2004). Majority of these are free-living or live in favourable or harmless associations with animals, while some are opportunistic pathogens. They are found in milk, milk products and in decaying plant resources. They are normally found inside the oral cavity, the intestinal tract and the vagina of the humans, where they play some important roles. (Kenneth, 2011).

Lactobacillus plantarum, Streptococcus pyogenes, Enterococcus faecalis, and *Lactococcus lactis* are some of the main species of the LAB, belonging to the phylogenetic order Lactobacillales (Kleerebezem *et al*., 2000; Siezen and Van HylckamaVlieg, 2011).

These bacteria have their natural habitats in rather different environments (*L. lactis,* milk; *L. plantarum, plants; E. faecalis,* faeces; *S. pyogenes*, skin and mucosal membranes) and interact differently with human beings (Siezen and Van

HylckamaVlieg, 2011). *L. lactis* and *L. plantarum* are bacteria of major importance for use in the food industry. *E. faecalis* is an important commensal of the human gut, a food contaminant, and a facultative pathogen (Fisher and Phillips, 2009). *S. pyogenes* is an exclusively human pathogen causing diseases like tonsillitis, pharyngitis, scarlet fever, and necrotizing fasciitis. Despite their different régimes, all four species predominantly obtain energy by homolactic acid fermentation. The free energy generated during homolactic acid fermentation is 2 mol of ATP/mol of glucose (Salit *et al*., 2013; Veith *et al*., 2013). Pyruvate kinase (PK) is an enzyme that catalyzes the final step of glycolysis i.e. the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), yielding one molecule of pyruvate and one that of ATP.

1.2 Knowledge gap:

Both fermentative and pathogenic lactic acid bacteria produce energy through anaerobic fermentation of sugar, but it is yet not clear how some are non-pathogenic, and others are pathogenic.

1.3. Hypothesis:

Can comparison of glycolysis of LAB answer the pathogenic nature of LAB? Pyruvate kinase is an enzyme that catalyzes the final step of glycolysis and produces energy. Therefore, comparison of pyruvate kinases across the LAB could help to understand pathogenicity associated with the LAB.

1.4. Objectives of the Study:

I. Expression and purification of recombinant Pyruvate Kinases of *Lactobacillus plantarum, Streptococcus pyogenes, Enterococcus faecalis,* and *Lactococcus lactis*.

II. Kinetic characterization of Pyruvate Kinases of objective 1 for their kinetic comparison to understand carbon flux through Pyruvate Kinase catalyzed reactions in the pathogenic and non-pathogenic LAB.

CHAPTER 2

REVIEW OF LITERATURE

There are four mammalian pyruvate kinase (PK) isoforms viz. PKM1, PKM2, PKR, and PKL, with unique tissue expression patterns and regulatory properties (Lunt *et al*., 2015). The PKM1 isoform is found in tissues with high catabolic demand, such as muscle, heart, and the brain, while PKM2 is expressed by most of the adult tissues. PKR is found exclusively in red blood cells, and PKL is major isoform in liver and minor in the kidney (Israelsen and Heiden, 2015).

2.1 Mechanism of catalysis

Pyruvate Kinase catalyzes the direct transfer of Pi (inorganic phosphate) from phosphoenolpyruvate (PEP) to ADP to produce ATP and pyruvate. This reaction is feasible due to the high energy of hydrolysis of PEP. During catalysis, the active site is occupied by both substrates (PEP and ADP, which is complexed with Mg^{2+}), one monovalent cation and one additional enzyme-bound divalent cation. The latter two metal ions assist with substrate binding and coordination, and a catalytic lysine residue stabilizes the penta-coordinate transition state that exists as the phosphate is transferred directly from PEP to ADP (Salit *et al*., 2013; Veith *et al*., 2013).

Transfer of the phosphate from PEP to ADP leaves the energetically less-stable enol form of pyruvate bound in the active site. The process of tautomerization of enol pyruvate to the more stable keto form of pyruvate, contributes to the favourable energetics of phosphate transfer from PEP to ADP. The Tautomerization occurs when enol pyruvate accepts a proton from a water molecule, that is held in position by conserved active site residues (T328 and S362 in humans). Following catalysis, the products leave the active site, and neither substrate binding nor release of products is thought to be well-organized (Israelsen and Heiden, 2015).

Glycolysis is the most fundamental energy yielding reaction in a living organism (Figure 1). Starting from simpler unicellular organism to complex multi-cellular organisms utilize carbohydrates as their energy source. Aerobes, as well as anaerobes, can perform glycolysis as the first step to consume energy because glycolysis is oxygen independent. Glycolysis is not a simple process, it is a multi-step reaction involving many enzymes. It is a ten-step reaction, and each is

catalyzed by a specific enzyme. The intermediate products of glycolysis are the key precursors to many cellular metabolic reactions (Garcia-Olalla and Garrido-Pertierra, 1987).

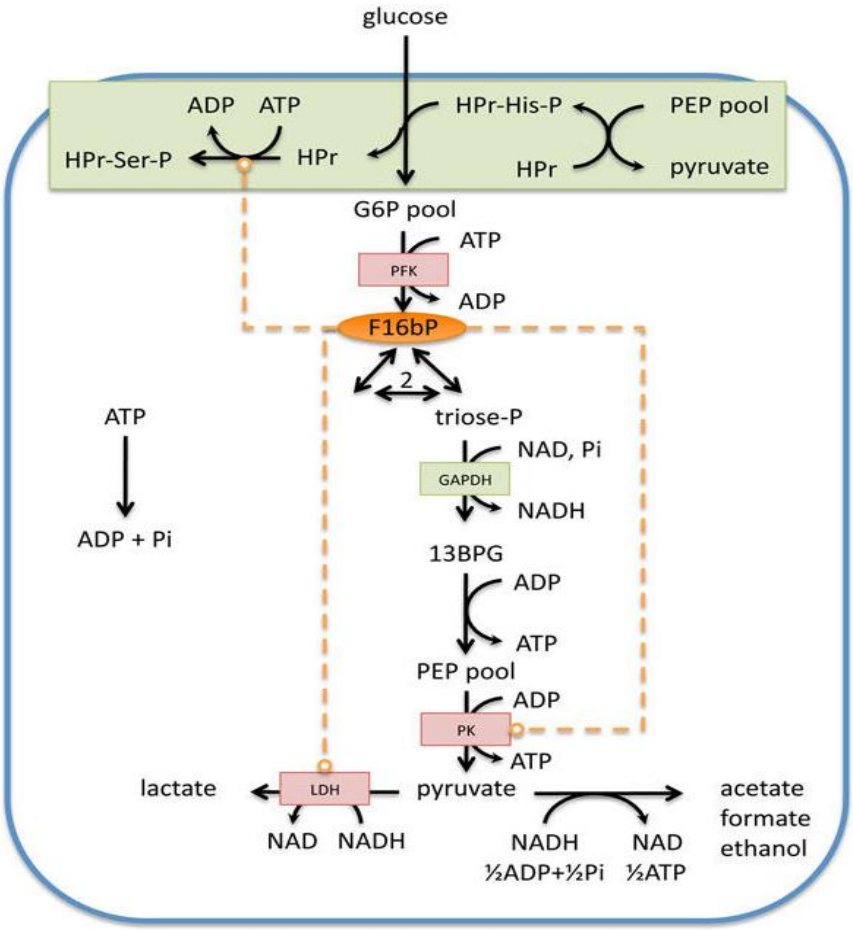


Figure 1. The pathway of Glycolysis (Garcia-Olalla and Garrido-Pertierra, 1987)

The enzymes and each step of the reaction are regulated differently. Here we have studied the recombinant enzyme Pyruvate Kinase (EC 2.7.1.40) in the LAB. Pyruvate Kinase is the last one to catalyze the reaction where Phosphoenol pyruvate is converted to Pyruvate and formation of an ATP molecule from the phosphorylation of an ADP molecule takes place (Ramos *et al*., 2004). The phosphorylation in this reaction is called as substrate level phosphorylation (Valentini *et al.,* 2000). The major part of the energy released from hydrolysis of phosphoenol pyruvate is conserved with the formation of high energy phosphoanhydride bond in ATP. The rest part of the energy helps the reaction for completion. Metal cation Mg^{2+} is required for optimum catalysis by the enzyme. The substrates for the enzyme are Phosphoenol pyruvate, ADP and Mg^{2+} (Munoz and

Ponce, 2003). The binding sites for the substrates are different and their binding is independent.

The quaternary structure of pyruvate kinase is a tetramer consisting identical subunits (Figure 2). Each subunit contains 500 amino acid residues. The monomer structure depicts three domains: domain A, B and C. Domain A bear the active site of the enzyme at the junction of domains A and B. Domain C contains the activator binding site. In bacteria, two isoforms of pyruvate kinase are found: pyruvate kinase I and pyruvate kinase II. They are called as isoenzymes. Although the isoenzymes share a conserved structure, their activation is regulated differently. Pyruvate kinase type I is activated by fructose 1,6-bisphosphate(FBP) and the type II is activated by AMP or monophosphorylated sugar (Zhu *et al.,* 2010).

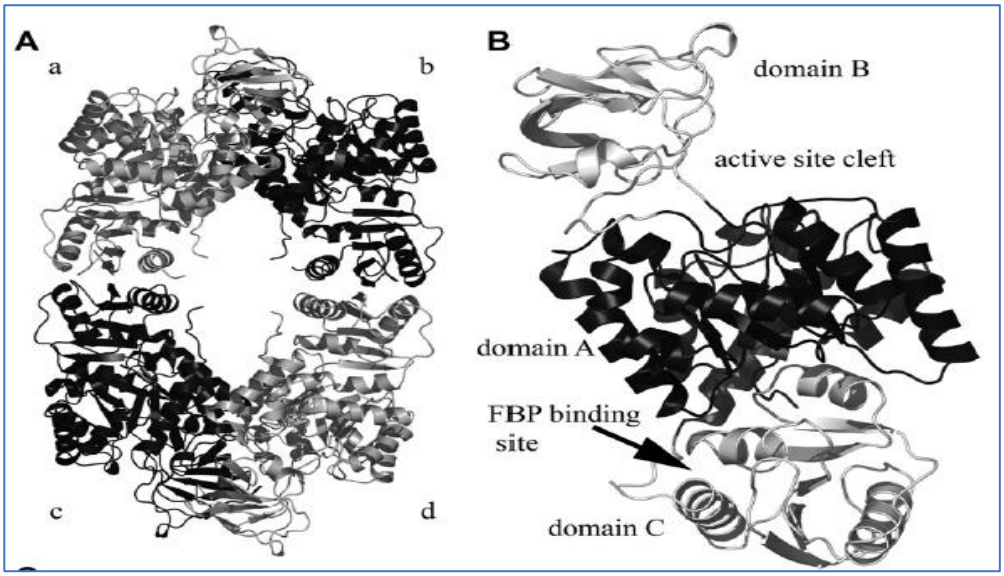


Figure 2. Tetrameric and monomeric form of pyruvate kinase

2.2 About the strain:

The present study comprises the bacterial strain of *E. coli*, BL21 (DE3). It is a gram-negative, facultatively anaerobic chemo-organotroph bacterium. It has a capacity for both respiratory and fermentative metabolism. The beauty of this strain lies in the enormous work it performs. It is widely adapted by many biotech industries as well as life sciences laboratories for studying the bacterial evolution, restriction-modification system, phage sensitivity, and most importantly recombinant protein expression, for which act it is known as the workhorse for protein production. There are certain factors which make BL21(DE3) as a desirable host for recombinant

protein production. Those characteristics are low acetate production at a high level of glucose, protease deficiency which would cleave over expressed proteins, and enhanced permeability which may be due to a simple cell surface. (Jeong *et al., 2009*).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials required

- **Chemicals:** Luria Bertani agar powder, Antibiotics (Ampicillin and Kanamycin), Isopropyl-1-thiogalactopyranoside (IPTG), Tris-HCl (pH 7.0), β-mercaptoethanol ,70% ethanol, Sodium chloride (NaCl), Metal ion resins, Distilled water, Imidazole, 2-N Morpholino ethane sulfonic acid (MES), Sodium hydroxide (NaOH), Acrylamide, Bis acrylamide, Sodium Dodecyl Sulphate (SDS), TEMED, Acetic acid, methanol and Coomassie blue.
- **Instruments:** Shaking incubator, BOD incubator, Autoclave, Weighing balance, pH meter, Refrigerator (-80˚C, -20˚C, 4˚C), Laminar air flow, Centrifuge, Column Stand, Sonicator, Rotor shaker, Spectrophotometer, SDS-PAGE assembly, 96 well plate reader and Gel doc.
- **Glassware and other equipments:** Conical flask (500 ml, 250 ml), Eppendorf tubes (2 ml, 1.5 ml), Beaker (1000 ml, 500 ml, and 200 ml), Reagent bottle (1000ml, 500ml), Falcon conical centrifuge tubes (50 ml and 15 ml) Tissue paper, Pipette and tips (1000 µl, 100 µl, 20 µl), Parafilm, Ice bucket, Glass Cuvette, Measuring cylinder, cotton and Aluminum foil.

3.2. Methodology:

3.2.1. Luria Bertani (LB) agar plates preparation:

LB media is the best suitable media for *E. coli* culture followed by Tryptone broth and terrific broth media. (Juliane, 2013). Four sets of 50ml of LB broth (20gm/L) were prepared in the 250ml conical flask. 1.3 gm of agar was added to each conical flask and then mixed properly. Each flask was tightened with cotton plugs on the top and enclosed with aluminium foil or any glossy papers. After that, the two flasks were autoclaved (at 121^{o}C for 15 minutes), and after cooling 50µl of 50 mg/ml antibiotics (Ampicillin and Kanamycin) were added to the separate flasks. All these procedures were carried out inside the laminar air flow chamber for avoiding the contamination. Now, petri plates for bacterial cultures were prepared by pouring the 25ml LB agar media. After the plates got solidified, streaking was performed with bacterial strains *(L. lactis, S. pyogenes, E. faecalis,* and *L. plantarum*) and then incubated for 24 hours at 37°C in the BOD incubator.

Figure 3.1. The Luria Bertani (LB) agar Plate

3.2.2. Bacterial Cell Cultivation:

After 24 hours of incubation, single isolated colonies were seen on the petri plates having the media with the respective antibiotics. Now for the seed culture process, two sets of LB broth media (50ml in the 250ml conical flask) were prepared by autoclaving. After cooling of the broth media in few minutes, 50 µl of media having bacterial growth was added to the conical flasks with the help of an inoculating loop. After that, the flasks were kept in the shaking incubator for overnight at 37ºC and 180 rpm.

After the incubation, media became turbid and it was transferred equally to four flasks having LB media with 100 µl of antibiotics. Later these four flasks were kept in the shaking incubator at 37ºC and 180 rpm. Then, the O.D of the culture was taken at 600nm at a regular time interval of half an hour as the doubling time of *E. coli* BL21 (DE3), is 17 minutes (Table 1) (Paliy and Gunasekera, 2007). When the required O.D range of 0.5-0.6 was achieved, 100 µl of 1mM IPTG was added to each of the four flasks, and then these flasks were kept in the shaking incubator for 4 hours at 37ºC and 180 rpm.

Table 3.1. Generation times (min.) for some common bacteria under optimal conditions of growth (Paliy and Gunasekera, 2007)

Bacterium	Medium	Mins.	Bacterium	Medium	Mins.
E. coli	Glucose-salts	17	*S. aureus*	Heart infusion broth	27-30
B. megaterium	Sucrose-salts	25	*L. acidophilus*	Milk	66-87
S. lactis	Milk	26	*R. japonicum*	Mannitol-salts-yeast extract	344-461
S. lactis	Lactose broth	48	*M. tuberculosis*	Synthetic	792-932

3.2.3. Cell Harvesting:

After completing cell cultivation, the induced media was centrifuged in 50ml falcon tube at 6000 rpm for 10 min at 4°C, for making the large collection of the pellet. Then, the pellet was washed two times by using 50mM suspension buffer (Tris HCl-NaCl β-mercaptoethanol buffer, pH 8.0). Next, the washed pellet was suspended in an equivalent amount of suspension buffer and stored at -80ºC for future use.

Figure 3.2. The framework of Cell harvesting

3.2.4. Protein Purification by Affinity Chromatography:

For the protein purification, the pellet suspended in the buffer solution was removed from the -80ºC freezer and was sonicated for 15 min with one min on and

off mode respectively, at 10 ºC in the ultrasonic cleaning bath tub. Then, the lysed cells were centrifuged at 7500 rpm for 30 min at 4°C and the supernatant was collected. For the chromatography process, the metal ion resin was washed with suspension buffer (3 ml) for 3 to 4 times. Next, the washed resins and the supernatant both were poured into a new sterilized falcon tube, sealed properly, and then placed on the rocker shaker for 1 hour. When the resins mixed properly with the supernatant, the resins were transferred into the column, and the supernatant was collected in another falcon tube which further kept at 4°C with proper labelling. Again, the resins were washed with the suspension buffer (3-4 ml) for 3- 4 times. Now the final wash was given by 20 mM Imidazole buffer and the washed-out solution was discarded. Afterwards, protein was eluted with 150 mM Imidazole buffer in three fractions of 2 ml each in the eppendorf tubes of 2ml, and then these eppendorf tubes were sealed and stored at 4°C. Subsequently, the used resins were recharged by washing it with 10ml of 20 mM MES buffer followed by 20 ml of distilled water, and then equilibrated with suspension buffer, and stored at 4°C for future use.

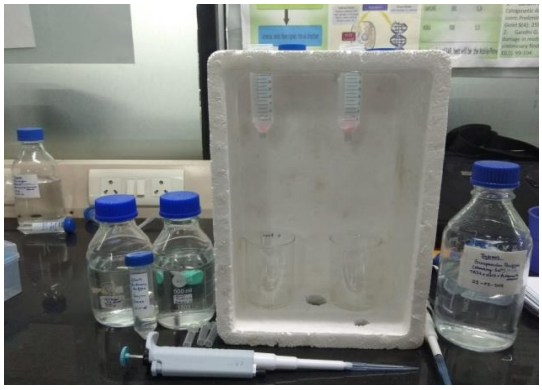


Figure 3.3. The Setup of Affinity Chromatography

3.2.5. Quantification of Protein by Bradford Assay:

Further the total protein concentration was quantified by Bradford method (Bradford, 1976), using bovine serum albumin (0.1µg/µl) as standard.

Bradford assay is a type of quantitative assay which provide the total protein content based on the absorbance shift. As an example of the colourimetric assay, here we use Coomassie brilliant blue (CBB) dye. Generally, CBB dye has two different colour formations in two different states. The first state is an acidic state or cationic state which is unstable in nature. Here the dye has dark reddish colouration.

On the other hand, if this dye is placed in basic condition (anionic state) then it becomes “Bound state” which is stable in nature. And here the colouration of the dye is blue, that’s why it is called as CBB. The amount of protein can be detected by using colourimetric assay or observing them in a spectrophotometer at 595 nm wavelength of light.

After addition of the protein of unknown concentration to the dye, the dye slowly starts to change the colour, which is because of the charged amino acids present in the protein that form bonds and interact to the dye by ionic interactions, hydrophobic interactions and Vander Waal’s interaction. So, the amount or concentration of protein present will be directly proportional to the intensity of the colouration.

The Bradford reagent was prepared by dissolving 100 mg Coomassie brilliant blue G-250 in 50 ml 95% ethanol. Then to this solution, an amount of 100 ml 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 in 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 the 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, to obtain a blue colour solution which was measured at 595 nm wavelength with a microplate reader.

3.2.6. Preparation of protein samples:

The calculated volume of protein samples was added and mixed with the loading dye. The samples were incubated for 2 min in a boiling water bath prior to loading. After the completion of polymerization, the comb and the lower spacer strip were removed gently. The gel was attached to the electrophoresis unit using appropriate clips/clamps and placed in the electrophoresis 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 supply. The gel was run at a constant current of 40 milli ampere 100 volts for 5-7 hrs. at room temperature. At the end of the run, the power supply was turned 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.

3.2.7. Protein Gel Electrophoresis: SDS PAGE

Biological macromolecules like nucleic acids and proteins are generally separated by Polyacrylamide gel electrophoresis (PAGE). This separation is based on molecular weight, shape, and size of the molecule (protein or nucleic acids) and the charge. A series of proteins of known molecular mass known as molecular weight markers or protein ladder is run on a gel in a fixed separated lane adjacent to the protein lane of unknown molecular mass under the effect of the electric field. Generally, sodium dodecyl sulfate (SDS) breaks the bond between the amino acids and make the protein linearized and it also puts a negative charge to the protein. In most of the proteins, the binding of SDS to polypeptide chain imparts an even distribution of charge per unit mass which makes separation much easier.

- Preparation of gel:

The glass plates were cleaned for the experiment by washing with detergent, rinsing subsequently in tap water, drying them and finally wiping with 70% ethanol. The solution of the separating gel was mixed well and poured into the space between the two plates. APS and TEMED were added just before pouring of the gel. 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, undisturbed at room temperature for 30 minutes. After separating gel was polymerized, the stacking gel solution was poured over it. Then an appropriate comb was inserted in between the plates.

In SDS-PAGE, 10% separating gel (Table 3.2) and 4% stacking gel (Table 3.3) was used for separation of prepared protein samples. Electrophoresis was done at 50V for two and half hour at room temperature. Coomassie staining was used for detection of protein bands in the gel after PAGE.

Table 3.2: List of chemicals required for 10% separating gel

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

Table 3.3: List of chemicals required for 4% stacking gel

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

- Coomassie Staining of the gel:

The gel was incubated in 10% trichloroacetic acid (TCA) for 5minutes and then washed with distilled water for blocking the band to the gel. After this, the gel was kept in the staining solution at a dancing shaker for overnight. Next day the gel was destained by destaining solution. The chemical composition of both the solutions is given in Table 3.4 and 3.5 respectively.

Table 3.4: List of chemicals required for the preparation of staining solution (100 ml)

Reagents	**Volume/ weight**
Acetic acid	10 ml
Methanol	20 ml
Distilled water	70 ml
Coomassie brilliant blue G-250	0.025 g

Table 3.5: List of chemicals required to make the destaining solution

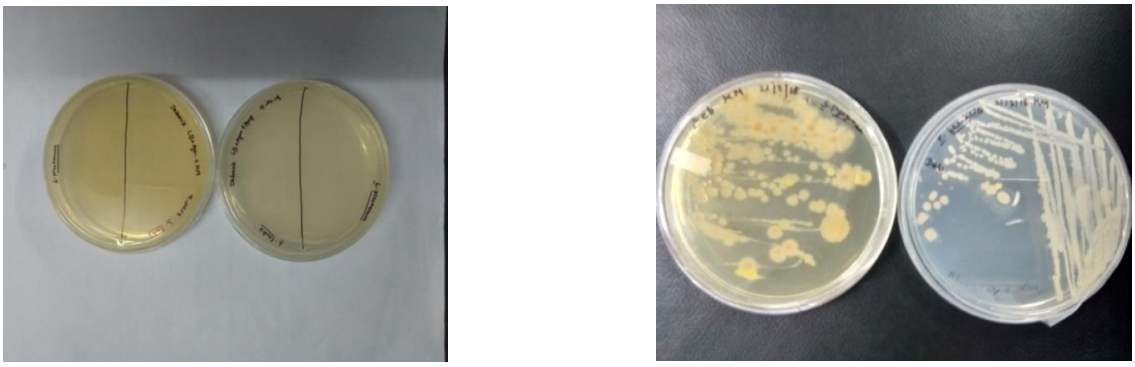
Reagents	**Volume**
Acetic acid	10 ml
Methanol	20 ml
Distilled water	70 ml

CHAPTER 4

RESULTS

4.1 Plate Culture:

The growth of recombinant *E. coli* strains containing PK genes of *S. pyogenes and E. faecalis were* observed on LB agar plate having antibiotic Kanamycin, which show the resistance of these bacterial strains for Kanamycin but not for the Ampicillin (Figure 4.1.B). Again, no growth of the recombinant *E. coli* strains (*L. lactis* and *L. plantarum)* was observed on the plates having Kanamycin and Ampicillin. The results showed more isolated colonies in case of *E. faecalis as* compared to *S. pyogenes,* from which we can say that in case of *E. faecalis,* it is easy to isolate single colony due to its better and fast growth.



(A) **(B)**

Figure 4.1. The Culture Of Recombinant *E. Coli* Growth (A: Negative Result; B: Positive Result)

4.2 Seed Culture and Protein extraction:

After the petri plate culturing, the cultures of *S. pyogenes and E. faecalis* were inoculated into the flasks containing LB media and Kanamycin. These flasks were kept in the shaking incubator shaker for 24 hours. Turbidity in the flasks shows the bacterial growth after 24 hours (Figure 4.2).

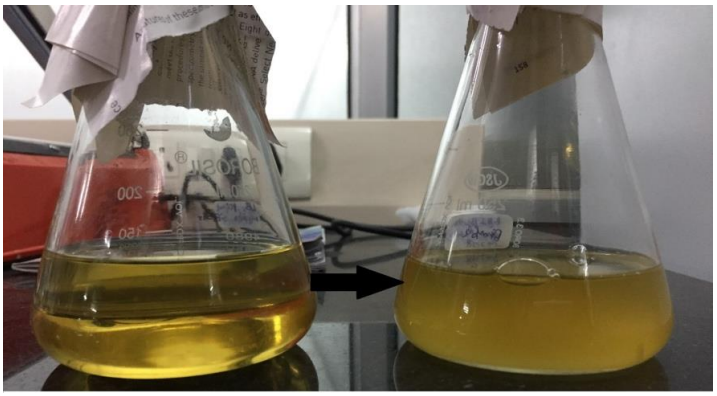


Figure 4.2. Turbidity shows the dense growth of both the bacterial strains after keeping the flask in a shaker for 24 hours

4.3 Protein Purification by Affinity Chromatography

The recombinant protein Pyruvate Kinases of *E. faecalis and S. pyogenes* were extracted from the recombinant strains of *E.coli* BL 21 (DE3) by affinity chromatography. For the protein quantification further assay was performed.

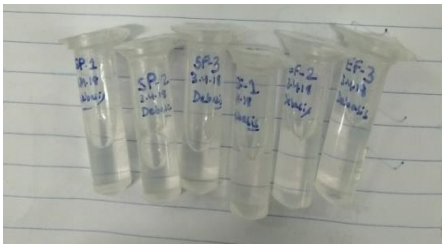


Figure 4.3. Recombinant PK protein collected purified by affinity chromatography

4.4 Bradford Assay

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

S. No.	BSA Conc. (µg/µl)	O.D. at 595nm
1	2 µg/µl	0.031
2	4 µg/µl	0.044
3	6 µg/µl	0.106
4	10 µg/µl	0.136
5	15 µg/µl	0.155

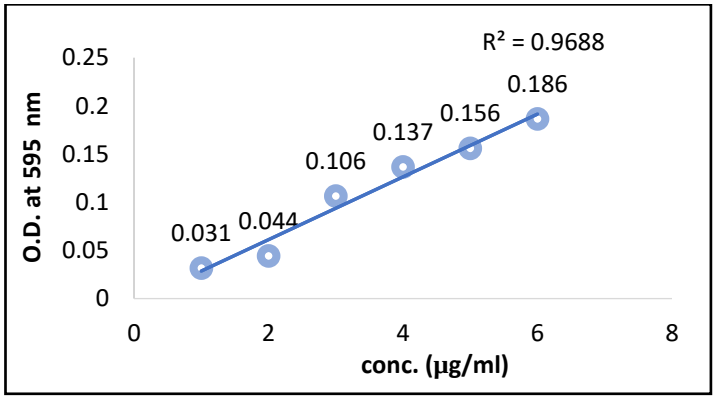


Figure 4.4. Standard curve of Bovine Serum Albumin (BSA)

The protein quantification was done by the Bradford assay using BSA as the positive control. Through this assay the concentration of unknown samples i.e. the pyruvate kinase extracted from the recombinant strains of *E.coli* BL 21 (DE3) were found to be **0.168 µg/µl** and **0.178 µg/µl** respectively.

4.5 SDS-PAGE Gel Electrophoresis

Since the protein quantification by Bradford assay is insufficient for a description of protein expression, hence supportive protein pattern analysis of Pyruvate kinases was done using SDS-PAGE. Separation of proteins was carried out in 10% separating gel at constant voltage (50 V for 180 minutes) and later the gel staining was done by Coomassie method.

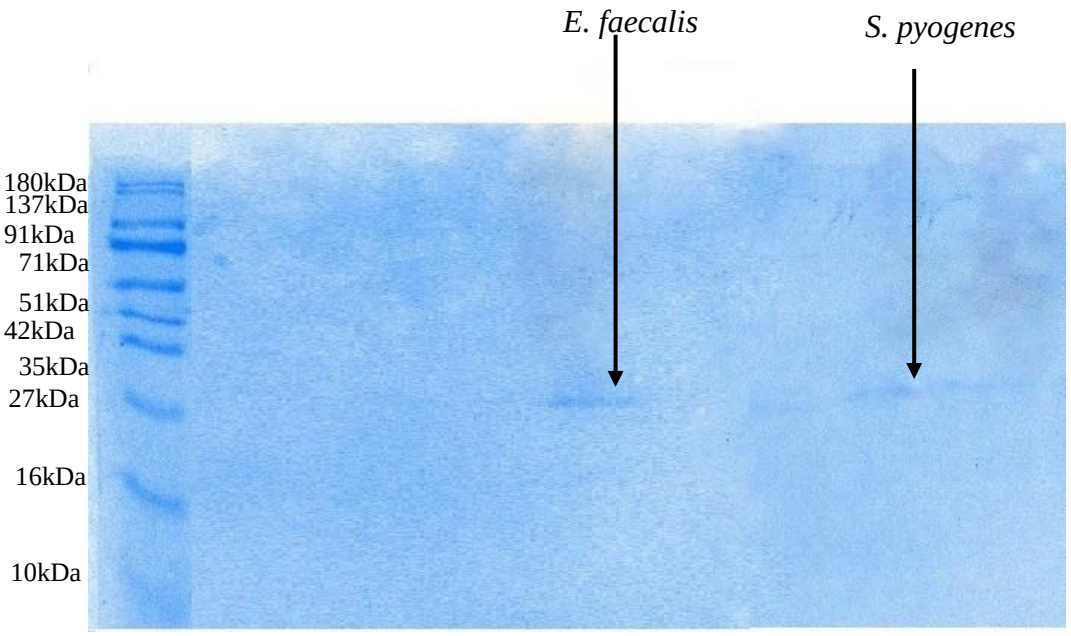


Figure 4.5. SDS-PAGE result with the very light smeared band (treatment with resins)

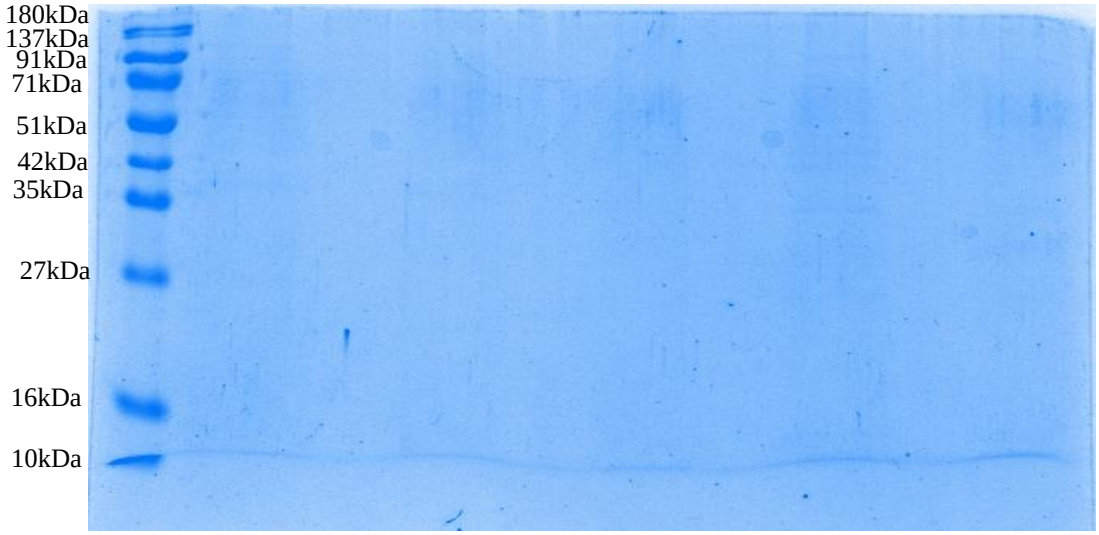


Figure 4.6. SDS-PAGE result with no band (treatment with resins)

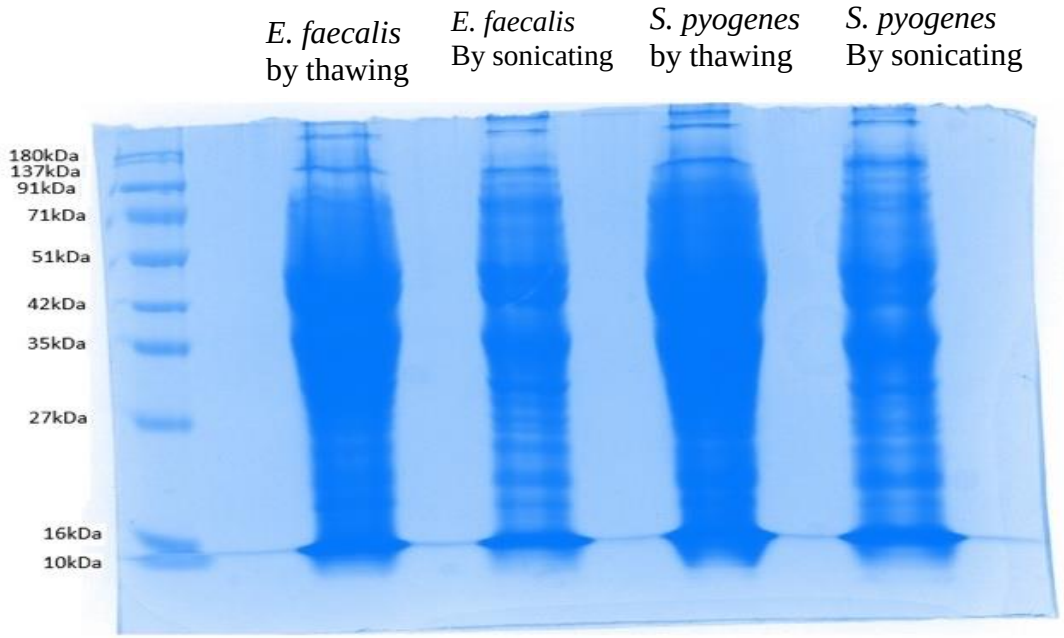


Figure 4.7. SDS-PAGE results with the non-differentiable band. (Treatment without Resins)

The bands on SDS PAGE gel were observed. Only a light smeared band was seen in PK of *E. faecalis* whose molecular weight was close to 27 kDa in comparison to the ladder. Again, in figure 4.6 no bands were found for the PK of *E. faecalis* and *S. pyogenes*, where as in figure 4.7 non-differentiable bands were seen for both *E. faecalis* and *S. pyogenes*.

CHAPTER 5

DISCUSSION

S. pyogenes and *E. faecalis* were the only two recombinant *E.coli BL21 (DE 3)* strains carrying PK genes, which were giving the positive results even after repeating the experiments again and again. Whereas in case of *L*. *lactis* and *L. plantarum* no growth was observed. Through this, we concluded that in both the recombinant *E.coli* strains recombinant plasmid were not transformed properly, or plasmids were lost during growth in multiple generations. In the case of recombinant PK of *S. pyogenes* and *E. faecalis,* it looks that empty plasmid pET 30 could got transformed into the *E. coli* BL21 (DE3) strains those were growing on kan antibiotic. The second reason could be that these proteins are toxic to the host strain, so it doesn't over-express these proteins. The third reason could be these proteins are difficult to over-express in the *E. coli* host strain.

CHAPTER 6

CONCLUSION

Comparison of Pyruvate Kinase among different strains of the LAB must reflect the similarity and differences at the level of expression of enzymes and also the rate of metabolism. Unfortunately, we couldn’t complete the kinetic characterization to make kinetics models due to the negative result (i.e. no target protein expression) but still have managed to handle different instruments which enhanced our knowledge of the better experimental design of the proposed project.

REFERENCES

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

Fisher, K. and 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.

Garcia-Olalla, C. and Garrido-Pertierra, A. (1987). Purification and kinetic properties of pyruvate kinase isoenzymes of *Salmonella typhimurium*. *Biochemical Journal* **241**(2): 573-581.

Israelsen, W. J. and Vander Heiden, M. G. (2015). Pyruvate kinase: function, regulation and role in cancer. In *Seminars in Cell & Developmental Biology* **43**:43-51.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **49**: 209-224.

Kenneth, T. (2011). *Bacterial Protein Toxins*. Kinase in Lactic Acid Bacteria. *PLoS Computational Biology* **9**(7): e1003159.

Kleerebezem, M., Hols, P. and Hugenholtz, J. (2000). Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. *Enzyme and Microbial Technology* **26** (2000) 840-848.

Lunt, S. Y., Muralidhar, V., Hosios, A. M., Israelsen, W. J., Gui, D. Y., Newhouse, L. and Hollern, D. P. (2015). Pyruvate Kinase isoform expression alters nucleotide synthesis to impact cell proliferation. *Molecular Cell,* **57**(1):95-107.

Munoz M.E. and Ponce, E. (2003). Pyruvate kinase: current status of regulatory and functional properties. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **135**(2):197-218.

Paliy, O. and Gunasekera, T. S. (2007). Growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources and salt contents. *Applied Microbiology and Biotechnology* **73**(5): 1169-1172.

Ramos, A., Neves, A. R., Ventura, R., Maycock, C., Lopez, P. and Santos, H. (2004). Effect of pyruvate kinase overproduction on glucose metabolism of *Lactococcus lactis*. *Microbiology* **150**(4): 1103-1111.

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

Siezen, R. J. and Van HylckamaVlieg, J. E. (2011). Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. In *Microbial Cell Factories* **10**(1): 3-10.

Valentini, G., Chiarelli, L., Fortin, R., Speranza, M. L., Galizzi, A. and Mattevi, A. (2000). The allosteric regulation of pyruvate kinase: A site-directed mutagenesis study. *Journal of Biological Chemistry* **275(**24): 18145-18152.

Veith, N., Feldman-Salit, A., Cojocaru, V., Henrich, S., Kummer, U. and Wade, R. C. (2013). Organism-adapted specificity of the allosteric regulation of pyruvate kinase in lactic acid bacteria. *PLoS Computational Biology* **9**(7): e1003159.

Wessels, S., Axelsson, L., Hansen, E. B., De Vuyst, L., Laulund, S., Lahteenmaki, L., Sven Lindgren, S., Mollet, B., Salminen, S. and Wright, A. (2004). The lactic acid bacteria, the food chain, and their regulation. *Trends in Food Science & Technology* **15**(10): 498-505.

Zhu, T., Bailey, M.F., Angley, L.M., Cooper, T.F. and Dobson R.C. (2010). The quaternary structure of pyruvate kinase type 1 from *Escherichia coli* at low nanomolar concentrations, *Biochimie* **92**(1):116-120.

APPENDICES

APPENDIX A

Sr. No.	Stock Solution	Methods of Preparation
1	Luria Bertani (LB) Broth	20 g of Luria Bertani broth was dissolved in 1 L of Distilled Water.
2	Agar	2.5 g of agar was dissolved in 100 ml of LB broth for plating.
3	Antibiotics (Ampicillin and kanamycin 50mg/ml)	250 mg of kanamycin and ampicillin were dissolved in 5 ml of distilled water separately using disc filter and stored in -20° C.
4	IPTG (Isopropyl β-D-1-thiogalactopyranoside)	2.4 g of IPTG was dissolved in 10 ml of distilled water and stored at -20° C.
5	Tris-Cl Buffer (0.5 M)	60.5 g of Tris-HCL, 90 g of NaCl and 36 µl of β-Mercaptoethanol was dissolved in 900ml of distilled water and make up the final volume 1000 ml, adjust the pH = 7.0 using concentrated HCl.
6	Washing/suspension buffer (50 mM)	100 ml of tris-HCl buffer was mixed in 900 ml of distilled water.
7	150 mM Imidazole	5.1 g Imidazole was dissolved in 500 ml of washing buffer.
8	20 mM Imidazole	27 ml of 150 mM Imidazole was mixed with 200 ml 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 30 ml of Acrylamide and bisacrylamide solution in 100 ml of distilled water. 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 9.085 g of Tris base in 20ml distilled water. Adjust the pH using conc. HCl. Make final volume 50ml using distilled water
12	10% APS	1.5 ng in 15 ml
13	10% SDS	1.5 g in 15 ml
14	0.5M Tris-Cl (pH6.8)	Dissolve 3.02 g of Tris base in 20 ml distilled water. Adjust the pH using conc. HCl. Make final volume 50 ml using distilled water.

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:

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

To prepare running buffer dissolve 30.2 g Tris base, 150 g glycine and 10 g 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:

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

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

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

URKUND

Urkund Analysis Result

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