IN-VITRO ASSAY OF BACTERIOPHAGE AGAINST *Salmonella* IN RAW FOOD PRODUCTS

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Letter of Recommendation

This is to certify that the research work entitled "**IN-VITRO ASSAY OF BACTERIOPHAGE AGAINST *Salmonella* IN RAW FOOD PRODUCTS**" was successfully carried out by Mr. Yujeen Chapagain under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

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Certificate of Evaluation

This is to certify that this thesis entitled **"IN-VITRO ASSAY OF BACTERIOPHAGE AGAINST *Salmonella* IN RAW FOOD PRODUCTS"** presented to evaluation committee by Mr. Yujeen Chapagain is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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ACRONYMS

^{o}C	:	Degree Celsius
ABR	:	Antibiotic Resistance
AMR	:	Antimicrobial Resistance
AST	:	Antibiotic Sensitivity Test
ATCC	:	American Type Culture Collection
ATP	:	Adenosine Tri-Phosphate
BLAST	:	Basic Local Alignment Search Tool
BLASTN	:	Basic Local Alignment Search Tool – Nucleotide
bp	:	base pairs
CBD	:	Cell Binding Domain
CDC	:	Centers for Disease Control and Prevention
CFU	:	Colony Forming Unit
CRISPR	:	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	:	CRISPR Ribonucleic Acid
dL	:	deciliter
DLAA	:	Double Layer Agar Assay
DNA	:	Deoxyribonucleic acid
dsDNA	:	Double stranded Deoxyribonucleic Acid
dsRNA	:	Double stranded Ribonucleic Acid
EDTA	:	Ethylenediaminetetraacetic acid
FDA	:	Food and Drug Administration
gDNA	:	Genomic DNA
H_2S	:	Dihydrogen Sulphide/ Hydrogen Sulphide
HIV	:	Human Immuno-deficiency Virus

ICTV	:	International Committee on Taxonomy of Viruses
KOH	:	Potassium Hydroxide
LB	:	Luria Bertani
LPS	:	Lipo-Polysaccharide
MDR	:	Multidrug Resistance
MHA	:	Muller Hilton Agar
ml	:	Milliliter
mmol/L	:	Milimole per Litre
mol/L	:	Mole per Litre
MR	:	Methyl Red
MOI	:	Multiplicity of Infection
NA	:	Nutrient Agar
NaCl	:	Sodium Chloride
NCBI	:	National Center for Biotechnology Information
nm	:	Nanometer (10^-9 meter)
OD	:	Optical Density
OD600	:	Optical Density at 600nm
PFU	:	Plaque Forming Unit
PG	:	Peptidoglycan
RNA	:	Ribonucleic Acid
RTE	:	Ready to Eat
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SM buffer	:	Sodium Magnesium buffer
ss DNA	:	Single stranded Deoxyribonucleic Acid
ss RNA	:	Single stranded Ribonucleic Acid

TAE	:	Tris-Acetate EDTA buffer.
TE	:	Tris-Chloride EDTA buffer.
TSA	:	Tryptic Soya Agar
TSB	:	Tryptic Soya Broth
TSIA	:	Triple Sugar Iron Agar
ul	:	microliter
UTI	:	Urinary Tract Infection
UV	:	Ultraviolet
VAD	:	Ventricular Assist Devices
VP	:	Voges Proskauer
WHO	:	World Health Organization

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Use of chemical agents for decontamination of food and poultry products has degraded the quality, texture and nutritional values of these foods and the chemicals have presented some serious health and environment hazards. Most common food and poultry borne pathogens include *Salmonella, Escherichia, Listeria,* etc. Bacteriophages or phages are natural predators of bacteria which are specific to their host and hence can be used against these bacteria. Use of bacteriophage as antimicrobial does not possess any health and environmental hazards. This study aims to find out if bacteriophage can be used for decontamination of *Salmonella* in food products (Sausage and Potato).

Salmonella-Shigella (SS) agar was utilized for the isolation of bacteria (*Salmonella*) which were confirmed by biochemical tests. Double Layer Agar Assay (DLAA) was performed to isolate phage particles from sewage samples. Phage streak was utilized for phage purification and suspension of phage in Sodium-Magnesium (SM) buffer was used as stock. Different concentration of metal ions were prepared taking human blood level of those ions in consideration and activity of phage in presence of those metal ions was identified by studying bacterial growth reduction at Optical Density (OD) 600. Activity of phage to diminish bacterial load in potato and sausage was studied by inoculating those foods with phage and bacteria at different MOI at room temperature for 4 hours and studying bacterial load afterwards.

Total nine isolates of *Salmonella* were isolated from poultry and river water samples and identified by biochemical tests. The Antibiotic Susceptibility Test (AST) pattern showed isolate S9 being resistant to third generation antibiotics (Cephalosporins). Four phages were isolated and phage P9 was found to be most effective against our MDR *Salmonella* S9. Phage P9 showed high reduction in bacterial load even at low MOI (0.001). Phage P9 was found to be active in pH range of 3-11 and active below temperature of 70^{o}C. The burst size was found to be 7 phages per infected bacteria. The phage showed increased activity in presence of calcium and iron ions while magnesium ion in higher concentration reduced phage activity. Phage P9 in presence of above metal cocktail showed large decrease in bacterial load (0.011 OD compared to 0.066 OD in absence of metal cocktail).

Similarly, on 4 hours incubation at room temperature, phage P9 showed more than 1.04 $\log_{10}$ CFU reduction in bacterial load in sausage at MOI 1 and above while 1.15 $\log_{10}$ CFU reduction in potato was seen in only MOI 10. This indicates that isolated phage P9 can have potential use in bio-control of *Salmonella* in raw foods.

In conclusion, phage P9 showed specific activity against *Salmonella* isolates. P9 also showed high pH tolerance and was active in temperature up to 70^{o}C. The activity of phage was greatly enhanced by the presence of metal ions in solution. Also, it can be concluded that phage P9 can be a potential bio-agent to decrease *Salmonella* load in food. Further study of its potential utilization in therapeutic purposes can also be done.

[Keywords: *Salmonella, Bacteriophage,* MDR, AST, metal cocktail, food products]

CHAPTER 1: INTRODUCTION

1.1. Background

1.1.1. Salmonella

Salmonella spp. has been regarded as an important foodborne pathogen and a major public health burden worldwide. There are more than 40,000 cases of salmonellosis in United States every year (CDC, 2016) and non-typhoid salmonellosis is the second most common cause of foodborne zoonotic infection in Europe (European Food Safety Authority- EFSA, 2019). Most human salmonellosis is caused by two common serovars of *Salmonella* viz. *Salmonella enteritidis* and *Salmonella typhimurium*. Most of the *Salmonella* infection starts from animal origin (mostly poultry meats and their by-products). Further contamination of *Salmonella* in other food products is due to poultry production chains and through the faeces, which can contaminate various water sources or the faeces might be used as fertilizers which allows transmission of *Salmonella* in food (Wiedemann et. al., 2015). Various chemical agents can be used to remove the bacterial contamination from food and meat surface, but the chemicals used can have unknown side effects thus hampering human health. Such use of chemicals is not allowed in European Union.

There is now prevalence of multidrug resistant (MDR) strains of Salmonella which can be a great threat to human health if solution for them can't be found. Bacteriophages are natural predators of bacteria and can be used to combat even the MDR strains. These phages are safe for use as they do not possess any health or environmental hazards (Verheust et. al., 2010). Application of such phages on the surface of food and meat products can cause drastic decrease in levels of bacteria in the surface thus promoting healthy life (Sillankorva et. al., 2012).

1.1.2. Decontamination of Food and Poultry Products

The global increase in the occurrence of foodborne infection and antibiotic resistance indicates that a new approach is necessary to deal with the ever-changing and ever-evolving bacterial strains. .“Antimicrobial Resistance (AMR) is an increasingly serious threat to global public health that requires action across all government sectors and society” (WHO Antimicrobial resistance fact sheet, 2016). Also, decontamination of food products and poultry carcasses are of major concern. Irradiation, water and steam based treatments are frequently used to decontaminate poultry carcasses (Buncic and Sofos, 2012; Mukhopadhyay and Ramaswamy, 2012). Several chemicals are also used in many countries to reduce microbial load in poultry carcasses during slaughter and processing.

Such chemicals include trisodium phosphate, chlorine-based compounds and organic acids which later on can have negative impacts on human health and environment (Dincer and Baysal, 2004).

Use of bacteriophage in bacterial decontamination can solve issues of both MDR bacteria and chemical use. Bacteriophage can effectively kill target bacteria in poultry carcasses and food surface without any negative impact on human health and environment. Being specific, they can also kill concerned MDR bacteria from food and poultry products thus decreasing our dependency on antibiotics or chemicals.

1.1.3. Antimicrobial, Antibiotic resistance and alternatives

Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Bacteria become antibiotic resistant on prolonged exposure to antibiotics. These bacteria may then infect humans and become harder to treat than non-resistant bacteria. Antimicrobial resistance is a broader term, encompassing resistance to drugs to treat infections caused by other microbes as well, such as parasites (e.g. malaria), viruses (e.g. HIV) and fungi (e.g. Candida).

Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. AMR occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death. As a result, the medicines become ineffective and infections persist in the body, increasing the risk of spread to others.

Antimicrobial resistance (AMR) has possessed a greatest challenge for effective treatment of infections globally, which is why it has become a growing concern to human, animal, and environment health. Excessive use of antibiotics, antibiotics sold over the counter, and increased release of non-metabolized antibiotics or their residues into the environment through manure/feces are the major causes of AMR (Aslam et. al., 2018). In developing countries, another cause of antimicrobial resistance is non-human use of antimicrobials such as to prevent and treat disease in animals, used as growth promoters in animal breeding and as additives in plant agriculture.

Multidrug-resistant (MDR) bacteria or “superbugs” are evolving at such a rate that the speed of production of novel antibiotics cannot keep up with the rate at which bacteria are developing and spreading resistance. WHO in its latest report of 2017 has clearly declared that the world is running out of antibiotics and therefore, the search for an

alternative is a matter of urgency (World Health Organization, https://apps.who.int/iris/handle/10665/258965, 2017).

Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these antibiotics. Antibiotic resistance leads to higher medical costs, prolonged hospital stays, and increased mortality. The world urgently needs to change the way it prescribes and uses antibiotics. Even if new antibiotics are developed, without behavior change, antibiotic resistance will remain a major threat. Behavior changes must also include actions to reduce the spread of infections through vaccination, hand washing, practicing safer sex, and good food hygiene (WHO, https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance, 2020).

Antibiotic resistance is rising to high levels in all parts of the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. A growing list of infections – such as pneumonia, tuberculosis, blood poisoning, gonorrhoea, and foodborne diseases – are becoming harder, and sometimes impossible, to treat as antibiotics become less effective. Where antibiotics can be bought for human or animal use without a prescription, the emergence and spread of resistance is made worse. Similarly, in countries without standard treatment guidelines, antibiotics are often over-prescribed by health workers and veterinarians and over-used by the public. Without urgent action, we are heading for a post-antibiotic era, in which common infections and minor injuries can once again kill (WHO, https://www.who.int/news/item/20-09-2017-the-world-is-running-out-of-antibiotics-who-report-confirms, 2020).

In searching alternative tools to treat infections, scientists from different parts of the world came up with different alternative tools such as bacteriophage therapy, predatory bacteria, bacteriocins, and competitive exclusion of pathogens all of which are highly specific in their action than antibiotics (Allen, 2017). Among all alternative options, bacteriophage therapy is among the most heavily researched and has the longest history. Phages in therapeutics have been investigated for over a century, now it has been developed as a revitalized therapy. Many researches regarding the use of phages in therapeutics are ongoing which has given a hope that phages can be a potent solution to battle the war of antibiotic resistance. For the phages to be commercialized as therapeutics, more insights into phages and their mechanism of infecting bacteria is to be studied. Using phages as cocktails to make the treatment more effective is one of the highlighted topics in phage therapy (Chan et. al., 2013).

1.1.4. Bacteriophage and its Application

Bacteriophages or phages are viruses containing either RNA or DNA covered by an external protein called capsid. They are generally icosahedral in shape. They have tail fibres which are capable of recognizing surface protein in the host and thus helps the bacteriophage to adhere to the host cell. As the name suggests, the host of a bacteriophage is a bacteria. The phage then inserts its genetic material into the host cell and replicates inside. The viral capsid and nucleic acid are synthesized and new viral particle is formed which are then released from the bacterium by lysis of cell wall.

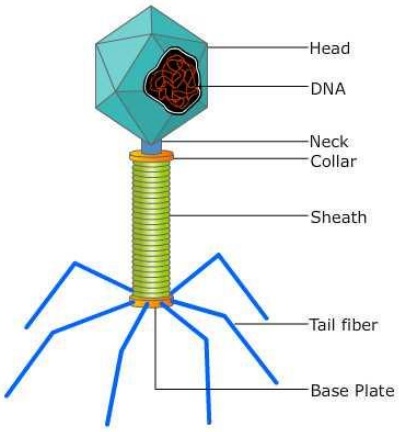


Fig 1.1: Structure of Bacteriophage

Phages are of two types: lytic and lysogenic and they have lytic and lysogenic cycle of infection.

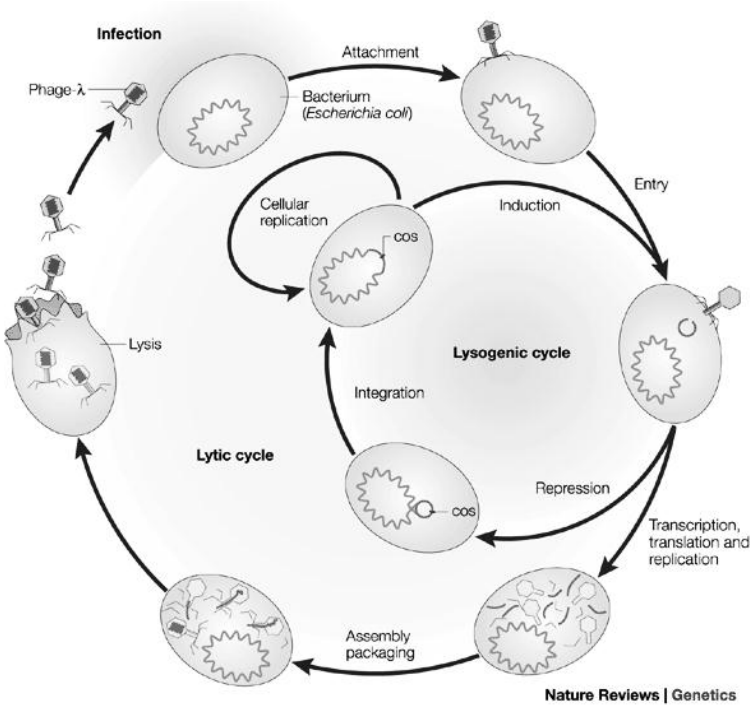


Fig 1.2: Life cycle of Bacteriophage

As shown in figure 1.2, in lytic cycle, phage infects the host, multiplies in it and lyses the host to release phage particles. In lysogenic cycle, phage DNA is integrated into the host genome and multiplies as a temperate phage. At some point, the integrated phage DNA starts manufacturing phages and finally kills the bacterium to release phage particles.

The widely used technique for the isolation of phage in laboratory is Double Layer Agar Assay (DLAA) method which is shown in figure 1.3. In this method, a hard layer serves as a base layer and the mixture of phage particles, host cell and soft agar forms the upper overlay in the petri plate. When the plate is incubated, host bacterium form a confluent growth and phages attached with the host lyses the bacterium releasing new phage particles which then infect neighboring bacterium. The spread of virus to the whole plate is limited by gel. The area in which phage kills the bacterium is seen as a clear plaque and hence this method is also called "plaque assay". The individual plaque in the plate is supposed to result from a single plaque forming unit and hence the plaque count gives the number of plaque forming units (PFU) in the original phage culture.

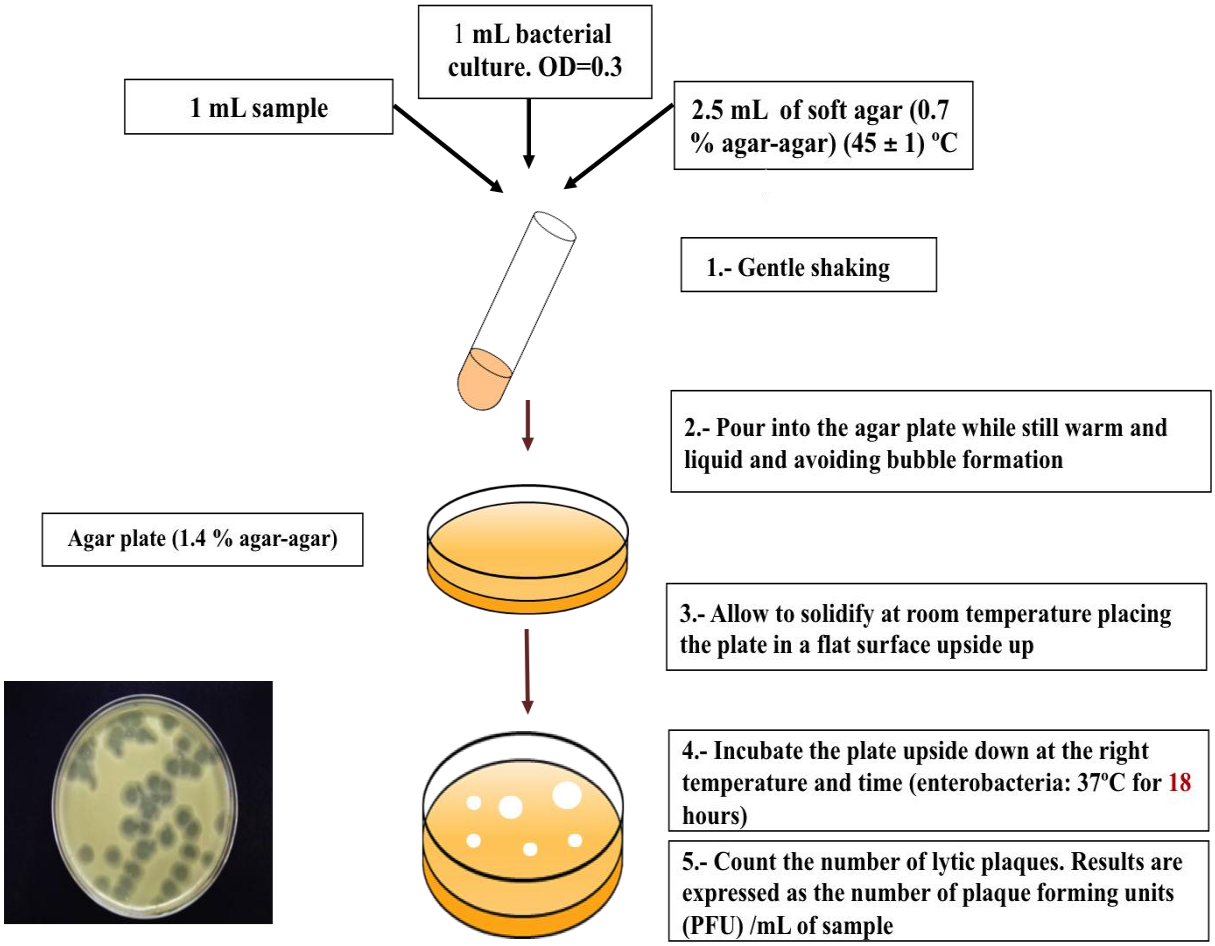


Fig 1.3: DLAA technique for phage isolation and culture

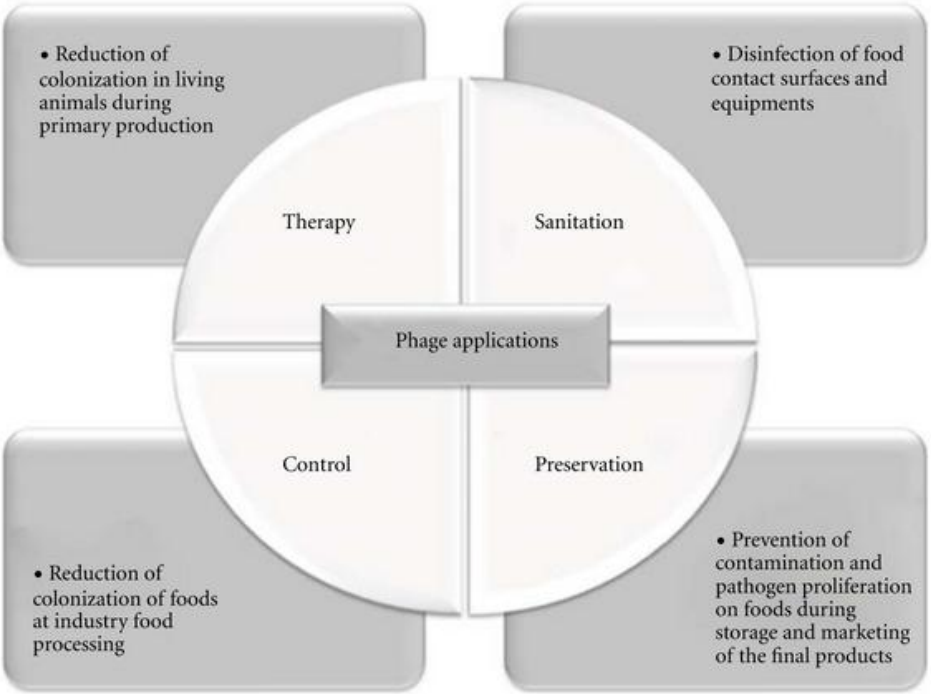


Fig 1.4: Application of Bacteriophage

Phages have wide variety of applications ranging from bio-control to therapeutics as shown in figure 1.4. In bio-control, phages can be used to reduce bacterial colonization of food. Phages can be used to prevent contamination and proliferation of pathogens on foods during storage and delivery. Phages can also be used for disinfection of food contact surfaces and equipments. In therapy, phages can be used to reduce bacterial colonization in living animal's body. Phages can also be used for the identification of strains of same organisms and this technique is called phage display. The therapeutic application of phage is linked with its ability to kill MDR bacteria which are a major health concern globally. Phages can also be used as decontaminants in food and equipments. They can be used to substitute antibiotics or vaccines in the future with extensive study and this process of phage therapy will be much cost effective than antibiotics.

1.1.5. Classification of Bacteriophage

Virus classification is based on characteristics such as morphology, type of nucleic acid, replication mode, host organism and type of disease. Over 40 criteria are engaged for phage differentiation into genera and species. Phages can be classified to a first approximation in terms of their genome type and virion morphology, with genome size representing an additional interesting means of distinguishing among phage. The International Committee on Taxonomy of Viruses (ICTV), latest scheme of classifying viruses, has produced an ordered system for classifying viruses. The ICTV taxonomic system requires visualization of the phage structure using electron microscopy. Nearly 5500 bacterial viruses have been characterized by electron microscopy. As of ICTV 2019 update, there are 22 families of phage that infect bacteria and archaea. Phages are found in a variety of morphologies: tailed phages, polyhedral phages, filamentous phages, phages with a lipid-containing envelope and phages with lipids in the particle shell. They have a genome, either DNA or RNA, which can be single or double stranded.

The genome contains information on the proteins that constitute the particles, additional proteins that are responsible for switching cell molecular metabolism in favor of viruses. The genome can be one or multipartite and is located inside the phage capsid. The shape of viruses is closely related to their genome, and a large genome indicates a large capsid and therefore a more complex organization. The most studied group of phages are the tailed phages of order Caudovirales.

1.1.6. Limitations of phage therapy

Being very specific, a single phage can't be utilized to get rid of variety of bacteria at once but this property of phage is useful in the sense that this therapy will not harm any helpful bacteria in the gut of human beings. Also, some phage contain integrase enzyme which enables it to integrate into host genome. This integration can lead to horizontal transfer of virulent genes in the phage genome and can be deadly when used in therapy. Thus, it is necessary to study phage genome for toxicity and lysogeny before its application on humans and phages with such property should not be used in therapy (Loc-Carillo & Abendon, 2011).

Another limitation of phage therapy is that there are increasing cases of phage resistance as well. Bacteria can have inherent phage resistance mechanisms that include Abortive infection mechanisms (ABIs), restriction methylation mechanisms (RM) and CRISPR-Cas systems. Bacteria may also acquire phage resistance through mutation when they are exposed to large number of similar phages.

1.2. Current Studies

With the quick rise of MDR microbes worldwide, the Western world has created brief interest in phages as option in contrast to antibiotics. In spite of the fact that bacteriophages can be appropriate in different fields, current examinations on bacteriophages center around clinical utilization of phages as helpful devices to treat bacterial diseases. Current studies in phage include:

- Application of phage for treating microbial infection in critical conditions. US clinical trial of intravenously regulated bacteriophage treatment has gotten FDA endorsement, for the treatment of members with ventricular assist devices (VADs) infected by resistant *Staphylococcus aureus*. Recently, various clinical trials have been enlisted; chronic otitis, infected burn wounds, diarrhea, UTIs, gastrointestinal disorders and others (Furfaro, et al., 2018).
- Utilization of phage as disinfectant in laboratory and contamination control.
- Study of host-phage interaction to develop similarly interacting drug molecules.
- U.S. Food and Drug Administration (FDA) has approved some phages mixture like SalmoFresh™ and PhageGuard S™ to help stop the growth of bacteria in food.
- First phage therapy center was started at University of California, San Diego, with the mission of running clinical trials in 2018.
- Phage Therapy Unit (PTU) was established in 2005 at the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland, which is regularly

working for phage therapy treatment in Europe and is one of Europe's first ethically approved treatment facility utilizing phage (Zaczek et. al., 2020).

- The European Medicines Agency (EMA) in 2015 held a workshop with participation of different scholars, universities and concerned stakeholders to identify possibilities for the development of bacteriophage-based therapies against bacterial infections (European Medicine Agency, 2015).
- Works have been done for the preparation of phage-displayed vaccines or phage DNA vaccines (Bao et. al., 2019).
- Phage4Cure program is being conducted in Germany which aims to study the utilization of phage against *P. aeruginosa* in chronic airway infection.

1.3. Research Hypothesis

1.3.1. Null Hypothesis, H0:

Metal ions do not enhance the activity of bacteriophage and bacteriophage cannot be used against pathogen in raw food products.

1.3.2. Alternative Hypothesis, H1:

Metal ions enhance the activity of bacteriophage and bacteriophage can be used against pathogen in raw food products.

1.4. Objectives

1.4.1. General Objectives

- To study the effect of metal ions in bacteriophage activity and potential use of bacteriophage to reduce *Salmonella* load in raw food products.

1.4.2. Specific Objectives

- To isolate *Salmonella* determine its antibiotic susceptibility pattern.
- To isolate bacteriophages against MDR *Salmonella* and study their lytic effect.
- To determine host range of individual phage using different bacterial isolates.
- To determine MOI, burst size, pH and temperature sensitivity of phages
- To assess the activity of phage in presence of different metal ions.
- To evaluate bacterial load reduction in raw food using phage.

1.5. Rationale of the Study

The increasing numbers of multidrug resistant bacteria to existing antibiotics add serious challenge to human health. Diseases that were easily treatable few years back are now difficult to treat or even some are untreatable with the similar therapeutic agent. Bacteria are evolving more rapidly with an unusual spread of resistance genes resulting in the outbreak of superbugs. It is a known fact that resistance genes of last resort antibiotics have been spreading worldwide (Reese et al., 2011).

Phages are anti-bacterial agents which multiply at the site of the infection until there are no more bacteria. In the past, use of phages as antimicrobial agents was limited due to lack of knowledge and sophisticated instruments to study about bacteriophage (Donlan, 2009). But now, various researches have been done and many advantages of using phages in therapy have been studied. Using single phages to treat specific infection has a chance to develop phage resistance so the concept of phage cocktails in therapy has been recently developed.

Analysis of phage activity, in presence of metal ions has also been studied quite extensively in the past. This study aims to focus on the study of phage activity in presence of 3 metal ions (Calcium, Magnesium and Iron) which are abundantly present in the human blood. Study of phage activity in the presence of such metal ions helps us to study the effects the human blood might have on phage activity. Also, we can administer phages in presence of activity enhancing metal ions to get better results during study of phage in-vivo.

Food borne bacterial infections are very common in both developing and developed countries of the world and there has been search for natural or biological way to fight these food borne bacteria rather than using antibiotics. This study focuses on utilizing bacteriophage for utilization in load reduction of *Salmonella spp.* in two raw food products; potato and sausage.

CHAPTER 2: LITERATURE REVIEW

Bacteriophages or phages are bacterial viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and cause the bacterium to lyse (Sulakvelidze et. al., 2001). They are ubiquitous in nature and are natural enemies of bacteria. They use the bacterial cell to replicate and multiply. They are commonly of two types: lytic and lysogenic phages. Lytic phages invade bacterial cell and multiply inside bacteria and later burst out of bacteria thus killing the bacteria in the process. Lysogenic phages however, integrate their DNA into bacterial genome and exist as prophage, which allows the phage to multiply synchronously with the host bacterium. Distinct phage isolates achieve antibacterial activity by differing in their mechanism of entry or strategy of replication (Young, 1992).

2.1. Bacteriophage Discovery

Ernest Hankin, in 1896, reported the presence of antibacterial agent in the water of Ganges and Yamuna river in India against strains of *Vibrio cholerae,* which was an epidemic at that time (Wittebole et al., 2014). He used porcelain filters to retain suspension of water which could kill the strains of *Vibrio cholerae* and concluded that the particles in that water might be responsible for the decreased cases of cholera in the villages around the river (Abendon et. al., 2011).

Bacteriophages or phages are thought to be one of the most ancient species in the earth and are able to infect and kill bacteria (Clokie et al., 2011). Although Ernest Hankin found presence of bacteriophage in water of Ganges and Yamuna, he was not able to isolate and truly discover phages. He simply suggested the presence of certain particles able to lyse cholera causing bacteria. Officially, bacteriophage was discovered by Felix d'Herelle, a French-Canadian microbiologist at Institute Pasteur in Paris in 1910. He discovered them while testing fecal filtrates from soldiers infected with *Shigella.* During culture, he found kill zones on culture plates and proposed them as 'ultraviruses'.

Frederick Twort, a British bacteriologist, in 1915 also found bacterial lysing agent while working with *Micrococcus* cultures but did not hypothesize them to be viruses. Later, most scientists accepted the discovery of phages by both Frederick Twort and Felix d'Herelle and called it the 'Twort-d'Herelle phenomenon' which was later termed as 'Bacteriophage phenomenon'.

2.2. MDR Bacteria

Bacteria defend themselves from the environment by using many different regulatory pathways, molecular machinery, shielding and by rapidly evolving. In MDR strains these defense mechanisms are the top of the line and constantly upgraded. The key to the rapid increase of MDR bacterial strains lies in the bacterial capability to rapidly reproduce and adapt with the ability to incorporate useful genes into their plasmid DNA repertoire almost effortlessly (Alberts et al., 2008; Reese et al., 2011). These genes are referred to as virulence factors, which include genes residing in plasmids, transposons, pathogenicity islands and lysogenic bacteriophages (Bae et al., 2006). As a consequence of careless use of powerful antibiotics for decades, we have provided the perfect environment i.e. breeding grounds for the bacterial species to evolve and to gain resistance against even the most potent antimicrobial drugs. Nowadays, the antibiotic resistance is no longer uncommon outside hospital settings. Because of this the risk of spreading the antibiotic resistance to new strains due to horizontal gene transfer and transfer of pathogenicity and virulence factors is imminent and will cause severe problems in the near future (Alibayov et al., 2014).

2.3. Interaction of Phage and its Bacterial host cell

The life cycle of phage dictates its role in bacterial and archaeal biology (Campbell, 1988). Three life cycles of phages have been reported: lytic, lysogenic and chronic phages. In general, once a virulent phage (a phage that follows the lytic cycle) has attached to its host cell, the phage's nucleic acid enters the cell and causes the bacterium to produce hundreds of phage copies. This results in the lysis of the cell and the newly formed phages are released into the surrounding environment.

Temperate phages (phages that follow the lysogenic cycle) may follow one of two scenarios. The first scenario results in the lysis of the host cell and release of newly formed phages, similar to the lytic life cycle outlined above. In the second scenario, phage DNA may be integrated into the bacterial chromosome. The integrated DNA (prophage) is non-infectious and replicates as part of the bacterial chromosome. Incorporation of the phage DNA into the bacterial chromosome can be beneficial for the evolution of the bacteria as useful genes may be transferred to the bacteria (Campbell, 1988). These prophage-mediated changes have been termed lysogenic conversion. In this state of symbiosis, both phage and the host cell experience an increased level of fitness. Under UV light or certain chemical treatments, the prophage is excised and causes the bacteria to produce phage particles.

The third life cycle is the chronic lifecycle which occurs in archaeal viruses and some filamentous and temperate phages. These viruses do not cause cell disruption or cell death, but instead the newly formed virions are continuously released from the cell. The infected host cells are capable of growing but at a much slower rate (Munson-McGee et. al., 2018).

Generally, the infection process begins with the phage attaching to the surface of the host cell via particular host cell surface receptors. As a consequence of infection, the genetic material of the phage is injected into the cytoplasm of the bacterial cell. The initiation of phage infection is triggered by the specific recognition between the phage's RBPs located at the tip of the tail and a receptor located on the surface of the host cell. This specificity is directly related to the specificity of adsorption, which correlates to the structure of receptors located on the host's cell surface (Bertozzi et. al., 2016). The localization, volume and density of these receptors play a pivotal role in the recognition process. Cell surface receptors recognized by the phage may include protein receptors (OmpA and OmpC), lipopolysaccharide (LPS) receptors, receptors located in capsular polysaccharides (Vi-antigen), pili and flagella (Rakhuba et. al., 2015). Proteins that act as receptors for phages may carry out a variety of functions in the host cells (i.e., enzymes, transport proteins, structural proteins, porins and flagella). Once successful binding to the host receptor has occurred, a conformational alteration in the phage's baseplate occurs and consequently results in sheath contraction and injection of the phage's nucleic acid into the host cell.

To begin the replication process, a phage may first have to overcome a variety of carbohydrate boundaries present on the surface of the bacterial cell. These carbohydrate moieties include capsular polysaccharides which can mask the host cell receptors and extracellular polysaccharides that may be secreted during biofilm production (Labrie et. al., 2010). Phages have evolved a variety of carbohydrate active enzymes (polysaccharide depolymerases, a common component of the tail in bacteriophages that function to recognize, bind and degrade carbohydrate components and gain access to a once inaccessible host cell receptor. In accordance to their mechanism of action, phage depolymerases can either be hydrolases or lyases, each of which causes the breakdown of polysaccharides into soluble oligosaccharides. The vast majority of hydrolases are members of the O-glycosyl hydrolases group which function by using a water molecule to cleave the O-glycosidic bonds of the polysaccharide. To form soluble oligosaccharides, lyases cleave a glycosidic bond through β-elimination resulting in the introduction of a new double bond and, unlike hydrolases, they do not use water. Hydrolases include sialidases that breakdown capsular polysialic acid and rhamnosidases that hydrolase O-antigen of LPS. Lyases include pectin lyases which

degrade extracellular polysaccharides and hyaluronidases that degrade hyaluronate-based capsules (Fernandes et. al., 2018).

Following a lytic life-cycle, the newly formed phages within the bacterial cell must lyse the cell in order to release these virions into the surrounding environment. Tailed phages accomplish this lysis through the use of the phage-encoded enzyme endolysin and the protein holin (Latka et. al., 2017). Endolysins are peptidoglycan (PG) degrading enzymes synthesized during the late phase of gene expression in the lytic cycle. At a time where it is critical for lysis to occur, endolysins degrade the bacterial cell wall from within (Loessner et. al., 2002). The most commonly found catalytic domains in these enzymes have muramidase or amidase activity. For phages infecting Gram-negative bacteria, the endolysin is generally a monomeric and globular polypeptide. Endolysins of Gram-positive phages are usually modular in structure with the catalytic domain (N-terminal) connected to the cell binding domain (CBD) (C-terminal). Research carried out by Loessner and team on the phages A188 and A500 that infect the Gram-positive bacterium Listeria monocytogenes (L. monocytogenes) indicated that the CBDs of these phages function in directing the phage endolysins Ply118 and Ply500 to their substrates present on the bacterial cell wall. Endolysins are granted access to the bacterial PG through holins which oligomerize in the cytoplasmic membrane and thus create small pores in the membrane and allow the endolysins to reach their substrates (Eugster et. al., 2011). Degradation of the outer membrane of the bacterial host is usually required for lysis also. This is carried out by a spanin complex which is composed of an outer membrane lipoprotein (o-spanin) and an integral cytoplasmic membrane protein (i-spanin) (Berry et. al., 2012).

2.4. Phage as Antimicrobial agent

The ability of bacteriophage to undergo lytic cycle and kill a bacterial host makes it a potent antibacterial agent. It is believed that for every bacteria, there exists a phage against it. It is also notable that as bacteria evolve to gain resistance against phage, phages will also evolve to infect the resistant species of bacteria. Thus, phages will act as self-evolving antibiotics against bacteria. The evolutionary competition between phages and bacteria makes phages a potent drug against the bacteria.

2.5. Bacterial Resistance to Phages

Bacteria can evolve resistance to phages. These resistance mechanisms are manifested when an interruption occurs during phage development, through specific molecular mechanisms, which have evolved in bacteria throughout their coevolution with phages. Bacteria are able to defend against phage infection almost in every stage of the infection

process. By blocking phage receptors, producing an extracellular matrix and competitive inhibitors, bacteria prevent the phage from adsorbing to their surface. This is termed phage adsorption inhibition. Injection of the phage genome can also be inhibited through a process known as injection blocking (Coffey and Ross, 2002).

Phage inhibition can also occur after phage genome injection into a host as a result of bacterial-encoded endonucleases that recognise and destroy foreign DNA, a phenomenon known as restriction-modification. Bacterial protection of its own DNA is based on modification by methylation at specific points on its DNA sequence, which concomitantly will give protection against restriction endonuclease cleavage. Restriction results in the cleavage of foreign DNA that does not carry the corresponding methylation pattern.

There are four types of restriction modification systems: type I, type II, type III and type IV. All of these types have some restriction enzyme activity and a methylase activity (except type IV which do not have any methylase activity). The restriction modification systems were named in the order of discovery. Type II restriction modification system is the most common among the four types.

- Type I systems are considered the most complex restriction modification system. It consists of three polypeptides: R (restriction), M (modification), and S (specificity). The resulting complex can cleave as well as methylate DNA. Both of these reactions require ATP for completion and cleavage often occurs at a considerable distance from the recognition site. The S subunit determines the specificity of both restriction and methylation reaction. Cleavage occurs at variable distances from the recognition sequence, so discrete bands are not easily visualized by gel electrophoresis.
- Type II systems are the simplest and the most prevalent in nature. Instead of working as a complex, the methyltransferase and endonuclease are encoded as two separate proteins and act independently (there is no specificity protein). Both proteins recognize the same recognition site, and therefore compete for activity. The methyltransferase acts as a monomer, methylating the duplex one strand at a time. The endonuclease acts as a homodimer, which facilitates the cleavage of both strands. Cleavage occurs at a defined position close to or within the recognition sequence, thus producing discrete fragments during gel electrophoresis. For this reason, Type II systems are used in labs for DNA analysis and gene cloning.
- Type III systems have R (res) and M (mod) proteins that form a complex of modification and cleavage. The M protein, however, can methylate on its own.

Methylation also only occurs on one strand of the DNA unlike most other known mechanisms. The heterodimer formed by the R and M proteins competes with itself by modifying and restricting the same reaction. This results in incomplete digestion (Wilson, 1991).

- Type IV systems are not considered true RM systems because they only contain a restriction enzyme and not a methylase enzyme. Unlike the other types, type IV restriction enzymes recognize and cut only modified DNA (Loenen, 2013).

Some unmodified phage genomes physically avoid host-mediated restriction (possibly by encountering the methylase enzyme molecule in advance of meeting the endonuclease), and, on being replicated, their genome becomes modified. This enables resulting phage to evade restriction by a particular host restriction/modification system in subsequent infective cycles (Bohannan and Lenski, 2000).

Another mechanism of phage resistance termed abortive infection represents a broad range of diverse phage resistance mechanisms whereby the phage-infected cells often die before completing the lytic cycle, thus containing the virus and preventing it from proliferating. Abortive infection mechanisms frequently have a different primary function in bacteria.

CRISPRs (clustered regularly interspaced short palindromic repeats) are loci containing multiple, short direct repeats, which are found in the genomes of approximately 40% of sequenced bacteria and 90% of sequenced Archaea (Mojica et. al., 1995). CRISPRs function like a prokaryotic immune system in that they confer a form of acquired immunity to exogenous genetic elements such as plasmids and phages. Short segments of foreign DNA, called spacers, are incorporated into the genome between CRISPR repeats and serve as a “memory” of past exposures. CRISPR spacers are then used to recognise and silence exogenous genetic elements in a manner analogous to RNA in eukaryotic organisms. The mechanism of CRISPR/Cas interference involves three phases (Fig 2.1)

Overview of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) adaptive immunity. (a) Adaptation. The CRISPR arrays are composed of short repeats and intervening sequences derived from foreign invaders. Upon infection with a foreign element (e.g., phages), part of the genome is typically incorporated into the leader end of the CRISPR array and the repeat is duplicated. The CRISPR arrays are located adjacent to a cluster of Cas genes. (b) crRNA generation. The CRISPRs are transcribed into pre-crRNAs that are then processed into mature crRNAs. (c) Interference. The crRNA, in a complex with Cas proteins, binds and degrades the target nucleic acid of the invading element.

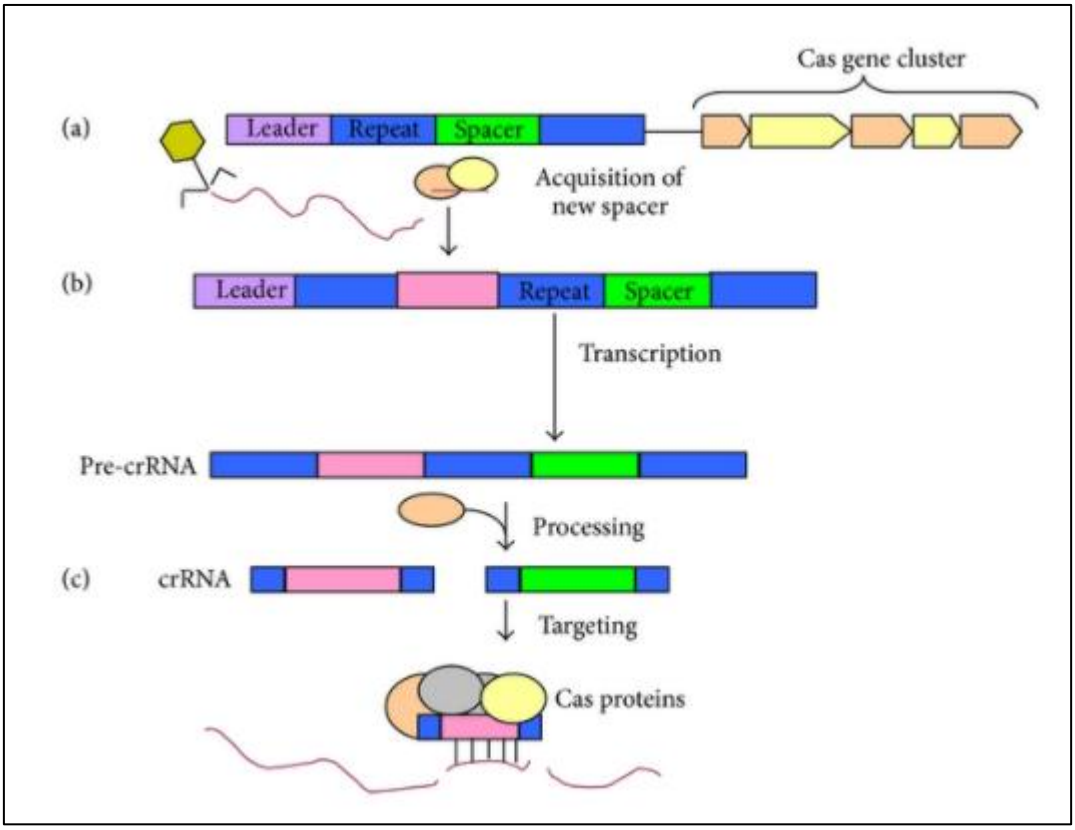


Fig 2.1: CRISPR mechanism

Firstly, resistance is acquired via the integration of short sequences from foreign genetic elements (termed spacers) into repetitive genetic elements known as CRISPR arrays. Secondly, CRISPR arrays are then transcribed and processed into small RNAs (crRNAs) by Cas proteins. In the third and final step, targeting of the invading phage or plasmid is mediated by a Cas protein complex that contains crRNAs. During this stage, the crRNA-Cas protein complex then interferes, in a sequence-specific manner, with the foreign nucleic acids (Makarova et. al., 2011).

2.6. Safety issues of Bacteriophage

Bacteriophages are very unique to their host (bacteria). They are so unique that phages can be used to identify different strains among the same bacterial species. Some phages however, have broad host range than others but phages cannot replicate in eukaryotic cells which makes them non-harmful/non-infectious for human cells and tissues. Some evidences have shown that bacteriophages interact with non-target cells and tissues of human. However, these interactions have no side effects recorded so far (Merril, 2008). When delivered by intravenous route, they are quickly removed from circulatory system and predominantly stored in liver and spleen. One major safety issue with bacteriophage

is that lysogenic phages can integrate their genome into host bacteria which can cause a normal bacterium to be pathogenic. Thus, it is very important to avoid lysogenic phages for phage therapy (Jassim and Limoges, 2014).

2.7. Salmonella Phage and use in raw food products

Various phages have been isolated against Salmonella spp and have been used for reduction of Salmonella from various sources. Hungaro et. al. in 2013 studied the reduction of Salmonella in chicken skin by use of bacteriophage and compared the reduction with the reduction in Salmonella by use of chemical agents. He found out that MOI (Multiplicity of Infection) of 10 or more was required to significantly reduce the Salmonella count as that of chemical agent. He also found that most phages isolated lysed all the S. enteritidis used in the study.

Atterbury et. al. in 2007 studied on Bacteriophage therapy to reduce Salmonella colonization of broiler chickens. He isolated the phages against Salmonella and found that they reduced bacterial load significantly even at 100 MOI. More reduction was seen when the phages were given to chicken just prior to slaughter than after slaughter.

Huang et. al. in 2018 studied the Application of Bacteriophage LPSE1 Against Salmonella enterica in Ready to Eat (RTE) Foods in which they studied the application of bacteriophage in milk, sausage and lettuce. Out of 35 isolated phages, LPSE1 demonstrated a broad *Salmonella* host range, robust lytic ability, extensive pH tolerance, and prolonged thermal stability. The capacity for phage LPSE1 to control *Salmonella enteritidis*-ATCC13076 in milk, sausage, and lettuce was established. Incubation of LPSE1 at 28°C in milk reduced recoverable *Salmonella* by approximately 1.44 log10 CFU/mL and 2.37 log10 CFU/mL at MOI of 1 and 100, respectively, as relative to the phage-excluded control. Upon administration of LPSE1 at an MOI of 1 in sausage, *Salmonella* count decreased 0.52 log10 at 28°C. At MOI of 100, the count decreased 0.49 log10 at 4°C. Incubation of LPSE1 on lettuce reduced recoverable *Salmonella* by 2.02 log10, 1.71 log10, and 1.45 log10 CFU/mL at an MOI of 1, 10, and 100, respectively, as relative to the negative control.

2.8. Phage activity in presence of Salinity and Metal Ions

Osmotic shock has been shown to inactivate bacteriophages. Whitman and Marshall (1971) observed that psychrophilic Pseudomonas phages (wy and ps1) had reduced persistence in highly concentrated solutions of NaCl or sucrose. The phage ps1 diluted in 4 mol/L NaCl showed a 99% decrease in viability, while the viability of the phage wy was reduced by only 26%. However, a 2-mol/L sucrose solution caused a decrease in viability

of ps1 by 50% and of wy by 48%. The same investigators observed that in 0.1% citrate in soft agar medium, the viability of both phages was reduced by 30%.

Several bacteriophages were isolated from marine water of different salinities. Wichels et al. (1998) studied 22 phages which they found in water near Helgoland in the North Sea. All of them had tails and icosahedral heads of 50.2 to 99.3 nm, and they were classified into three different families: 11 phages to Myoviridae, 7 to Siphoviridae, and 4 to Podoviridae. No similarity in DNA structure was shown among phages belonging to different families present in this area. Also, Hidaka (1971) tested the stability of five marine bacteriophages in media with the addition of different inorganic salts (distilled water, 0. 5% NaCl solution, 3% NaCl solution, artificial seawater diluted sixfold, artificial seawater, and seawater broth). They observed that all phages were most inactivated in a medium containing 0.5% NaCl than in the other media. It suggests that the phages had the highest activity in salt concentrations roughly equivalent to seawater. Seaman and Day (2007) successfully isolated bacteriophages from a soil sample of salt plains in OK (USA). The salinity of the groundwater in this area varies between 4% and 37%, and soil salinity, between 0.3% and 27% (Wilson et al. 2004). One of those phages, ΦgspC, a member of the Myoviridae family, has an unusually large genome (340 kb). The authors suggested that this large genome may encode environmentally relevant genes that probably increase the phage adaptation to some environments. Interesting observations made by Leibo and Mazur (1969) revealed that when the T4B phage was rapidly transferred from a concentrated to a dilute solution, the phage activity depended on the initial salt concentration of the solution in which the phage was suspended. The phage inactivation occurred by rapid dilution, but it did not decrease when the phage was slowly diluted. A rapid change in osmotic pressure may cause phage DNA to extrude from the tail or their heads to break. This occurred when phages were diluted from high salt concentration to low concentration solutions (Lark and Adams 1953). Yamamoto et al. (1968) who investigated the inactivation of the T5 phage similarly observed streaking decrease in phage activity achieved immediately after rapid dilution. Furthermore, the sensitivity of the T5 phage to chelating agent shock (sodium citrate or ethylenediaminetetraacetic acid) increased when the concentration of the chelating agent increased. Interestingly, higher inactivation was observed with low concentrations of chelating agents. The same authors observed that the inactivation of the phage by chelating agents was reduced by ionic solution (such as 0.85% NaCl).

Adams (1949) checked the stability of bacteriophage T5 incubated at 37°C in salt solutions (phosphate buffer, buffer plus citrate, and buffer plus calcium). He observed that the phage was stable in the calcium ion solution but lost its activity in phosphate buffer, whereas it was rapidly inactivated in citrate solution. No phage particles were

detected after 2 h of incubation in 10 mmol/L phosphate buffer with 2 mmol/L citrate (pH 7). He also showed that divalent metals at millimolar concentrations might prevent phage inactivation. He supposed that the increase in T5 stability in the presence of different anionic solutions resulted from complex formation between the phage particle and ion. Mylon et al. (2009) studied MS2 phage stability in different solutions of LiCl, NaCl, KCl, and CaCl2 in a range of 0.01–1.0 mol/L. Their observations revealed that monovalent salts did not influence phage aggregation. In contrast, the growth rate of the phage aggregates increased with an increasing calcium salt concentration. It was suggested that this resulted from neutralization of the negatively charged moieties on the phage surface by cation binding. The chemical composition of water may also influence phage stability. The stability of five Flavobacterium phages (PFpW-3, PFpC-Y, PFpW-6, PFpW-7, PFpW-8) isolated from pond water collected from Japanese ayu farms was tested for 21 days at 18°C (Kim et al. 2010). There were no significant changes in the phage titer in pond water, autoclaved filtered water, or broth during the first 3 days of incubation, but their stability decreased below the detection limit in pond water after 10 days. The persistence of MS2 and PRD1 phages was compared in tap water and ultrapure water system samples at room temperature and pH increasing from 7.6 to 8.9 and at stable pH 7 (Governal and Gerba 1997). There was no significant decrease in concentration of PRD1 during the experiments, but MS2 showed a different decrease in survivability in different types of water. The highest inactivation was in post reverse osmosis water. These observations were explained as resulting from phage structure. The genetic material of PRD1 is DNA, which is generally a more stable acid than RNA of MS2. After removing contaminants, water becomes a “more powerful solvent,” and the possibility to degrade the phage genetic material increases. Moreover, phage PRD1 has internal lipids which increase its resistance to degradation in ultrapure water. It was suggested that ultrapure water, being an aggressive solvent, attacks the virus surface through a mechanism of direct oxidation. It causes head degradation, dispersion of capsids, tail fragmentation, and release of viral genetic material into the water environment. Jepson and March (2004) observed that phage was more stable at ambient temperature when stored in distilled water than in tap water in which its titer decreased by 2–3.5 log after 2 weeks. It was suggested that halogenating agents in tap water may inactivate phages. According to Thorne and Holt (1974), the addition of 10 mmol/L Mg2+ to the NBY may protect CP-51 phage against inactivation under unprofitable temperature. A 1-h incubation of phage lysates at 0°C at pH 6.8 with magnesium ions caused no detectable loss in phage activity. In comparison, 60% decrease in initial phage titer was observed when there was no Mg2+. Similarly, other authors suggest that some metal ions may protect phages against inactivation.

Interestingly, Ca2+ (1 or 5 mmol/L) could protect Xp12 phage particles suspended in 10 mmol/L Tris buffer solution at pH 8.0 against inactivation by heating at 60°C (Chow et al. 1971). On the other hand, Mg2+ in a concentration of 5 mmol/L increased thermal inactivation of the phage. Therefore, it was suggested that addition of 5 mmol/L CaCl2 to solutions may prevent a loss of phage titer during the purification process. Kuo et al. (1971) observed phage Xp12 dissociation by sodium citrate in Tris buffer at pH 7.5 at room temperature. Phage particles exposed to 3 mmol/L sodium citrate presented decomposition in DNA and empty heads and tails. Gupta and Yin (1995) showed that bacteriophage T7 lost its activity with half life after 30 s when was exposed to 6 mol/L urea used as denaturing component. Whang et al. (1996) presented that 1 mmol/L metal ions may slow or accelerate T7 phage inactivation by urea. As they observed, divalent metal ions (Mg2+, Ca2+, Co2+, Ni2+) stabilized activity in the presence of urea, in contrast to trivalents (Al3+ and Au3+) which destabilizated phages. The presence of either of the ions caused loss of phage titer more than 50-fold even at concentrations of 0.25 mmol/L.

2.9. Application of Bacteriophage

2.9.1. Phage Therapy

Phage therapy has many advantages over antibiotics for therapy, but there are also concerns. This approach has been used in animal, plants, and human beings with varied degrees of success. One of the advantages of using phage is their specificity to the target bacteria and lack of interference with the host normal flora. After phage administration, they spread quickly through the human body reaching every organ. However, the immune response generates antibodies that clear the systemic phage and that is one of the major concerns about using phage therapy. One way to circumvent this is to use the phage's lytic enzymes such as endolysins and holins for therapy instead of using the whole viron particle (Haq et al., 2012).

2.9.2. Phage Display

In the phage display technique, DNA, which encodes the desired polypeptide, is fused within the coat protein genes for the phage. Then the desired protein is produced and expressed on the surface of the phage (Smith, 1985). Phage display can be used to generate antibody fragments libraries using the filamentous phage such as M13 phage. These display libraries have been involved in many applications such as in the treatment of cocaine addiction. First, the phages are administrated nasally until it reaches the central nervous system where the displayed antibody binds to the molecules of cocaine and prevent its effect on the brain (Dickerson et al., 2005).

2.9.3. Phage Typing

The use of phages as a diagnostic tool or for phage typing depends upon the sensitivity patterns of bacteria to certain phages. There are various methods that can be used to detect pathogenic bacteria. For example, phages can be used to deliver reporter genes, which can be detected post infection. Phage adsorption can be detected by using phage that have fluorescent dye attached to their coats (Goodridge et al., 1999). Detection of released bacterial proteins following bacterial lysis due to phage infection, such as adenylate kinase, can be detected by using antibodies produced by phage display that will bind specifically to these complexes (Petrenko and Vodyanoy, 2003). The phage amplification assay is the most technique that have been used to detect bacteria such as Pseudomonas, E.coli, Mycobacterium tuberculosis, Salmonella, Campylobacter and Listeria species (Barry et al., 1996).

2.9.4. Bioprocessing and Biocontrol

Bacteriophages are used in bioprocessing to decrease the bacterial load in foods. Especially foods that are minimally processed to avoid cooking associated texture or flavor (Garcia et al., 2010). Phage bioprocessing has been employed to reduce the growth of many food pathogens such as *Salmonella enteritidis* in cheese, *Campylobacter* and *Salmonella* on chicken skin, and *Listeria monocytogenes* on meat. In addition, this approach can be used to extend the shelf life of animal products (Dykes and Moorhead, 2002).

Phages can be used as well as predators of bacteria that associated with fungi, plants or their products. Phage biocontrol of plants pathogens has been a successfully used against *Xanthomonas pruni* on peaches, peppers, and cabbage plants. These methods also worked against *Ralstonia solanacearum* on tobacco and *Xanthomona campestris* on tomatoes.

2.10. Advantages of using phage therapy over antibiotics

The advantages are given below on the basis of phage properties:

- Phages can self-propagate thus multiplying their number and thus infection rate to bacteria. Due to this, even low initial doses of phage can be used to combat bacterial infection (Donlan, 2009). It is termed as “auto-dosing”.
- Mutation is very common in phage thus they can be effective against new resistance acquired by the bacteria.
- Phages can be used to clear biofilms (Harper et al., 2014).
- Being highly specific, phages are unlikely to kill any normal flora in the human body (Skurnik & Strauch, 2006).
- Phages can effectively be used against Antibiotic resistance bacteria as phages have developed themselves to combat all forms of Antibiotic-resistant bacteria.
- Bacteriophage isolation and use is less costly than antibiotic generation and preparation.

2.11. Drawbacks associated with the use of bacteriophages

- **Phage specificity:** Phages are highly specific so their medical utilization is limited. Even a slight mutation in bacteria may render the phage useless against that bacteria.
- **Bacteriophage resistance:** Bacteria may be able to develop resistance against phage but the rate of phage resistance acquiring is around 10 times lower than that of antibiotics resistance. However, using phage cocktail (a mixture of phages) can be used to combat phage resistance (Labrie et al., 2010).
- **Phage inactivation:** Phage inactivation by human serum can pose a limitation in phage therapy.
- **Endotoxins:** During cell lysis by bacteriophage, various lipopolysaccharide molecules are released from bacterial cell wall and cell membrane. Various toxic proteins may also be released from bacterial cytoplasm. When present in high concentration, they can trigger a coagulation cascade, invoke fever, endotoxic shock, and hypotension (Dabrowska et al., 2004). Endotoxin can be avoided in phage preparation by purifying phage preparation using chromatography and ultrafiltration (Boratyński et al., 2004).
- Obligate lytic property as well as full characterization of phage along with its thermal stability, efficacy and absence of any virulence gene is needed for phage therapy (Loc-Carrillo & Abedon, 2011). This limits the use of phage in phage therapy.

CHAPTER 3: MATERIALS AND METHODS

3.1. Isolation and Identification of *Salmonella spp.*

Faecal and water samples were collected from different poultry farms and rivers respectively. Collected poultry faeces were suspended in 10 ml of sterile water. Hundred (100) microliters of the sample was taken in Salmonella-Shigella(SS) agar plate and spread plating was done. The plates were incubated at 37 degree Celsius for 24 hours.

After incubation, pale colony with black center in the plate was suspected as *Salmonella spp* and individual colony was sub-cultured in *Salmonella*-Shigella (SS) plate to prepare pure culture of that colony. The isolates were named as S1, S2, S3 and so on. Further analysis and identification of bacteria was done by Gram staining and biochemical tests (Indole, Methyl Red (MR), Voges-Proskauer Test (VP), Citrate, Triple Sugar Iron Agar (TSIA) and Urease test.

3.2. Antibiotic Susceptibility Test (AST)

A small quantity of bacterial culture was inoculated in LB broth and incubated at 37 degree Celsius. A 0.5 McFarland was prepared by adding 9.95ml of 1% H_2SO_4 and 0.05ml of 1% Barium chloride. Bacterial density in the culture was compared with 0.5 McFarland and when similar turbidity was achieved, the bacterial culture was taken for AST.

For AST testing, 100 microliters of above culture was spread plated in Muller Hinton Agar (MHA) plate and antibiotic discs (ampicillin AMP10, ciprofloxacin CIP5, ceftazidime CAZ30 and colistin CL10) were aseptically put firmly in the plates. The plates were then cultured at 37 degree Celsius for 24 hours. After incubation the clear zone around antibiotics (zone of inhibition) was measured in millimeters using ruler. The size of zone of inhibition was compared with provided standard data to determine if the organism was sensitive, intermediate or resistant with that antibiotic.

3.3. Determination of Bacterial Growth Curve

A colony of bacteria from Nutrient Agar (NA) was inoculated into a Luria Bretini (LB) broth and incubated at 37 degree Celsius. A solution of sterile LB broth without any bacteria was also incubated and labeled blank. After each 20 minutes, spectrophotometric reading of the bacterial broth with respect to blank solution was taken at optical density (OD) of 600nm. The reading was taken every 20 minutes till 2-3 readings of nearly equal OD were obtained which indicated the stationary phase in the growth cycle and the experiment was stopped.

A graph was plotted as OD value v/s time (in minutes) to obtain growth curve with various phases like lag phase, log (exponential) phase and stationary phase.

By taking any two OD values from log phase, doubling time can be calculated as,

$$\text{Doubling Time} = \frac{T2 - T1}{3.3 \log (OD2/OD1)}$$

Doubling time is generally calculated in minutes. The unit of doubling time is same as the unit of time (T1 and T2) taken during calculation.

3.4. Isolation and Purification of Bacteriophage

Sewage samples from various nearby rivers in Balkhu, Teku, Chabahil, Narayantar, Maitidevi, Sankhu were taken and centrifuged at 4000 rpm for 30 minutes to settle down the bacteria and residues from sewage. The supernatant was syringe filtered with 0.22 micrometer syringe filter. 100 microliter host culture (*Salmonella*) with OD around 0.1 was taken and mixed with 1ml of syringe filtered sewage sample in a sterile falcon tube and allowed to stand for 5 minutes to allow attachment of phage with the bacteria. Double Layer Agar Assay (DLAA) technique as described by Adams, 1959 with the utilization of Tryptic Soya Broth (TSB) and agar was used for the isolation of phage. The petri dish was then incubated at 37 degree Celsius for 24 hours. After incubation, clear zones (plaques) in the plate were assumed to be bacteriophages liberated by lysis of the bacterium.

For purification of phage, isolated plaque was taken and then streaked continuously in a TSB agar plate (1.5% agar). Then, 100 microliter of *Salmonella* (S9) culture was added with 3ml of soft agar and poured gently on top of hard agar and distributed evenly on the plate by shaking. The plate was then left for some time to allow the soft agar to solidify. Then, the petri dish was incubated at 37 degree Celsius for 24 hours (Chase & Bradley, 2011). This process was repeated at least 5 times to obtain pure isolates of bacteriophage. Pure isolates of phage were amplified by similar streaking method for stock preparation.

3.5. Stock Preparation and Titre Determination of Phage

Sodium Magnesium (SM) buffer was used for preparation of phage stock culture. SM buffer was prepared by adding NaCl (5.8 gm per litre), $MgSO_4.6H_2O$ (2 gm per litre), 1M Tris-Cl (pH 7.5, 50ml) and 2% gelatin (5ml). 3-4 ml of SM buffer was flooded in each phage-streaked petri plate and then put in a shaker at 90-100 rpm for 8-10 hours. After shaking, SM buffer was pipetted out into a sterile falcon tube. The falcon tube was

centrifuged at 4000 rpm for 30 minutes to allow bacterial and agar debris to settle down. The supernatant was filtered through 0.22 micrometer syringe filter into a sterile falcon tube and the filtrate was taken as phage stock culture.

For the titre determination of phage, the phage stock was diluted into different dilution ranging from 10^{-1} to 10^{-12}. The stock culture is the 10^{0} dilution. To dilute stock, 100ul of stock was added to 900ul SM buffer and labeled 10^{-1} dilution and from each upper dilution 100ul was added to 900ul SM buffer to produce 10-fold lower dilutions.

Spot assay was utilized for titre determination. In this, grids were drawn on the bottom of TSA plate for spot test of each dilution. Then the bacterial lawn was prepared by pouring the mixture of 100 µl active log phase bacteria (S9) in 3ml soft agar into the labelled TSA plates. After allowing to dry, 5 µl of respectively prepared phage dilutions were spotted aseptically onto corresponding grids as labelled. Only SM buffer was used as negative control. The droplets were allowed to soak into the agar and plates were incubated at 37°C for 24 hours in inverted position. Next day, the plates were observed for the clear zone of bacterial lysis/plaques on spots.

For determining the concentration of phage in stock solution, the last three dilutions which showed clear lysis on spot assay were used. DLAA was performed taking individual dilutions and host bacteria (S9) and incubated overnight after solidification. After overnight incubation, the plates were observed for plaque formation and the distinguishable plaques were counted. The plaque forming unit per ml was calculated by using the following formula:

$$\text{PFU/ml} = \frac{\text{number of plaques observed}}{\text{dilution} \times \text{volume of sample}}$$

3.6. Activity of Different Phages on S9 Bacteria

To determine activity of different phages on S9 bacteria, different solution of S9 bacteria and phage at 1 MOI (as shown in table 3.1 below) were prepared and added to 10 ml of sterile LB broth and allowed to incubate in shaking incubator at 37^{o}C for 6 hours. Spectrophotometer reading at OD600 was taken every hour and bacterial density was identified as compared to non-phage treated bacterial solution (S9). The phage solution with lowest OD after 6 hours is the phage with highest activity.

Table 3.1: Phage titre and bacterial concentration used to prepare 1 MOI

Phage	Stock Titre (PFU/ml)	Volume	S9 concentration (CFU/ml)	Dilution used	Volume	Final Concentration of S9
P1	10^6	1 ml	2×10^8	10^{-2}	0.5 ml	10^6
P3	10^4	1ml	2×10^8	10^{-4}	0.5 ml	10^4
P5	10^5	1ml	2×10^8	10^{-3}	0.5 ml	10^5
P9	10^7	1ml	2×10^8	10^{-1}	0.5 ml	10^7

3.7. Host Range Analysis of Phages

Both intra specific and inter genus host range analysis was done by spot assay. Different samples of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter, Proteus* and *Salmonella* available in the CDBT laboratory were used for spot assay. Total 13 bacterial samples were revived (1 *E. coli*, 2 *Klebsiella*, 4 *Salmonella,* 2 *Pseudomonas aeruginosa*, 2 *Proteus* and 2 *Acinetobacter* spp.) were used for intra and inter species host range analysis. At first, lawn culture of all 13 bacteria was prepared by mixing 100 µl active log phase bacteria with 3ml soft agar (Verma et al., 2009). The mixture was then poured into TSA plates and allowed to set for few minutes. Like the spot assay performed above, blocks were already made for different phages to be spotted. 5 µl of each phage was pipetted on corresponding blocks and allowed to soak for about 15 minutes. Then the plates were incubated overnight at 37°C. Presence of clear spot after incubation shows lysis of bacteria by corresponding phage.

3.8. Determination of Optimal Multiplicity of Infection (MOI)

MOI is defined as the ratio of the number of phage infecting a number of bacterial host cells. To determine the optimal MOI of phage, different dilution of bacteria and phage are mixed with each other so that the ratio between the number of phage to number of bacteria gives MOI.

$$\text{MOI} = \frac{\text{number of phage}}{\text{number of bacteria}}$$

Different MOI of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 were prepared and added to 10 ml of sterile LB broth and allowed to incubate in shaking incubator at 37^{o}C for 24 hours. The solution was observed for clarity as turbidity refers to growth of bacteria. The

lowest MOI at which clear solution without bacterial growth was observed was taken as optimal MOI.

3.9. Determining Phage Lytic Activity (P9) at different times

Phage lytic activity refers to the lytic activity of phage at certain time against a certain bacteria. It is important to determine if the phage has a prospect of being used in therapeutic purposes because this will show how long the phage remains active and needs to be administered again.

Table 3.2: Preparation of different MOI for analysis of phage activity (P9)

MOI	P9 Stock Titre (PFU/ml)	Dilution used	Volume	Final Conc. of P9 (PFU/ml)	S9 conc. (CFU/ml)	Dilution used	Volume	Final Conc. of S9 (CFU/ml)
100	2×10^6	10^0	0.5 ml	10^6	2×10^8	10^{-4}	0.5 ml	10^4
10	2×10^6	10^0	0.5 ml	10^6	2×10^8	10^{-3}	0.5 ml	10^5
1	2×10^6	10^0	0.5 ml	10^6	2×10^8	10^{-2}	0.5 ml	10^6
0.1	2×10^6	10^{-1}	0.5 ml	10^5	2×10^8	10^{-2}	0.5 ml	10^6
0.01	2×10^6	10^{-2}	0.5 ml	10^4	2×10^8	10^{-2}	0.5 ml	10^6
0.001	2×10^6	10^{-2}	0.5 ml	10^4	2×10^8	10^{-1}	0.5 ml	10^7
0.0001	2×10^6	10^{-2}	0.5 ml	10^4	2×10^8	10^0	0.5 ml	10^8

To determine phage lytic activity, different MOI of 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 were prepared (by mixing different dilution and volume of phage stock and bacteria as given in table 3.2 above)and added to 10 ml of sterile LB broth and allowed to incubate in shaking incubator at 37^{o}C for 4-6 hours. Every hour, bacterial density in each MOI solution was identified by taking optical density (OD) with respect to blank (LB only) at 600nm. The graph of OD vs time was plotted and lytic activity of phage was studied.

3.10. pH Sensitivity and Heat Sensitivity of Phage

Stability of isolated phages was tested against a wide range of pH (1–12) and temperature range (30°C to 80°C) to determine their survival under acidic, alkaline and high temperature conditions.

Stability studies for acidic and alkaline conditions were conducted according to the methods described by Niu et al., 2012. Briefly, phages were suspended in LB broth

(adjusted with 1 M NaOH or HCl, to yield a pH range of 1–12) for 1 hour. DLAA was performed to determine the survival of phage (shown by presence of plaques).

For thermal stability, working stock of each phage (100 µL) was suspended in 900 µL LB and the suspension was incubated at 30, 40, 50, 60, 70 and 80°C for 10, 30 and 60. Survival of phage at each temperature and time was determined by DLAA after incubation.

3.11. One-Step Growth Curve

For one-step growth curve, 0.01 MOI of bacteria and phage were prepared and added to 30 ml sterile LB broth. The LB broth was then incubated in shaking incubator at 37^{o}C for 1.5 hour and during this time, samples were taken at 5 mins, 10 mins, 20 mins, 30 mins, 40 mins, 50 mins, 60 mins, 70 mins and 80 mins. The samples were then centrifuged at 12000 rpm for 2 mins to settle bacterial debris. The supernatant was taken and mixed with 3ml soft agar, 100ul bacterial culture and plated on TSA plate. During plating after 30 mins, the supernatant was diluted 10 times and for both diluted and original supernatant, DLAA was performed. The plates were incubated at 37^{o}C for 24 hours and number of plaques were observed.

The data was used to plot graph of PFU v/s time which shows the one-step growth curve indicating adsorption time and number of phages released per phage infected cell. The burst size was identified by dividing the average of PFU/infected-cell in the post-rise period of growth curve by the average of PFU/infected-cell in the pre-rise period of the growth curve (Ellis & Delbrück, 1939).

3.12. Phage Protein Visualization Using SDS-PAGE

Sample for SDS-PAGE was prepared by direct heating method and acetone precipitation method.

3.12.1. Sample preparation

- **Direct heating method:** In this method, 25µl of purified phage was mixed with equal volume of 2X sample buffer and heated in heating mantle at 95°C for 10 minutes to denature protein.
- **Acetone precipitation method:** For acetone precipitation method, purified phage solution and ice-cold acetone were mixed in the ratio of 1:4 (200 µl:800 µl) and vortexed. Then, the vortexed sample was incubated for 60 minutes at -20°C for phage precipitation (Urban-Chmiel, et al., 2018). After incubation, the sample was centrifuged at 13000rpm for 10 minutes. The supernatant was decanted and pellet was air dried and was resuspended in 50µl PBS buffer (8 g l^{-1} NaCl, 0.2 g l^{-1}

KCl, 0.2 g l^{-1} KH_2PO_4, 1.44 g l^{-1} $Na_2HPO_4.2H_2O$, pH7.5). Acetone precipitation of phage helps to concentrate the different proteins of phage particles so that proteins can be easily visualized in the gel. Denaturation of protein was performed as in direct heating method.

3.12.2. SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) with slight modification. Separation was carried out in 12% resolving gel (Tris–HCl buffer with pH 8.8), and 4% polyacrylamide in Tris–HCl buffer (pH 6.8) was used as a stacking gel. Electrophoresis was carried out in standard Tris–glycine chamber buffer at a constant current of 400 mA till the tracking dye reached the bottom of the gel. A molecular weight standard (Protein Ladder, (Genei) with a molecular weight range from 20 to 250 kDa was used as protein marker (Urban-Chmiel, et al., 2018). After electrophoretic separation, the gels were stained with Bio-Safe Coomassie brilliant blue solution for certain hours in a shaker and then suitably destained with destaining solution for best visibility of protein bands. The gel was scanned in scanner and photograph of separated protein bands was taken (Sangha, et al., 2014).

3.13. Activity of Phage in Presence of Metal Ions

The activity of phage was tested in presence of 3 metal ions viz. Calcium, Magnesium and Iron which are the most abundant metal ions present inside the human body. The purpose of this analysis is to determine whether any of these ions negatively or positively hamper the phage activity. This study can be used to primarily analyze the activity of phages in human blood and if the phage holds possibility of human/animal testing.

30 to 60 mg/L of calcium ion solution was prepared by mixing sterile solution of calcium salt with 10 mL sterile LB. Similarly, 13-25 mg/L of magnesium ion solution was prepared by mixing sterile solution of magnesium salt with 10 mL sterile LB and 30-300 ug/dL of iron ion solution was prepared by mixing sterile solution of iron salt with 10 mL sterile LB.

Table 3.3: Various concentration of Metal ions used for study and their preparation from stock

Metal Ion	Stock Concentration	Volume of Stock added to 10mL LB	Final Concentration of Metal Ion in Solution
Calcium	10mg/mL	30uL	30 mg/L
		40uL	40 mg/L
		50uL	50 mg/L
		60uL	60 mg/L
Magnesium	10mg/mL	13uL	13 mg/L
		17uL	17 mg/L
		21uL	21 mg/L
		25uL	25 mg/L
Iron	10mg/100mL (0.1mg/mL)	30uL	30 ug/dL
		120uL	120 ug/dL
		210uL	210 ug/dL
		300uL	300 ug/dL

These concentrations were taken as they are close to the natural higher and lower concentration of these respective metal ions in human body. Normal ionized calcium level in adult human blood ranges from 4.64-5.28 mg/dL (46.4-52.8 mg/L) (healthline.com, 2021). Also, Normal Magnesium ion level in adult human is 0.65-1.05 mmol/L (15.8-25.5 mg/L) (emedicine.medscape.com, 2021). Similarly, normal ionized iron level in adult human blood is 60-170 micrograms per deciliter (ug/dL) (ucsfhealth.org, 2021).

After preparing various concentrations of metal ions (as given in table 3.3 above), bacteria and phage at 0.001 MOI were added into each solution. The cocktail of all metal ions was also prepared adding 45uL calcium stock, 20uL magnesium stock and 150uL iron stock to 10 mL LB broth and to it, bacteria and phage at 0.001 MOI was added. LB inoculated with bacteria only (positive control), phage only (negative control) and bacteria and phage at 0.001 MOI without any metal ions (for comparison) was also prepared.

After this, all solutions were incubated at 37^{o}C for 6 hours and spectrophotometric reading at OD600 was measured for all solutions after each hour and reading was recorded.

The reading recorded was utilized in final comparison and evaluation of efficiency of metal ions for phage activity.

3.14. Assay of Phage P9 in Raw Food Products

Raw sausage and potato were taken to study the activity of phage in food products. Sausages were of 2cm diameter and cut into 0.5cm thick pieces and potatoes were cut into 2cmx2cm pieces. The raw foods were then UV-sterilized in the laminar hood by exposing to UV-light for 1 hour.

After sterilization, different MOI (0.01, 0.1, 1, 10 and 100) of bacteria (keeping bacteria concentration at 25 CFU per cm^2 in potato and 100 CFU in each sausage piece) and phage were inoculated onto the surface of food kept inside sterile petri dishes. Positive control (only bacteria) and negative control (only phage and without both bacteria and phage) were also kept. They were allowed to stand for 4 hours under room temperature (25 ^{o}C).

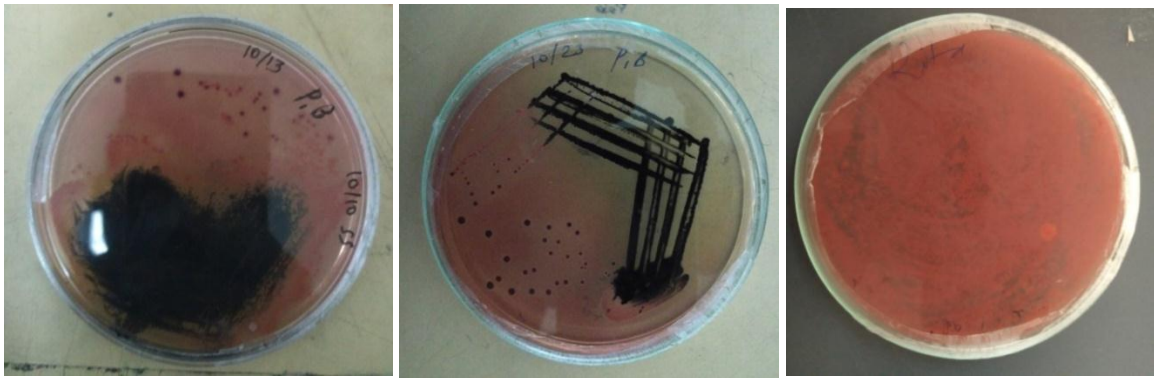
After 4 hours, the samples were added in 5 ml of sterile water and mixed thoroughly. The mixture was then serial diluted to 10^{-1} dilution and 100ul form the non-diluted and 10^{-1} dilution were poured and spread plated into LB agar.

The agar was incubated at 37^{o}C for 24 hours and the number of bacterial colonies were counted after incubation. The bacterial growth in phage treated and non-treated samples gives the indication of phage activity in raw food products.

CHAPTER 4: RESULT AND DISCUSSION

4.1. Isolation of *Salmonella*

Nine samples of presumed *Salmonella* were isolated from poultry (3) and river water (6) samples. The colony with black center was presumed to be *Salmonella* which was then picked and sub cultured in SS agar. Cultures were named as S1, S2 and so on. For biochemical identification, culture was also taken in NA plate.



(1) (2) (3)

Fig 4.1: Culture of black centered colony on SS agar (1 and 2) and plate with no black centered colony (3)

The selectivity of SS medium is based on the presence of brilliant green and bile salts which totally inhibit the growth of Gram positive (+) bacteria and partially inhibit the growth of *Enterobacteriaceae* and swarming of *Proteus*.

The differentiation of *Shigella* and *Salmonella* is based on the ability of *Salmonella* to ferment lactose (colourless colonies) and produce black-centered colonies. The black center is due to the reduction of sulphates (Thiosulphate) into sulphide in the presence of ferric citrate by H2S.

The *Salmonella* colonies are colourless with black center in SS agar. In mac-conkey agar, it shows colourless colonies or sometimes medium colour from amber to orange. However, in Xylose Lysine Deoxycholate (XLD) agar, *Salmonella* shows yellow coloured colony due to the fermentation of sugar to produce acid, which turns phenol red present in agar into yellow.

4.2. Gram staining and Biochemical tests

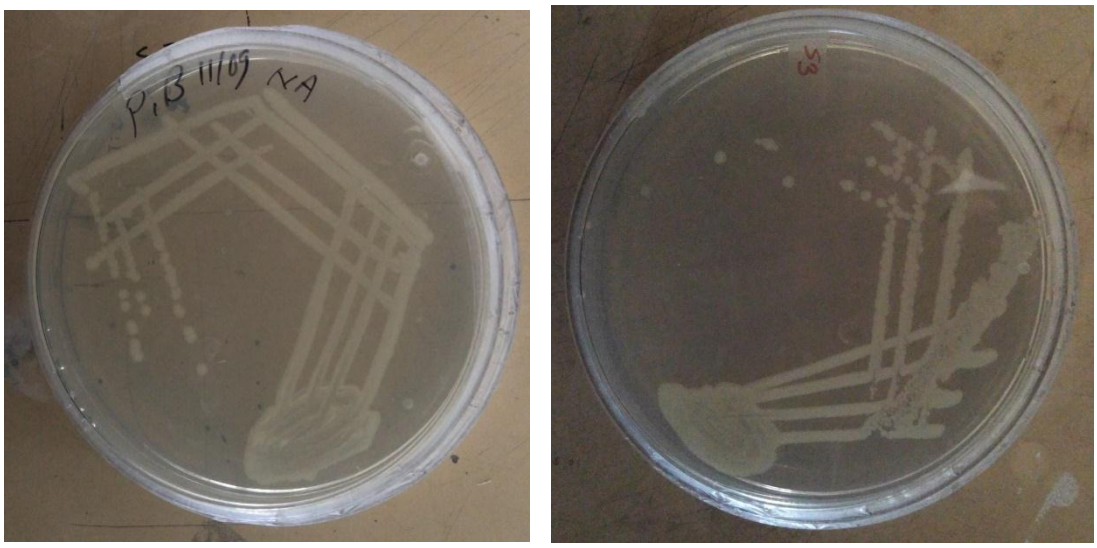


Fig 4.2: Sub Culturing on NA for Biochemical Identification

From the colony on NA plate, the morphology of different isolates were found as:

Table 4.1: Colony Characteristics on NA

Sample	Isolated From	Shape	Size	Color	Margin	Elevation	Transparency
S1	River	Round	4mm	Pale white	Rough	Elevated	Translucent
S2	River	Round	3mm	Pale white	Rough	Elevated	Translucent
S3	River	Round	3mm	Pale white	Rough	Elevated	Translucent
S4	Poultry	Round	3mm	Pale white	Rough	Elevated	Translucent
S5 (P1B)	Poultry	Round	2mm	Pale white	Rough	Elevated	Translucent
S6	River	Round	3mm	Pale white	Rough	Elevated	Translucent
S7	River	Round	2mm	Pale white	Smooth	Elevated	Translucent
S8	River	Round	2mm	Pale white	Rough	Elevated	Translucent
S9	Poultry	Round	3mm	Pale white	Rough	Elevated	Translucent

The colony from NA plate was Gram stained and all the organisms were found to be: **Gram Negative, Rod Shaped**

Fig 4.3: Gram Staining

The biochemical tests of all the isolates showed following result:

Indole: Negative

Motility: Motile

Methyl Red (MR): Positive

Voges Proskauer (VP): Negative

Citrate: Positive

Triple Sugar Iron Agar (**TSIA) test:** H_2S producer. Color of slant and butt couldn't be determined due to H2S production

Negative indole test thus indicated that our organisms did not produce tryptophanase. This ruled out the possibility of our organism being coliform as coliforms are indole positive. Positive MR test and Negative VP test along with positive citrate test and H2S production in TSIA showed our isolate to be either *Proteus* or *Salmonella.*

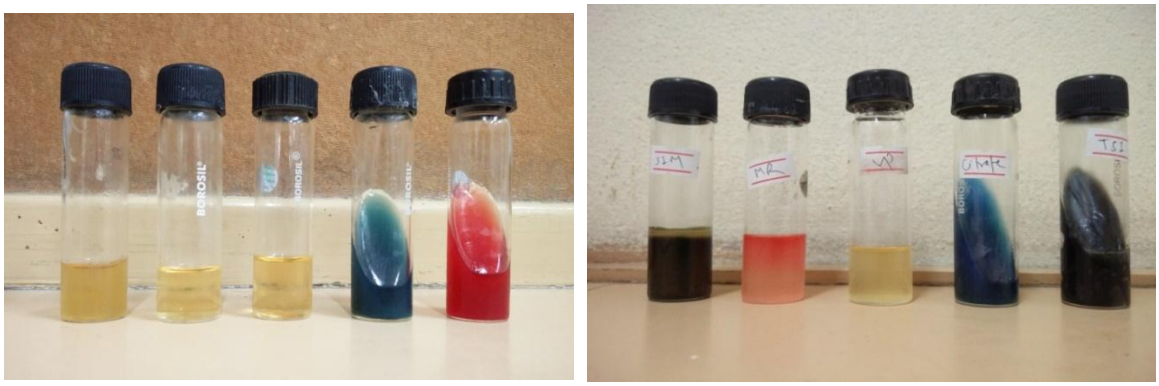


Fig 4.4: IMViC and TSIA test result (Left: Control, Right: with test organism) Media are SIM (Simons Indole Motility), MR, VP, Citrate and TSIA from left to right in each individual figures.

To distinguish among these two, Urease test was done in which *Proteus* is a rapid urea hydrolyzer thus giving positive urease test while *Salmonella* is urease negative organism. The negative urease test by *Salmonella* is due to its inability to produce urease enzyme. *Proteus,* on the other hand produces urease which hydrolyzes urea into ammonia, thus increasing the pH of the medium. The increased pH (basic pH) thus changes the color of phenol red in medium to pinkish red.

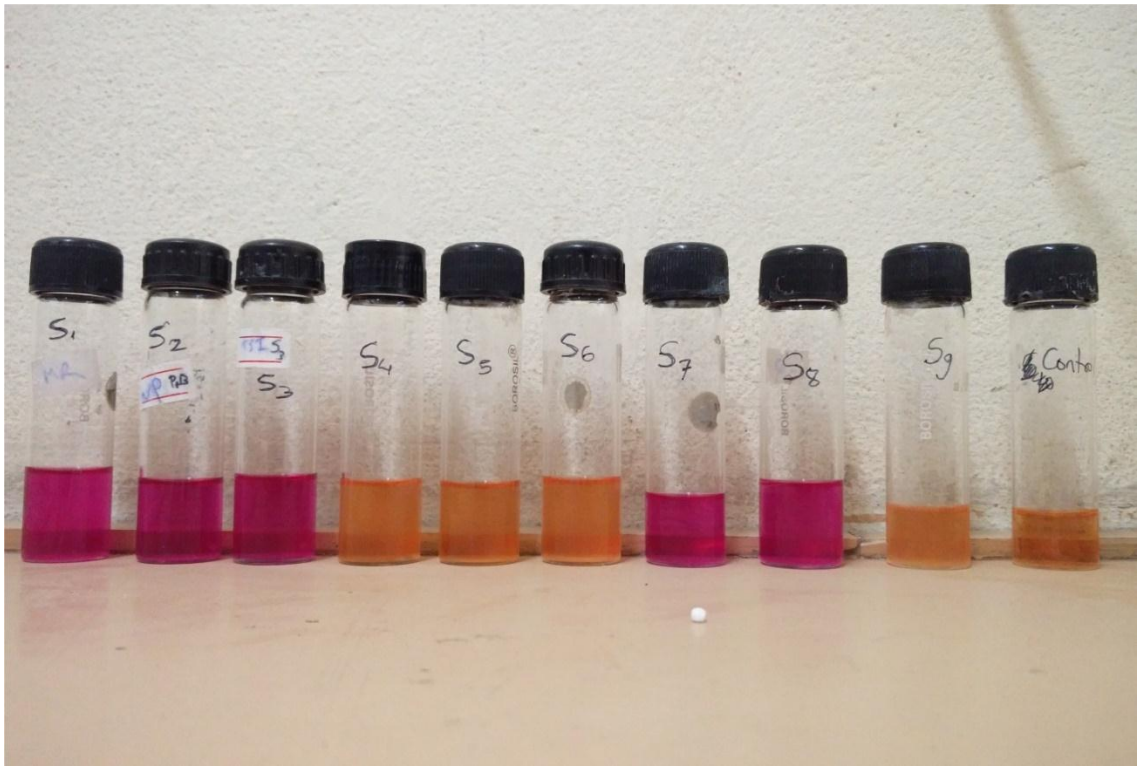


Fig 4.5: Urease test result for samples S1 to S9 and control on far right

Thus, only samples with urease negative result were confirmed to be *Salmonella and taken* for further study.

4.3. Antibiotic Susceptibility Test (AST)

Antibiotic Susceptibility Test (AST) helps us to identify if our bacteria is susceptible to certain antibiotic or not. The Zone of inhibition is a circular area around the spot of the antibiotic in which the bacteria colonies do not grow. The zone of inhibition can be used to measure the susceptibility of the bacteria towards the antibiotic. Higher zone of inhibition indicates greater sensitivity of the bacteria towards the antibiotic and vice-versa.

AST performed on our samples, in presence of antibiotics Ampicillin, Ciprofloxacin, Ceftazidime and Colistin gave the following results as listed in table below.

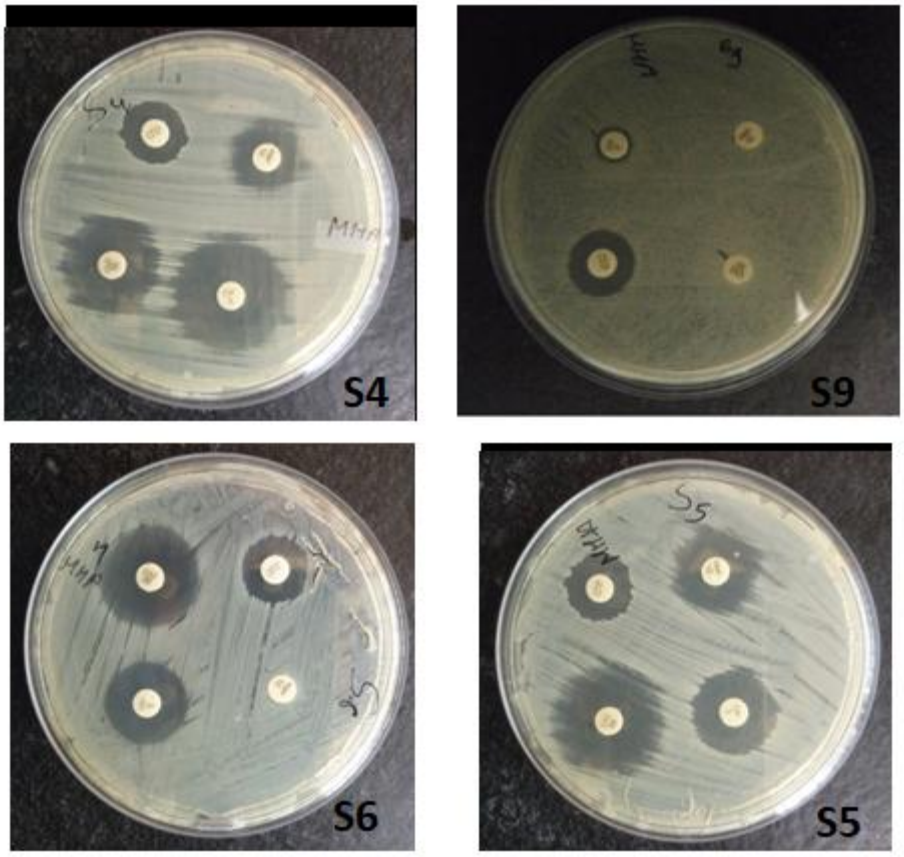


Fig 4.6: AST for *Salmonella* with AMP 10, CIP 5, CAZ 30 and CL 10 from Himedia.

Table 4.2: AST zone diameter for different antibiotics (HiMedia).

Antibiotics (ug)	**Reference Zone (mm)**			**Zone of Inhibition Diameter (mm)**			
	S	**I**	**R**	**S4**	**S5**	**S6**	**S9**
Ampicillin 10	>17	14-16	<=13	15 (I)	19 (S)	0 (R)	0 (R)
Ciprofloxacin 5	>31	21-30	<=20	25 (I)	20 (R)	19 (R)	0 (R)
Ceftazidime 30	>21	18-20	<=17	22 (S)	26 (S)	23 (S)	0 (R)
Colistin 10	NA	NA	NA	15	15	15	15

[Ref: R indicates Resistant, I indicates Intermediate and S indicates Sensitive.]

Above table 4.2 shows that, Sample S9 was resistant up to third generation antibiotic Ceftazidime and was a Multi-Drug Resistant (MDR) isolate. Thus, S9 was taken for further study and analysis. The resistance with colistin could not be cnfirmed because disk diffusion with colistin is not taken as reliable test and Minimum inhibitory

Concentration (MIC) of >4 microgram per ml colistin is taken as colistin resistant strain (EUCAST, 2018).

Antibiotic resistance is primarily due to mutation in bacterial DNA (Bayot and Bragg, 2020). The presence of certain plasmids or gene in a bacteria leads to resistance to certain antibiotic. Genes for such antibiotic resistance can also be acquired from environment by horizontal gene transfer via transformation, transduction or conjugation (MSU, 2021).

4.4. Growth Curve Analysis

The spectrophotometric reading at optical density of 600nm at different time intervals for growth curve analysis was obtained as below:

Table 4.3: Spectrophotometric reading at OD600 at different time intervals

Time(min)	0	20	40	60	80	100	120	140	160	180
Abs (OD 600)	0	0.04	0.061	0.12	0.206	0.371	0.733	0.933	0.95	0.952

From the data above, a graph was created which shows different phases of growth cycle of *Salmonella* S9:

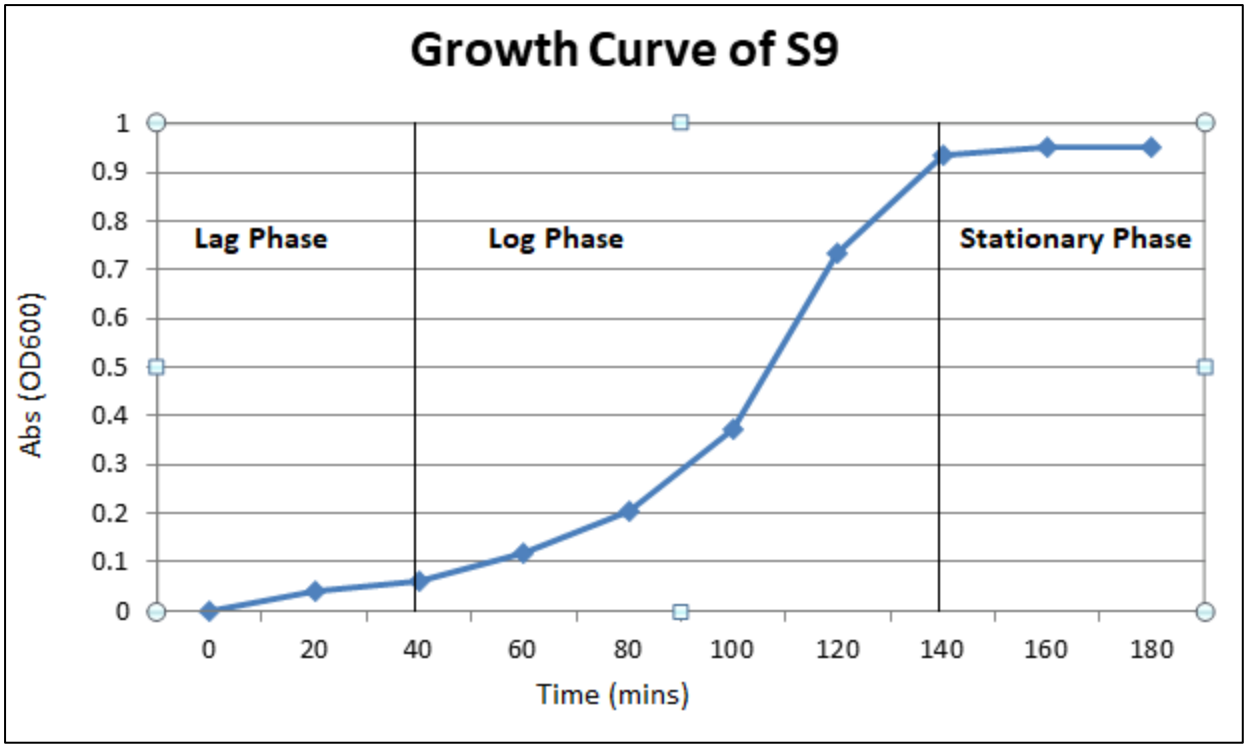


Fig 4.7: Growth curve for S9

Doubling time= (100-80)/3.3 log(0.371/0.206) = 23.8 mins

Using the data obtained as per table 4.3, the doubling time was determined to be 23.8 minutes i.e. nearly 24 mins. The graph also clearly shows the latent (lag) phase, log phage and stationary phase. During lag phase, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs. During the lag phase cells change very little because the cells do not immediately reproduce in a new medium. This period of little to no cell division is called the lag phase and is the initial phase represented by relatively no growth in the chart above. The lag phase is followed by log phase or exponential phase in which rapid multiplication of bacteria occurs. Thus, this period is characterized by exponential growth of bacteria which can be seen by exponential increase in OD of bacteria. Log phase is followed by stationary phase in which the number of bacteria remain relatively constant due to the nearly equal number of bacterial death and bacterial multiplication. Stationary phase is due to limitation in food source for bacteria in the medium.

According to Gibson et. al. (2018), the doubling time of *Salmonella* in laboratory condition is around 0.5 hrs (30 mins) which is near to our calculated doubling time. Also, Molloy S. (2010) identified hyper-replicating *Salmonella* which had a doubling time of 20 mins.

4.5. Isolation of Bacteriophage

Four bacteriophage samples were isolated from sewage samples from rivers in Balkhu, Teku, Chabahil and Narayantar by using *Salmonella* S9 as host bacterium. The plaques formed by different phages are shown in figure 4.9 below and morphology of the isolated phages is listed in table 4.4 below.

Fig 4.8: Sewage sample collection sites (Left: Teku, Right: Balkhu)

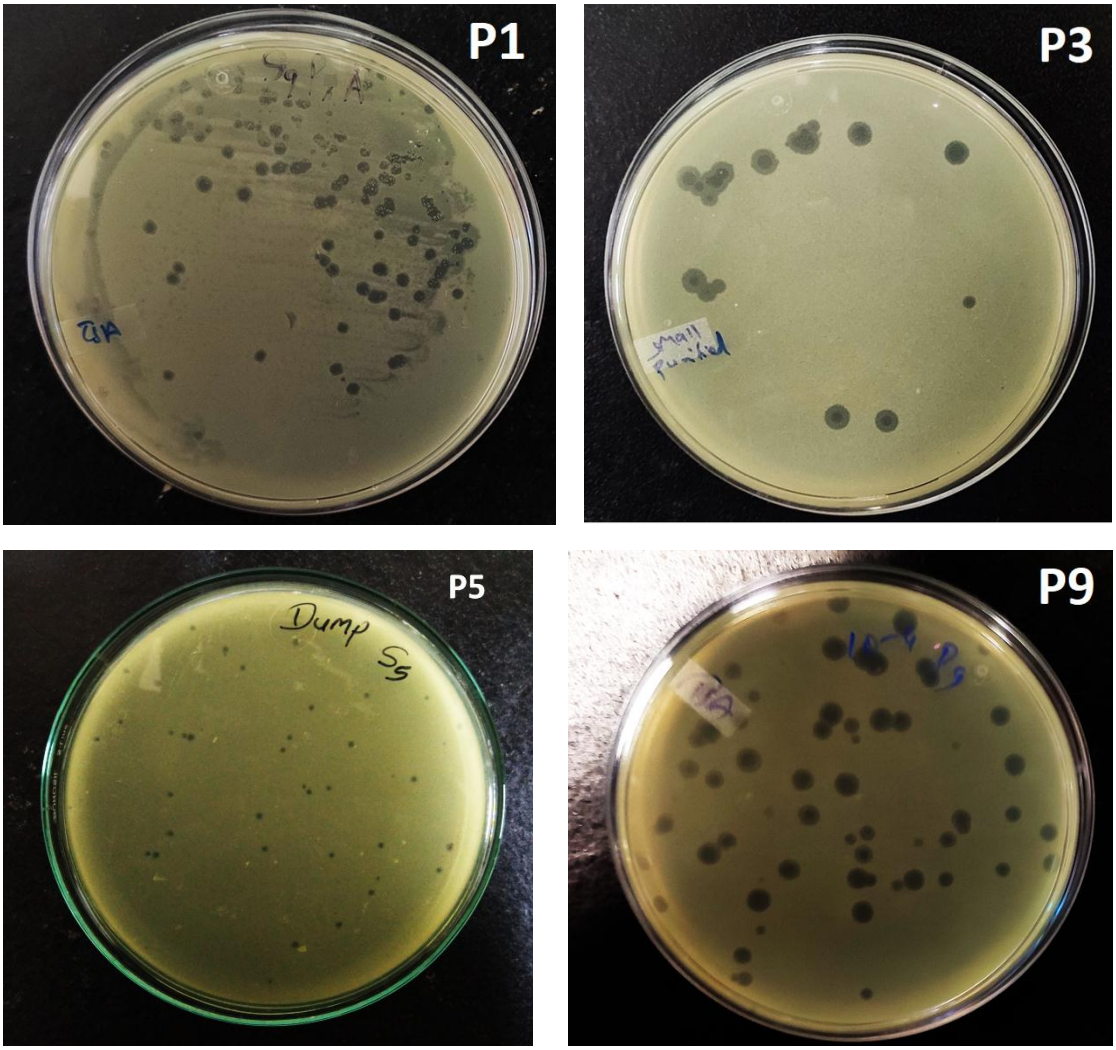


Fig 4.9: plaques seen on TSA agar plate after DLAA and 24 hrs incubation at 37 degree Celsius

Table 4.4: Morphology of different isolated phages

Name of Phage	Source	Plaque size	Plaque type
P1	Balkhu river	4mm	Bull's eye
P3	Narayantaar river	3mm	Bull's eye
P5	Teku river (Dump site)	1mm	Pinhead
P9	Chabahil river	5mm	Bull's eye

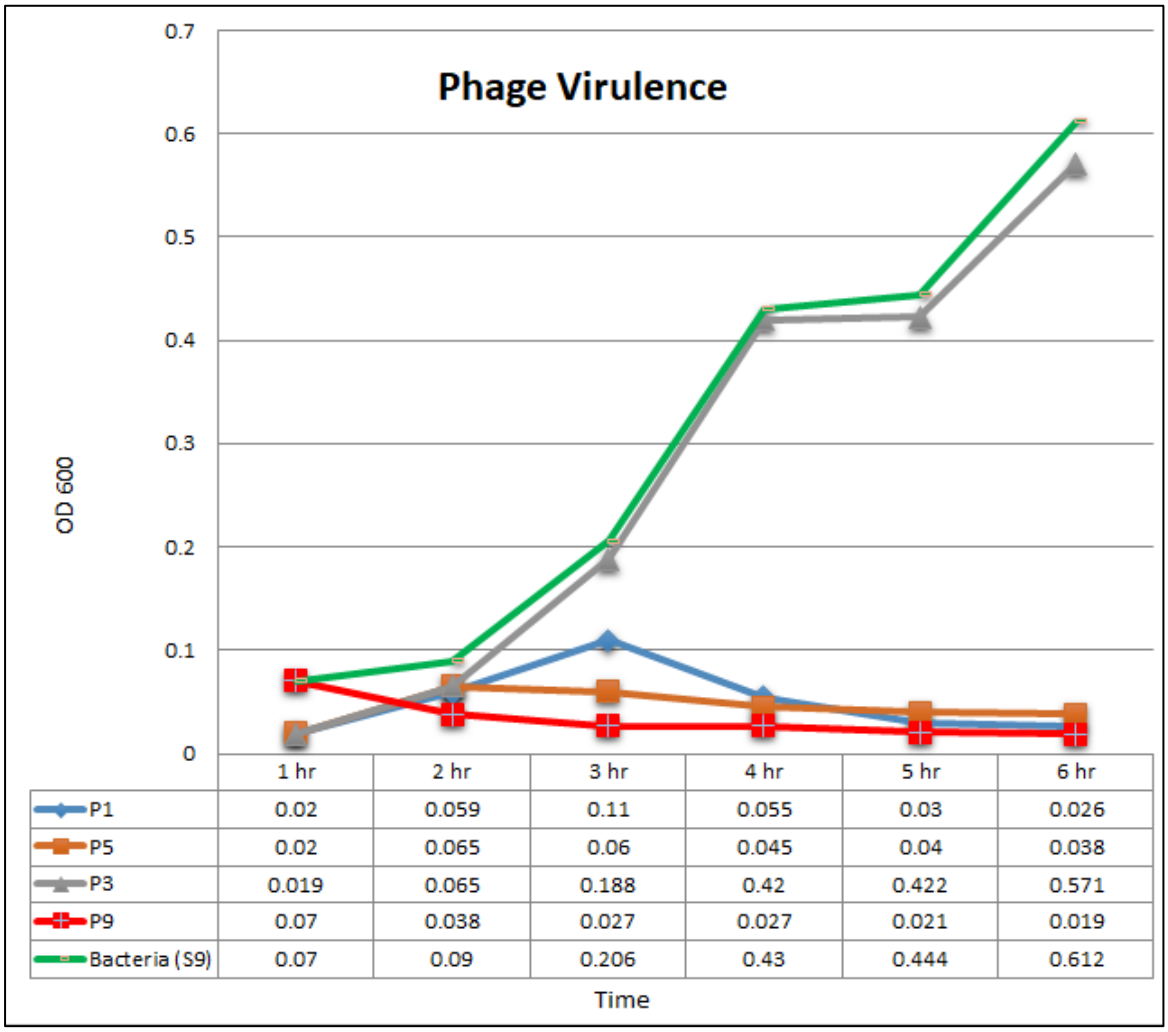
Most of the isolated phages were of Bull's eye type and their diameter ranged from 3-5mm. One phage P5 was found to be pinhead type with diameter of 1mm. The appearance of the plaque depends on the host strain, virus and the conditions. Highly virulent or lytic strains create plaques that look clear (due to total cell destruction), while strains that only kill a fraction of their hosts (due to partial resistance/lysogeny), or only reduce the rate of cell growth, give turbid plaques. Some partially lysogenic phages give bull's-eye plaques with spots or rings of growth in the middle of clear regions of complete lysis. Phage that adsorb early make larger plaques than those that adsorb later (sdsu.edu, 2021). When temperate phage infects a population of exponentially growing cells, each phage produces a plaque with "bulls-eye" plaque morphology, a turbid center surrounded by a ring of clearing. This characteristic plaque morphology is due to the role of the MOI and cell physiology on the lysis-lysogeny decision. Lytic growth is favored when cells are growing rapidly and the MOI is low. Lysogeny is favored when cells are growing slowly and the MOI is high. This is why temperate phage typically have plaques with turbid centers.

The stock of phage was prepared after purification and stock titre was determined using spot assay. The calculated stock of different phages is given in table 4.5 below.

Table 4.5: Stock concentration of phage determined by spot assay

Name of Phage	**Titre (PFU/ml)**
P1	10^6
P3	10^4
P5	10^5
P9	10^7

4.6. Activity of different phages on S9 bacteria



	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
P1	0.02	0.059	0.11	0.055	0.03	0.026
P5	0.02	0.065	0.06	0.045	0.04	0.038
P3	0.019	0.065	0.188	0.42	0.422	0.571
P9	0.07	0.038	0.027	0.027	0.021	0.019
Bacteria (S9)	0.07	0.09	0.206	0.43	0.444	0.612

Fig 4.10: Activity of different phages on bacteria S9

The plot of OD600 v/s time (Fig 4.10) showed that the bacterial density without any phage particle went up to 0.612 after 6 hours of incubation. On the phage treated samples, phage P1, P5 and P9 showed higher reduction in bacterial load even after 6 hours (OD value around 0.019-0.038 for all 3 phages) while phage P3 did not show any considerable reduction in bacterial load as compared with the non-phage treated bacterial sample (S9). The OD for P3 was lower than that of bacteria which may be due to some extent of phage activity at initial phase of inoculation, but the phage activity did not persist longer and the bacterial population in P3 treated solution surged nearing the bacterial population of S9 only.

The large reduction of bacterial load (nearly 90% reduction in bacterial load) indicates that the phages P1, P5 and P9 are good for the anti-bacterial activity and have high potential to be used in the therapy against *Salmonella spp.* (S9).

4.7. Host Range Analysis of Phage

Table 4.6: Activity of different phages on multiple bacterial hosts.

Bacteria Genus	Code	Lysis by phage			
		P1	P3	P5	P9
Pseudomonas	Pseudo-3	X	x	X	X
	Pseudo-53	X	x	X	X
Escherichia	E. coli	X	x	X	X
Klebsiella	K57	X	x	++	X
	K41	X	x	X	X
Salmonella	S4	++	++++	++++	++
	S5	+++	+	++++	++++
	S9	++++	+	+++	++++
	S6	+	+	+	+++
Proteus	S7	X	x	X	X
	S8	X	x	X	X
Acinetobacter	A8	X	x	X	X
	A56	X	x	X	X

[Note: 'x' indicates no lysis of bacteria, '+' indicates very slight lysis with turbidity on lysis zone, '++' indicates better lysis with slightly larger lysis zone but irregular with turbid areas, '+++' indicates clear lysis zone largely visible but not uniform and '++++' indicates clear lysis zone with uniformity in lysis (best lysis).]

During the multi-host range analysis of the bacteriophage, out of 13 bacterial samples taken, *E.coli, Pseudomonas, Proteus* and *Acinetobacter* were not lysed by any of the 4 phages taken for study. The largest host range was shown by phage P5 which lysed all 4 isolates of *Salmonella* and 1 *Klebsiella* (K57) as shown in table 4.6.

All isolates of *Salmonella* were lysed by the phages taken for study. Isolate S6, on the other hand was lysed by all the phages, but only P9 produced more clear lysis zone while others showed incomplete lysis (with higher turbidity). This data shows that most of our phage isolates are highly selective to genus *Salmonella.* Phage P9 produced high

degree of bacterial lysis in all *Salmonella* isolates. Thus, phage P9 was taken for further analysis.

Host range analysis is an important factor in the study of bacteriophage. The host range analysis indicates the specificity of phage. Phages are generally considered to be very specific to their host and generally do not infect other bacteria except their host. But there has been increasing cases of broad host range phages too. Unfortunately, 'broad host range phage' is not a well-defined term in phage biology (De Jonge et. al., 2019). Ross et al. (2016) considered that the term should be reserved for the phages that can infect multiple species and some genera. Hyman and Abedon (2010) discussed the importance of defining the methodology used for host range determination, as different methods can bias the findings. A productive host range is based on the production and release of phage progeny, and while the formation of a plaque is indicative of a productive host, the absence of plaques does not confirm a lack of a productive infection. The study of phage host range has been largely skewed towards spot tests, plaque assays, or clearing of liquid cultures to indicate bacterial lysis (Xie t. al., 2018).

The advantage to narrow host range is that the phage is very specific to its host and can be utilized without worrying about the damage phage may cause to other bacterial species. But narrow host range also makes phage obsolete in many conditions as it cannot be utilized against similar strains with slight mutations.

Broad or Multi host range, on the other hand can be utilized to fight multiple strains or in some cases genus of bacteria. But utilizing such phage in treatment may lead to killing or lysis of unwanted bacteria or useful bacteria of body and may cause further complications.

4.8. Phage Titer/Concentration of P9

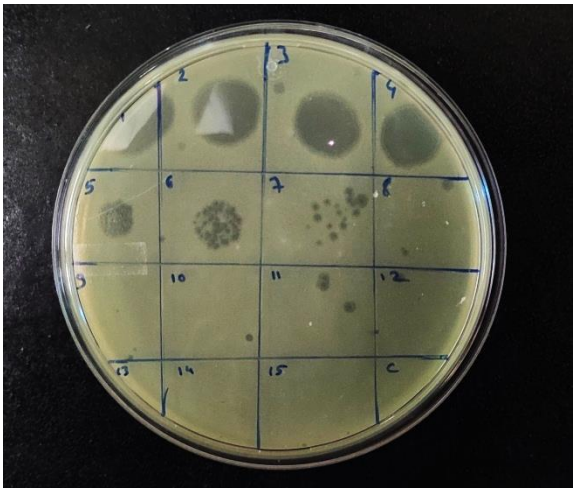


Fig 4.11: Spot Assay for calculating titer of phage P9

The spot assay was used to determine the titre/concentration of phages in the original stock solution. Figure 4.11 above shows clear lysis of bacterial lawn by phages at different dilutions up to dilution 10^{-7}. Some plaques are even seen in 10^{-8}, 10^{-11} and 10^{-10} dilutions but they have high chance of being false positive as these plaques are not in the area where the phage solution was pipetted (in the center).

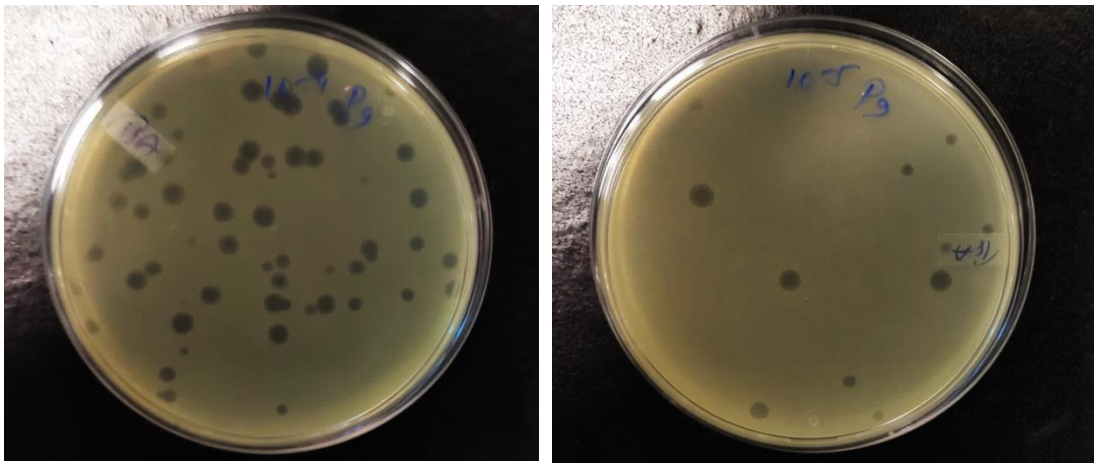


Fig 4.12: Plaques seen after performing DLAA of diluted phage stock: 10^{-4} (left) and 10^{-5} (right).

For further confirming the presence of phage as shown by spot assay, individual dilutions were taken and plated on individual plate (500uL phage dilution + 100uL log phase bacterial culture + 3mL SM buffer). After incubation the plaques on each plate were counted (countable plaques on 10^{-4} and 10^{-5} dilution are shown in figure 4.12) and total concentration of phage was calculated as shown in table 4.7.

Table 4.7: Plaque count table at different dilutions to determine phage concentration

Dilution	Volume used for plating	Number of plaques in plate after incubation			Average number of plaques	PFU/mL= Plaques number (Dilution x Volume)	Average
		Test 1	Test 2	Test 3			
10^{-4}	500uL (0.5ml)	60	64	61	62	$1.24x10^6$	$2x10^6$
10^{-5}		10	12	9	10	$2x10^6$	
10^{-6}		1	1	1	1	$2x10^6$	
10^{-7}		0	0	0	0	-----	

The concentration of bacteriophage in the stock was found to be $2x10^6$ PFU/mL.

4.9. Optimal Multiplicity of Infection (MOI)

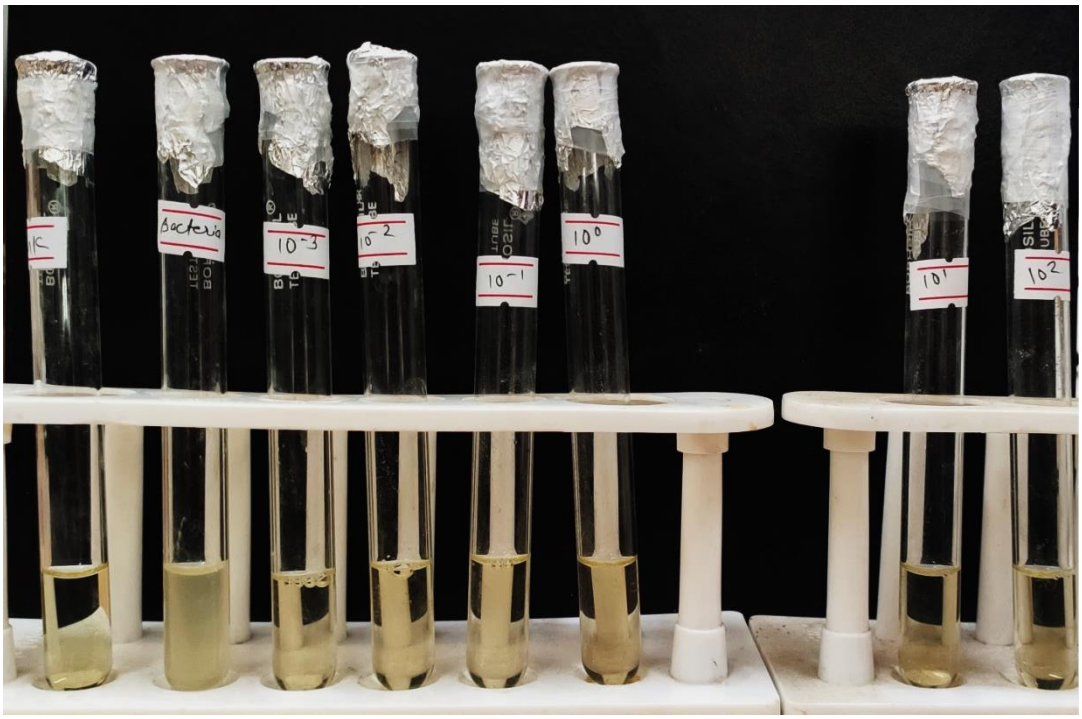


Fig 4.13: Culture of Bacteria (S9) and Phage (P9) at different MOI. Tubes are Blank (no bacteria or phage), bacteria only (no phage), bacteria and phage at MOI 0.001, 0.01, 0.1, 1, 10 and 100 from left to right. Presence of turbidity denotes lesser activity of phage.

The tubes incubated for 24 hours with different MOI showed some turbidity in 0.001 and 0.01 MOI, though it was not as turbid as the bacterial culture. This shows incomplete but high degree of bacterial lysis even in lower MOI.

Clear solution, like blank (no bacteria and phage, only sterile LB broth) were seen in the tubes with MOI 0.1 and above. This indicates the optimal MOI for the complete lysis of bacteria is 0.1 MOI. The result indicates that although there is high degree of bacterial lysis even at MOI 0.001, these MOI cannot completely lyse the bacteria and some bacterial population still remains in solution which can further grow and multiply, after the activity of phage is decreased. But at MOI 0.1 and above, complete bacterial lysis can be obtained by the phage particles, showing the ratio of 1:10 for phage : bacteria is enough to completely get rid of bacteria in-vitro in the LB broth.

4.10. Lytic Activity at Different Time Intervals

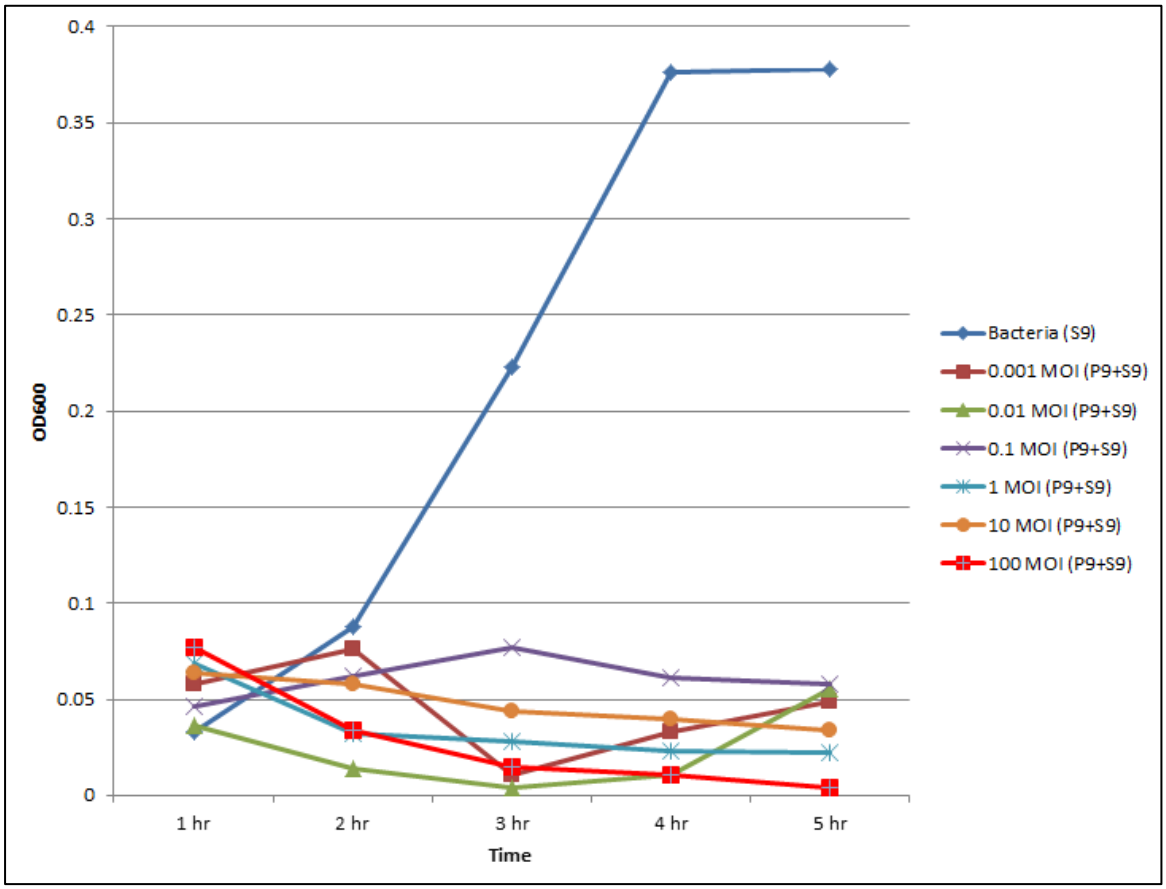


Fig 4.14: Lytic activity of phage P9 at different MOI at different time intervals

To further elaborate the MOI data, lytic activity of phage at different time interval was taken which showed that all MOI of phages were successful in largely reducing bacterial load as compared to the bacteria only sample.

The OD600 of bacteria after 5 hours of growth was 0.378 while the OD600 of bacterial and phage solution after 5 hours was 0.049 (0.001 MOI), 0.055 (0.01 MOI), 0.058 (0.1 MOI), 0.022 (1 MOI), 0.034 (10 MOI) and 0.004 (100 MOI). This data shows that phage P9 was able to cause high degree of bacterial lysis even at 0.001 MOI and was most effective at higher MOI.

In a similar experiment by Konopacki et.al.(2020), they found that the bacterial growth under various MOI mostly followed bell growth, with bacterial population increasing for the first 1-2 hours and then lowering due to lysis activity of phage. My experiment however, found that the bacterial growth under different MOI did not follow bell curve growth mechanism, but rather steady decrease or in some cases decrease at first and slightly increase afterwards. This might be due to our phage activity being high from

initial time, resulting in steady decrease or our bacterial solution dividing slowly so giving phage more time to attach and infect bacteria from initial time resulting in lysing more bacteria due to more time for attachment and infection.

4.11. pH Sensitivity of Phage

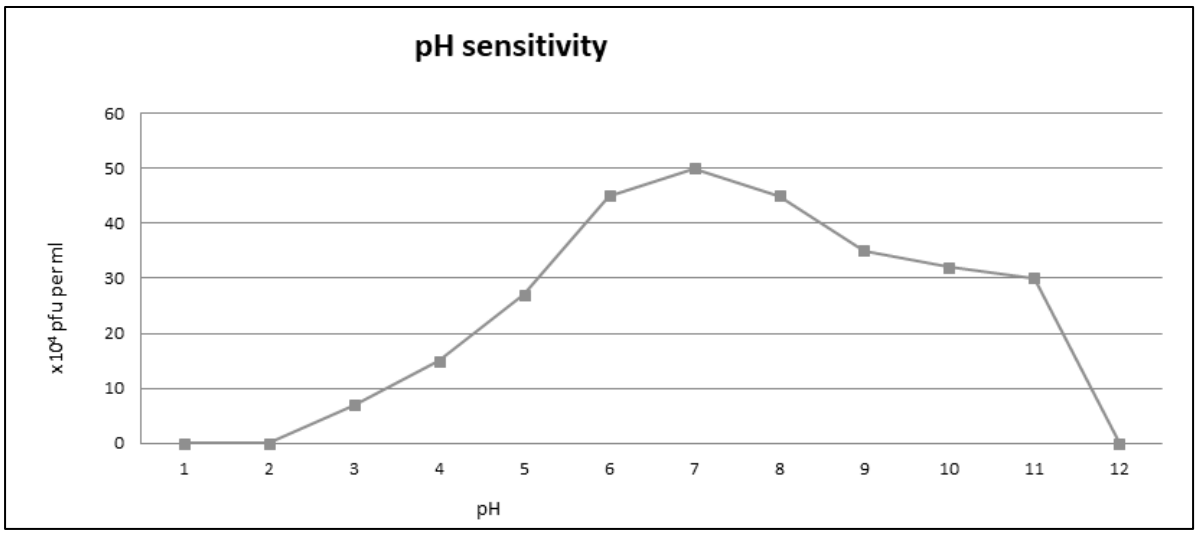


Fig 4.15: P9 Phage survival at different pH

The pH sensitivity of our phage P9 showed that our phage survival was high from pH 6-9. The phage survived from pH as acidic as 3 to pH as basic as 11. Below pH 3 and above pH 11, none of the phage survived resulting 0 PFU on DLAA. It was also seen that the phage was more stable towards basic pH with more than 30 PFU/ml phage surviving up to pH 11, while the survival rate was lower towards the acidic side with less than 30 PFU/ml in pH 3, 4 and 5 (7, 15 and 27 PFU respectively).

This indicates that the phage can tolerate high range of pH and can be utilized in acidic and basic conditions as well. The basic pH tolerance gives the phage usability to be used in mixture with hand-wash and other soapy materials to disinfect hands, floors, etc.

In a similar experiment performed by Huang et. al. (2018), they found out that their phage against *Salmonella* was active at pH range of 4-13. Their phage survival rate was nearly equal at pH 4-12 while the phage survival was highly diminished at pH 13. Although, our phage survived wide pH range (3-11), the survival of our phage greatly varied between the pH range while theirs remained stable.

However, in another experiment by Ateba and Akindolire (2019), the survival of phage was only seen between pH 6-10, and the phages were more susceptible to inactivate on acidic pH rather than basic pH. Although, our phage survived higher pH range than this, but the statement of phages being more susceptible to inactivate at acidic pH than basic pH remains true in our experiment too.

4.12. Heat Sensitivity of Phage

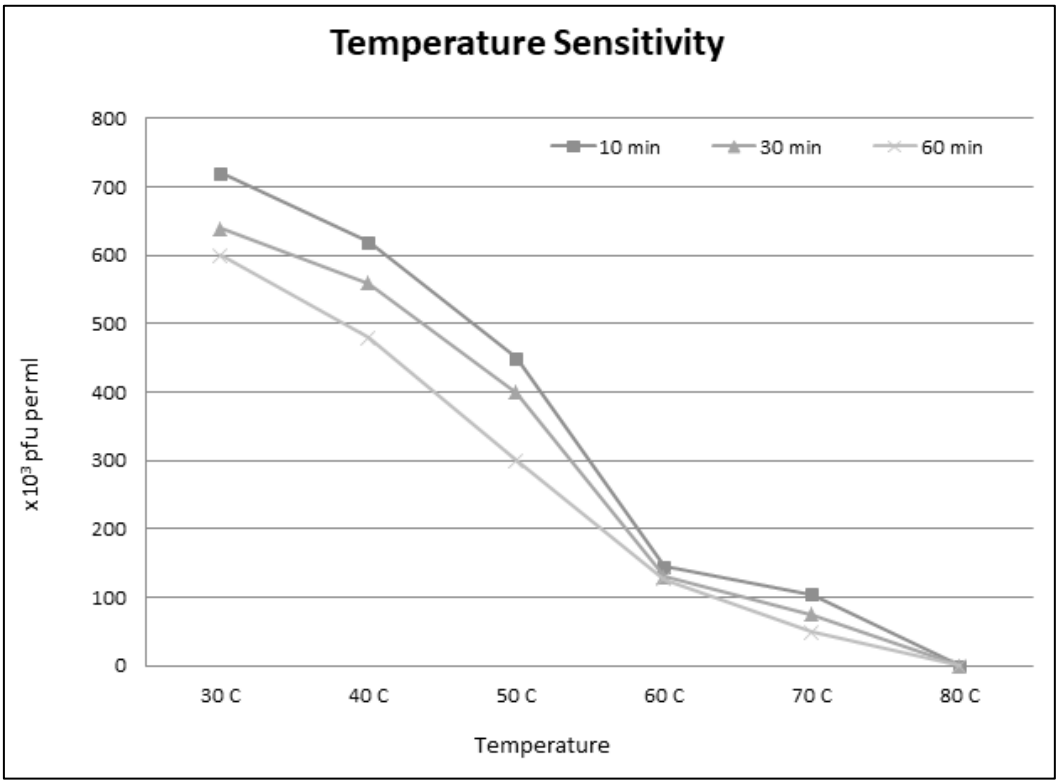


Fig 4.16: P9 Phage survival at different temperature.

The heat sensitivity of our phage P9 showed that our phage survival was high in the temperature range of 30-40^{o}C. The phage survived upto 70^{o}C with very low active phage particles in 70^{o}C. At 80^{o}C, none of the phage survived resulting 0 PFU on DLAA.

Phages were exposed to relative temperature for 3 time periods (10 min, 30 min and 60 min) and the time difference did not make any notable difference in the survival of phage particles. The phage particles followed a descending trend of survival from 30^{o}C to 70^{o}C in which the survival greatly reduced after 50^{o}C. The survival of phage decreased 7 times from 30^{o}C to 60^{o}C.

In a similar experiment performed by Huang et. al. (2018), they found out that their phage against *Salmonella* was active at temperature between 30-80^{o}C on 30 min exposure while active only between 30-70^{o}C on 60 minute exposure. The exposure time in that experiment also did not make relative difference because of exposure time except at 80^{o}C. This is consistent to our result too.

However, in another experiment by Ateba and Akindolire (2019), the survival of phage was only seen below 60^{o}C and even at temperature around 45^{o}C, there was significant reduction in phage titre.

4.13. One-Step Growth Curve

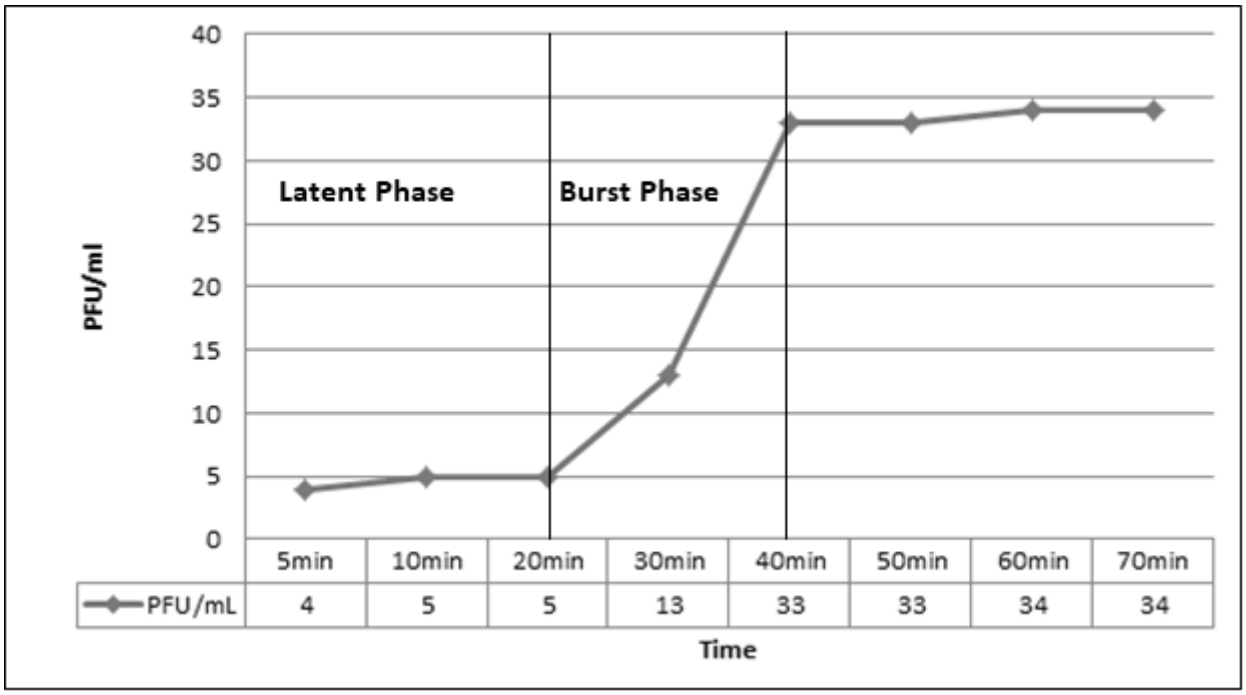


Fig 4.17: One-Step growth Curve of phage P9.

The one-step growth curve of phage P9 showed a latency period of 20 minutes after which rapid infection takes place and phage virions are released afterwards. The latency period means the time for adsorption and infection by phage particles which happened in about 20 minutes during our experiment.

The rapid explosion of plaque forming units at 40 mins is due to bursting and release of phage virions after infecting the host. From the experiment, the burst size was calculated to be 33/5 i.e. 6.6, which is around 7 phage particles per infected bacterial cell (7 PFU/CFU).

In a similar experiment performed by Huang et. al. (2018), they found out that their phage against *Salmonella* had a burst size of 94 PFU/CFU which is very high compared to our data. The relative low burst size of our phage might be due to larger genome size or early lysis of cell bursting of phage virions.

4.14.SDS-PAGE Protein Profiling

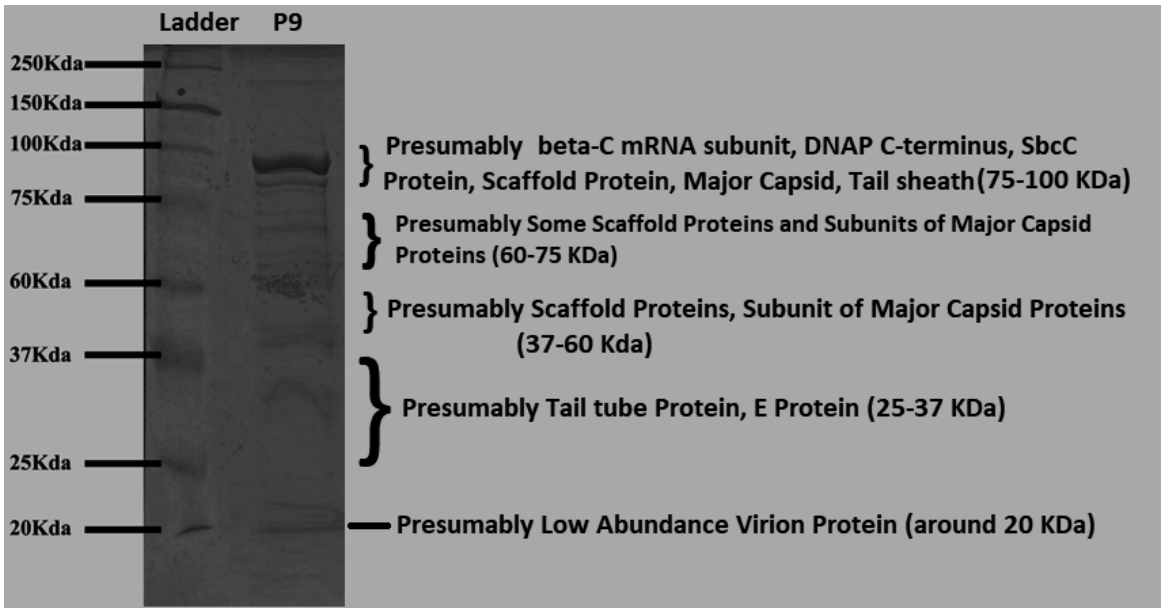


Fig 4.18: SDS PAGE and protein bands of phage P9.

Protein profiling was done by SDS-PAGE and the band size obtained was compared with standard protein marker (20-250 KDa protein ladder, Genei). Clear bands were seen below 100 KDa which is the largest protein and might be capsid protein present in phage. Multiple bands between 20 and 100 Kdal were observed.

Comparing our protein bands with the data of protein mass published on ASM (2018), Journal of Virology, we can make various presumptions about the proteins present in our phage P9. The presumed proteins along with their respective size are labeled in the figure above. Protein bands obtained in SDS show a wide variety of proteins present in our phage ranging from large capsid, scaffold, tail proteins to smaller tail tube proteins and low abundance virion proteins.

4.15. Activity of Phage in Presence of Metal Ions

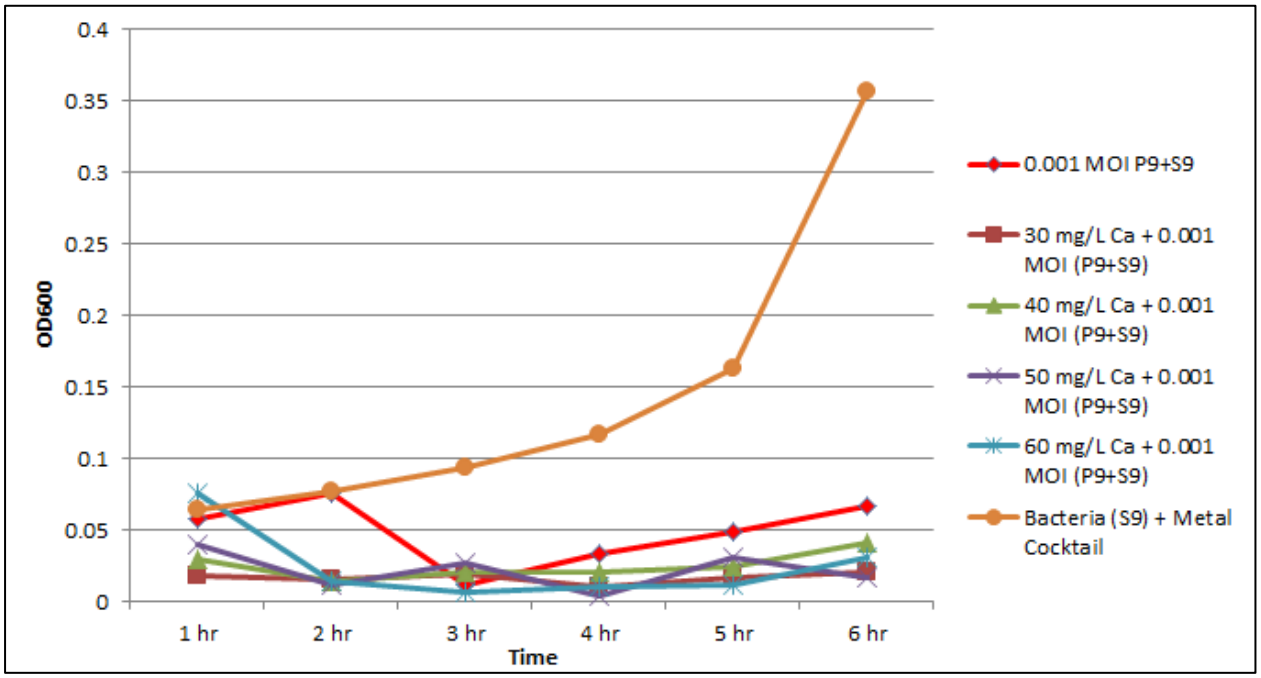


Fig 4.19: Activity of phage P9 against bacteria S9 at different concentrations of Calcium.

The phages in presence of Ca^{++} ions reduced the bacterial load after 6 hours of incubation as compared to the absence of calcium ions at MOI 0.001. Though the reduction was not very large as MOI 0.001 already caused high reduction in bacterial load (OD 0.066), it can be seen that the bacterial load in presence of calcium ions at all concentration is below the bacterial load without calcium ions (OD 0.02 at 30 mg/L Ca++, OD 0.041 at 30 mg/L Ca++, OD 0.017 at 50 mg/L Ca++ and OD 0.03 at 60 mg/L Ca++). The OD of bacteria in sample without phage was 0.357.

According to various previous studies, divalent metal ion like calcium increases phage activity and induce tolerance in phage particles. Gupta and Yin (1995) showed that divalent metal ions (Mg2+, Ca2+, Co2+, Ni2+) increased phage activity, in contrast to trivalents (Al3+ and Au3+) which destabilized phages. Our result is also in-line with the above finding due to increase in phage activity by calcium ions. Divalent ions are also utilized in the synthesis of phage where they act to promote topoisomerase and polymerase activity. This might be the reason for higher viral activity in the presence of metal ions.

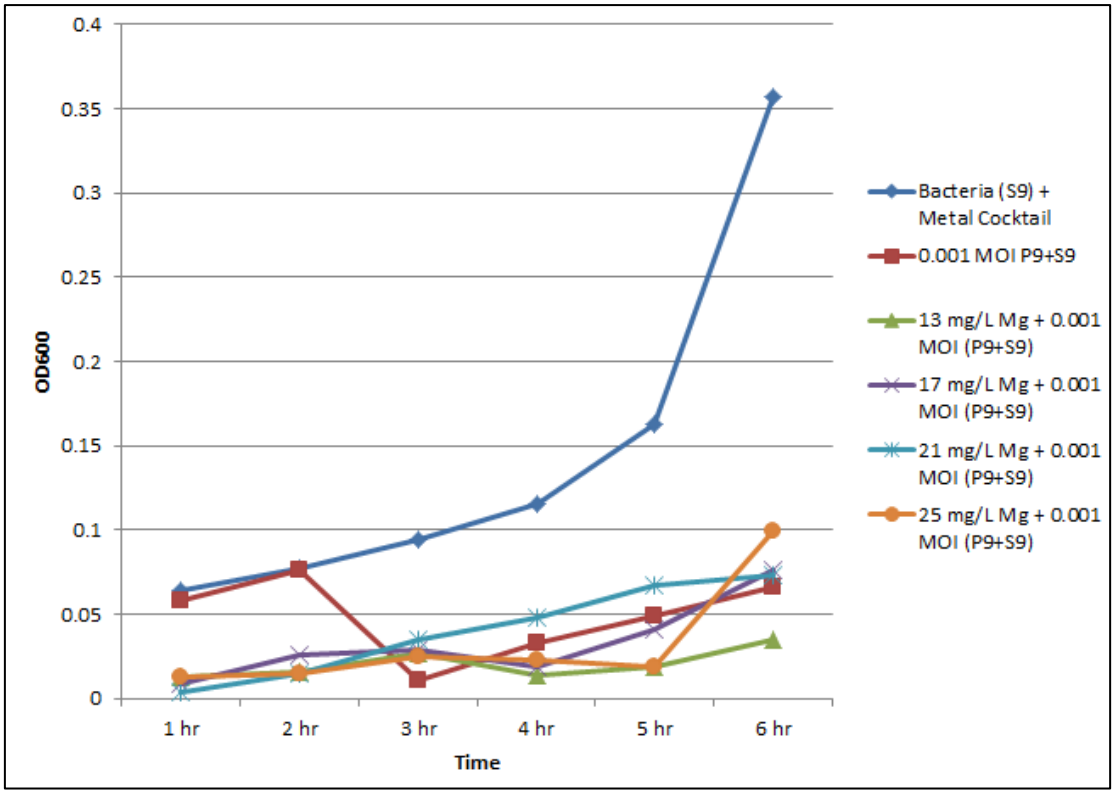


Fig 4.20: Activity of phage P9 against bacteria S9 at different concentrations of Magnesium.

In contrast to the increase in phage activity by calcium ion, magnesium (Mg^{++}) ion showed decrease in phage activity in higher concentrations. The phage activity only increased in presence of 13 mg/L (OD 0.035) magnesium while in presence of 17 (OD 0.076) and 21 mg/L (OD 0.073), the phage activity slightly decreased and in presence of 25 mg/L magnesium (OD 0.099), the phage activity actually decreased than that of non-metal treated sample (OD 0.066).

Although various experiments including experiments of Gupta and Yin (1995) indicated an increase in phage activity in presence of divalent metal ions like magnesium, our study showed different result.

The activity of phage was found to be inversely proportional to the concentration of magnesium ion in the solution. This result can be interpreted in two ways, one magnesium ion binds with phage and inhibits attachment or that magnesium ion binds in the receptor of bacterial membrane (guided by CorA, MgtA or MgtB transport systems in *Salmonella spp.*)(Moncrief & Maguire, 1998) and prevents phage binding. Either way, it is seen in the study that magnesium in higher concentration decreased the lysis of bacteria by phage.

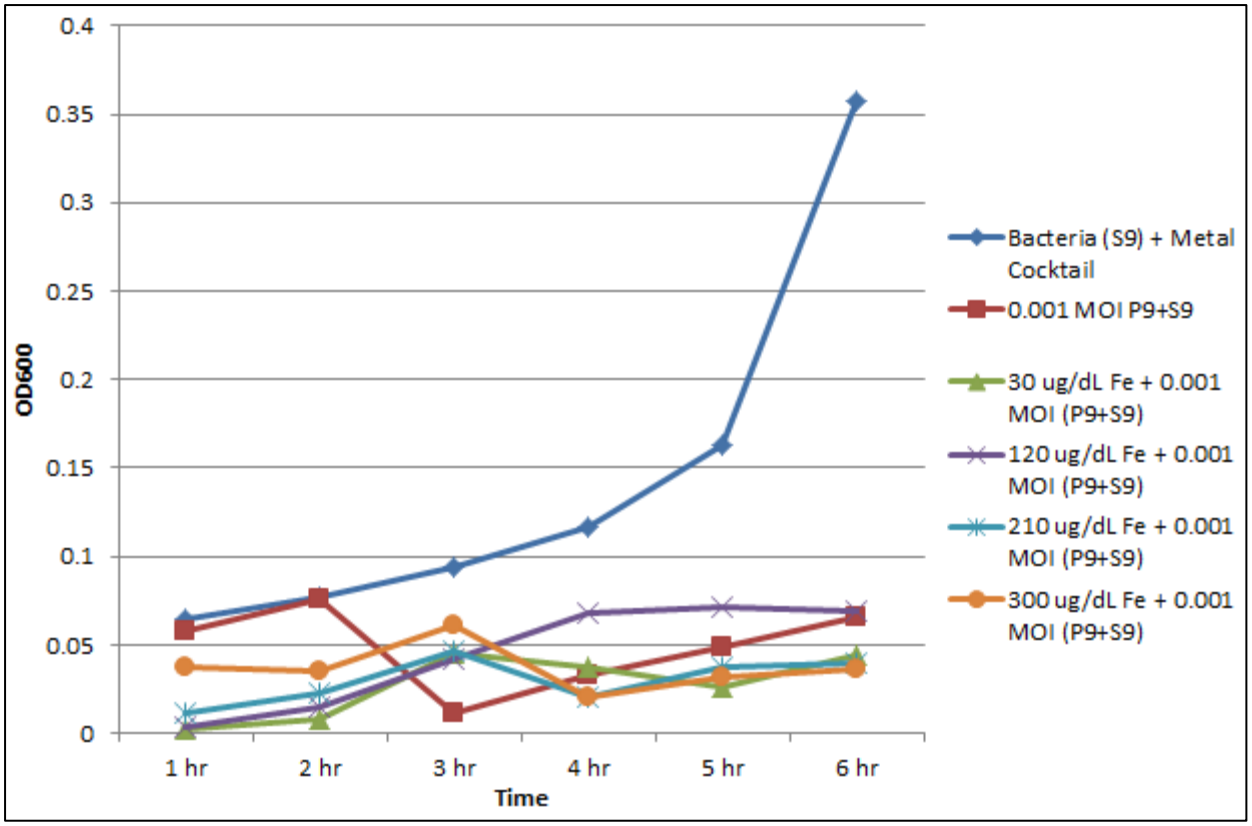


Fig 4.21: Activity of phage P9 against bacteria S9 at different concentrations of Iron.

The phages in presence of Fe^{++} ions reduced the bacterial load after 6 hours of incubation as compared to the absence of iron ions at 0.001 MOI. Though the reduction was not very large as MOI 0.001 already caused high reduction in the bacterial load, it can be seen that the bacterial load in presence of magnesium ions at all concentration is below the bacterial load without iron ions except at 120 mg/dL concentration in which the bacterial load seems same. The OD of bacteria in non-metal treated solution of phage at MOI 0.001 was 0.066, while OD in 30, 120, 210 and 300 ug/dL iron treated phage-bacterial solutions were 0.044, 0.069, 0.04 and 0.036 respectively.

Our result is in-line with the previous findings in different experiments which conclude that divalent metal ions increase phage lysis activity. The activity of phage remained particularly similar in presence of different concentrations of iron ions, which probably means that iron ion may act as catalyst to increase phage activity rather than binding in either bacteria or phage.

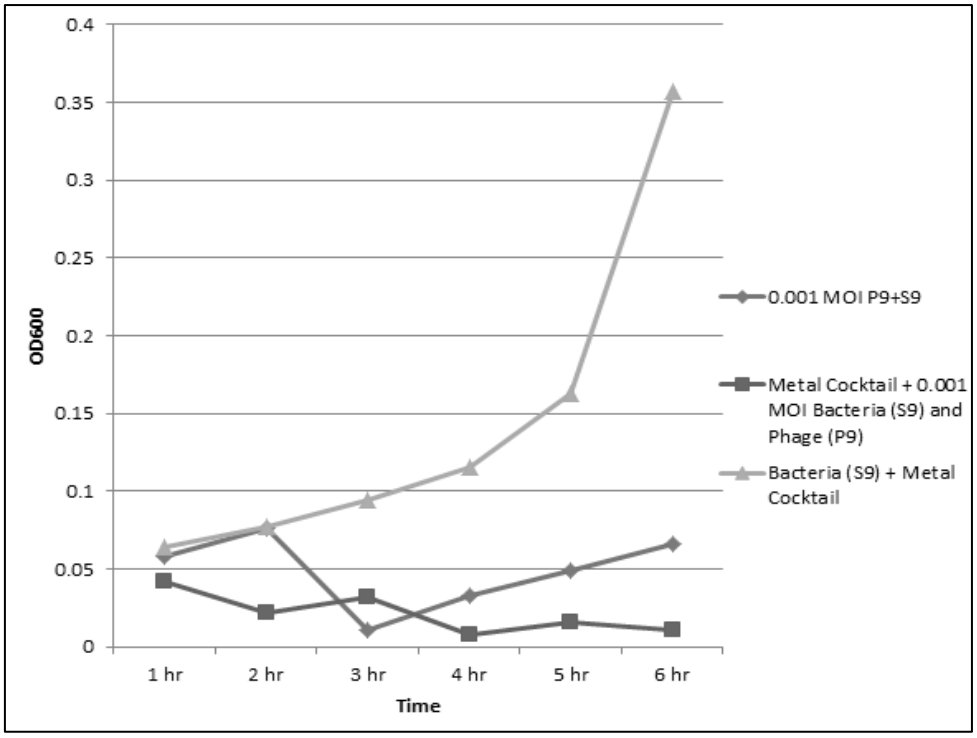


Fig 4.22: Activity of phage P9 against bacteria S9 at presence of metal ion cocktail and its comparison with bacterial growth without phage and without metal ions.

High difference in bacterial load can be seen in the presence of cocktail of metal ions in culture solution. The cocktail of all metal ions contained 45 mg/L calcium, 20 mg/L magnesium and 150 ug/dL iron ions. The figure above shows that there is large reduction in bacterial load in metal ion present solution (0.011 OD) as compared to phage treated bacteria without metal ions (0.066). This is a six fold decrease in bacterial load due to the presence of metal ions in the solution.

This shows that under the conditions of human blood in terms of presence of major metal ions, there can be great increase in phage activity, which will lead to rapid lysis of bacterial cells. This experiment also shows that metal ions in blood actually increases the phage activity and this phage can be further tested in blood provided that blood proteins and antibiotics do not render the phage inactive.

4.16. Activity of Phage in Raw Food Products

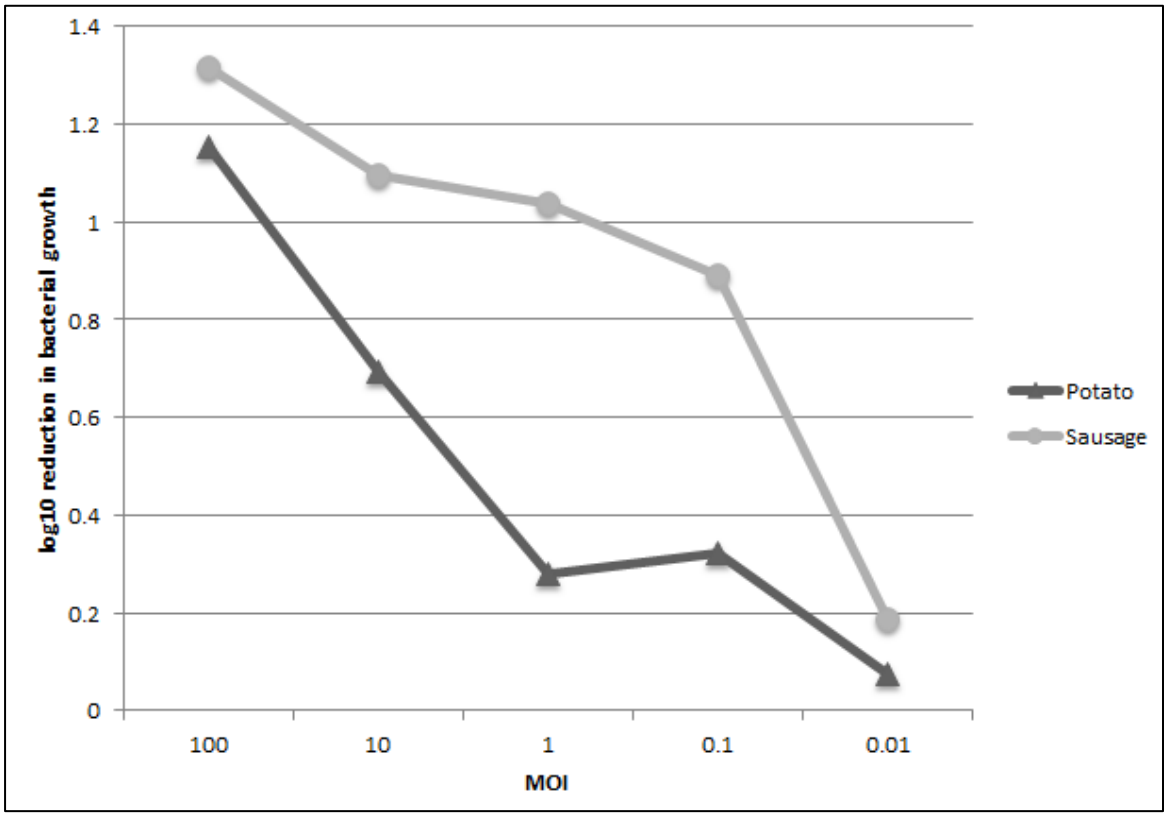


Fig 4.23: Comparative log Reduction of Bacterial growth (S9) in Potato and Sausage at different MOI of phage P9 and bacteria S9.

The initial load of bacteria taken was 100 CFU in both potato and sausage. After 4 hours of incubation, the bacterial load increased to 256 and 1200 CFU in untreated sample of potato and sausage respectively. At MOI 0.01 there was 0.19 $\log_{10}$ CFU reduction in bacterial load in sausage while there was only 0.07 $\log_{10}$ CFU reduction in bacterial load in potato. At MOI 0.1 there was 0.89 $\log_{10}$ CFU reduction in bacterial load in sausage while there was 0.32 $\log_{10}$ CFU reduction in bacterial load in potato. At MOI 1 there was 1.04 $\log_{10}$ CFU reduction in bacterial load in sausage while there was only 0.28 $\log_{10}$ CFU reduction in bacterial load in potato. At MOI 10 there was 1.1 $\log_{10}$ CFU reduction in bacterial load in sausage while there was 0.69 $\log_{10}$ CFU reduction in bacterial load in potato. Finally, at MOI 100 there was 1.32 $\log_{10}$ CFU reduction in bacterial load in sausage while there was 1.15 $\log_{10}$ CFU reduction in bacterial load in potato.

Huang et. al. (2018) in their study found out that at room temperature, the bacterial load reduction in sausage was 1.44 $\log_{10}$ CFU at MOI 1 and 2.37 $\log_{10}$ CFU at MOI 100. Our data, however, suggests only 1.04 $\log_{10}$ CFU and 1.32 $\log_{10}$ CFU bacterial load reduction at MOI 1 and MOI 100 respectively. An independent observation showed a 3

log_{10} reduction when a distinct phage was applied against Salmonella on Chinese cabbage and 1.7 log_{10} reduction on lettuce (Spricigo et al., 2013), 1.37 log_{10} on mustard and a 0.55 log_{10} reduction on broccoli (Pao et al., 2006).

This high degree of reduction of bacterial load in raw foods by our phage at room temperature indicates that our phage P9 is a potential candidate to be used as a disinfectant of *Salmonella* in raw food products. This bacterial load reduction along with heat and pH stability as well as activity against multiple isolates of salmonella makes phage P9 a highly usable biological agent to reduce *Salmonella* in raw food and prevent various food borne infections related to *Salmonella*.

CHAPTER 5: SUMMARY

Four samples of *Salmonella* were isolated from poultry and contaminated water sources from inside Kathmandu valley and biochemically identified and labeled S4, S5, S6 and S9. Antibiotic susceptibility test was done for all isolates and isolate S9 was found to be resistant up to third generation antibiotic Ceftazidime. The MDR isolate S9 was taken for further analysis and its doubling time was calculated and found to be around 25 minutes.

Bacteriophage isolation was done by taking water samples from polluted water sources and rivers around Kathmandu valley. DLAA method was used for bacteriophage isolation. For some water samples, pre-enrichment was done by adding S9 and LB in the supernatant of centrifuged water sample and allowing it to incubate at $37^{o}C$ for 24 hours. After DLAA, various bacteriophage plaques were seen and specific and single plaque was taken for further enrichment using phage streak method.

The phages were purified by phage streak method and later formed into stock phage by mixing the streaked pure phages in SM buffer. 4 phages were isolated and named P1, P3, P5 and P9. Activity of all these phages onto host S9 was studied and found that P3 phage was not very active in lysing the host. Phages P1 and P9 were found to be highly active in lysing the host S9. All the phage were also subjected to multiple bacterial isolates of *Acinetobacter, E. coli, Klebsiella, Pseudomonas, Proteus* and *Salmonella.* All phages lysed *Salmonella* isolates taken for study. *Klebsiella* K57 was lysed by phage Ph5. Phage Ph1, Ph5 and Ph9 showed highest activity against all *Salmonella* isolates. Out of these phages, phage Ph9 was taken for further analysis as it lysed only *Salmonella* isolates and it lysed them with very clear zone of plaque (minimal turbidity in plaques).

Titer of P9 stock was found to be $2x10^{6}$ PFU/ml using spot assay and DLAA afterwards. The Optimal MOI for complete lysis of host by phage infection was found to be 0.1 and higher. Effect of MOI on phage activity was also studied and all MOI up to 0.001 showed large decrease in bacterial load after 5 hours of incubation. pH and temperature sensitivity test was performed and our phage was found to be stable at wide range of pH and temperature from pH 3-11 and temperature up to $70^{o}C$. One-step growth curve assay was performed and the burst size of phage was found to be around 7 virions per infected host cell.

The activity of phage was tested in presence of various concentrations of metal ions (Ca^{++}, Mg^{++} and Fe^{++}) and it was seen that calcium and iron at all concentration enhanced phage activity while magnesium slightly inhibited phage activity at higher concentration. The cocktail of all metal ions showed high degree of enhancement in phage activity while comparing to the phage activity in solution without these metal ions. This result

shows that our phage will have increased activity in presence of metal ions level comparable to human blood. This in turn, indicates that our phage has potential to be used in therapeutic purposes and this can be tested in animal models for further verification and use cases.

Finally, activity of phage in raw food products was studied in which potato and sausage were taken as food products and various concentrations of phage and bacteria were inoculated into them and incubated at room temperature for 4 hours. Later the bacterial load in these food products was calculated by dissolving them in 5ml sterile water and spread plating. The data showed more than 1.04 $\log_{10}$ CFU reduction in bacterial load in sausage at MOI 1 and above while 1.15 $\log_{10}$ CFU reduction in potato was seen in only MOI 10. These high levels of reduction of bacterial load indicate that our phage has potential to be used as disinfectant in raw food products to control *Salmonella* infections.

This research will be primarily useful to food industries that are looking for an antibiotic or chemical alternative to reduce *Salmonella* load in their raw food products. This thesis will also be useful and can stand as a baseline to other researchers who aim to work in similar topics. Since, there are huge cases of increased *Salmonellosis* in Nepal as well as other countries, a biological remedy like this will be phenomenal and will also help to reduce dependency on chemicals (which may have unusual side effects).

CHAPTER 6: CONCLUSION

From this study, it can be concluded that divalent metal ion cocktail can be used to improve phage activity in lab condition. It can also be inferred that low concentration (around 13 mg/L) of magnesium should be used and higher concentration (around 60 mg/L) of calcium can be used to enhance phage activity. The increasing concentration of iron did not change phage activity but it was seen that iron even in low concentration (30 ug/dL) enhances phage activity.

It can also be concluded that the phage P9 has higher potential to be used in disinfection of raw food products either during storage or sale. Phage P9 showed large reduction (1.32 $\log_{10}$ in sausage and 1.15 $\log_{10}$ in potato) in *Salmonella* S9 load at room temperature. So, it can be used as a potential agent to reduce *Salmonella* load in raw food products and prevent food borne Salmonellosis.

CHAPTER 7: LIMITATIONS OF THE STUDY

As our study was conducted in the research lab of Central Department of Biotechnology, various requirements to conduct our study were not available in our lab and even due to time constraint various experiments could not be performed. Some limitations of our study are:

- Due to limited time, we could only collect small number of bacterial as well as phage samples.
- Only biochemical tests were performed to identify the isolated bacterial species. Molecular identification with specific primers could be used.
- Phage isolates were not purified with ultracentrifugation, so purified phage might contain small amount of impurities.
- Phages activities were only tested for a limited time frame (6 hours). A longer test time could have given us idea about degradation in phage activity over longer time periods.
- Activity of phage in food products in presence of metal ions could have been studied.

CHAPTER 8: RECOMMENDATION

Our study focused on isolation and identification of *Salmonella* and isolation and morphological characterization of phage only. Our phage P9 in this study has showed great potential to be used in raw food products to decrease *Salmonella* load. Also our phage has shown improved activity in presence of metal ions comparable to ionic composition in human blood. Based on these tests, further recommendations regarding this study are:

- Large scale assay on raw food products can be done and the change in texture, taste, smell and other characteristics of food products can be studied.
- In-vitro test of phage activity in whole human blood and blood plasma can be done to study the activity and efficiency of this phage in humans.
- Pharmacokinetic study of this phage can be conducted in mouse or animal model to study the response against phage by animals.
- Host pathogen interaction can be studied by gene analysis of phage and can be used in the synergistic application of this phage with other phages to improve efficacy and efficiency.
- This phage can be turned into a potential disinfectant to reduce *Salmonella* load in poultry farms and other areas.

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APPENDIX

1. Media composition/Reagent preparation

A) Luria Bertani (LB) Broth

Ingredients	Grams/litre
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Final pH (at 25°C)	7.5 ± 0.2

B) Tryptic Soy Broth (TSB)/Soybean-Casein Digest Medium – HiMedia

Ingredients	Grams / Litre
Pancreatic digest of casein	17.00
Papaic digest of soyabean meal	3.00
Sodium Chloride	5.00
Dextrose	2.50
Dibasic Potassium Phosphate	2.50
Final pH (at 25°C)	7.3 ± 0.2

C) Sodium-Magnesium (SM) buffer

Ingredients	Grams / Litre
Sodium chloride	100mM
Magnesium sulphate	10mM
Tris-HCL	50mM
Gelatin	0.01% (w/v)

D) Mueller Hinton Agar (MHA) – HiMedia

Ingredients	Grams / Liter
Meat, infusion solids from 300g	2.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3 ± 0.1

2. Reagents For SDS-PAGE

A) 30% acrylamide solution: (For 100ml)

Constituents	weight/volume
Acrylamide; C3H5NO	29g
Bis Acrylamide; C7H10N2O2	1g
TDW	Maintain upto 100ml

B) Casting Constituents

Solution components	Resolving gel (12%) :10ml	Stacking gel (5%) :3ml
TDW	3.3	2.1
30% Acrylamide	4	0.5
1.5% Tris (pH 8.8)	2.5	-
1.5% Tris (pH 6.8)	-	0.38
10% SDS	0.1	0.03
10% $(NH_4)_2S_2O_8$	0.1	0.03
TEMED	0.004	0.003

C) Tris buffer

Lower tris pH 8.8: for 100ml		
S.N.	**Constituents**	**Amount**
1	Tris (Tris base)	1.5M/18.17g
2	TDW	Maintain 100ml

Upper tris pH 6.8: for 50ml		
S.N.	**Constituents**	**Amount**
1	Tris (Tris base)	0.5M/3.03g
2	TDW	Maintain 50ml

D) Loading (Sample) buffer (pH 6.8): For 10ml

S.N.	Constituents	Amount (ml)
1	Upper Tris pH 6.8	1.25
2	10% SDS	3.0
3	Glycerol	4.75
4	Beta-mercaptoethanol	0.5
5	0.1% bromothymol blue	0.5

E) Staining solution CBB G-250: 500ml

S.N.	Constituents	Amount
1	CBB G-250	500mg
2	Glacial acetic acid	25ml
3	Methanol	250ml
4	TDW	225ml

F) Destaining solution: 500ml

S.N.	Constituents	Amount (ml)
1	7% glacial acetic acid	37.5
2	5% methanol	25
3	TDW	437.5

G) Running buffer/ Electrolysis buffer (pH 8.4): 1000ml

S.N.	Constituents	Amount
1	39mM tris	4.724g
2	48mM glycine	3.603g
3	0.1% SDS	0.37g