Characterization of newly identified bacterial biofilm degrading bacteriophage obtained from Dhaka city lake water samples

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelors of science in Biotechnology

Biotechnology Program

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Declaration

It is hereby declared that;

1. The thesis submitted is our original work while completing the Bachelor of Science in Biotechnology at BRAC University;
2. The thesis does not contain material previously published or written by a third party except for where this is appropriately cited through full and accurate referencing;
3. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has clearly been stated;
4. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution
5. All main sources of help have been acknowledged.

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Approval

The thesis titled **"Characterization of newly identified bacterial biofilm degrading bacteriophage obtained from Dhaka city lake water samples"** submitted by Munif Mahdee(ID: 19236007) and Syed Ridwan Abdullah(ID: 19236008) of Fall 2019, has been accepted as satisfactory in partial fulfillment of requirement for the degree of Bachelors of science in Biotechnology.

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Dedication

This thesis is dedicated to our respected supervisor, Dr. Iftekhar Bin Naser.

Acknowledgement

To begin, we would want to offer our deepest appreciation to God for blessing us with the chance to pursue this line of inquiry and for giving us the courage to see it through to fruition. Our thanks go out to Dr. Iftekhar Bin Naser, Associate Professor in the Biotechnology program, Department of Mathematics and Natural Sciences in BRAC University, without whom this study would not have been possible. We are really grateful to our supervisor for his unwavering support and enthusiasm throughout this study process, which has allowed us to develop as researchers. Thanks to his exceptional research skills, we were able to easily resolve some unexpected situations.

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Abstract

Recently acquired bacteriophages from water samples in Dhaka city which degraded the bacterial biofilms were characterized in this investigation. Bacteriophages unique to *E. coli* strain 9A(ϕ9A) and *Vibrio cholerae* strain 13B(ϕ13B) were identified. Throughout the process, strict laboratory protocols were followed, and the characterization was based on three variables: temperature, pH, and salinity. In the first step of the procedure, single colonies of host bacteria were obtained by streak plating, which was followed by enrichment of the corresponding bacteriophages. The Phage counts were then determined using the Double Layer Assay (DLA). To begin DLA, single colonies in LB broth were first taken and cultured in a shaker. The bacteriophages underwent a series of dilutions before the phages and the bacteria were added in the molten soft agar. After being centrifuged, the soft agar was quickly placed onto a Luria Bertani Agar plate. After evenly distributing the materials, the plate was swirled and until the agar solidified and was then placed in an incubator for 16 hours. The following day, the plaques were counted, and the number of Plaque Forming Units per Milliliter (PFU/ml) were determined. An average phage count was discovered after the experiment was conducted three times for each component. Both ϕ9A and ϕ13B displayed unusual results in temperature and pH readings in addition to salinity.

Chapter 1: Introduction

Escherichia and *Vibrio* are commonly detected in water sources that have been polluted with fecal matter from infected individuals or animals. These sources include private wells, lakes, rivers, and other bodies of water. There are various pathways via which waste can contaminate aquatic bodies, including sewage overflows, dysfunctional sewage systems, unclean storm water runoff, and agricultural runoff. According to research, the consumption of coliforms, including *Escherichia coli* (*E. coli*) and *Vibrio*, in contaminated water can lead to significant health implications such as diarrhea, enteritis, and potentially fatal outcomes. These results have been found to result in substantial economic losses.

The *Vibrio* genus of bacteria is a prominent constituent of aquatic environments, where it fulfills a significant ecological function in sustaining the equilibrium of the aquatic ecosystem. *Vibrio* bacteria are classified as gram-negative microorganisms, characterized by their elongated rod-like morphology, which can be either straight or curved. These bacteria typically range in size from 1.4 to 2.6 micrometers in length. *Escherichia coli* are gram-negative bacteria which do not form spores, and possess motility through the use of peritrichous flagella. Its presence is indicative of fecal pollution and through its introduction to water bacteriology, it serves as an important marker of food and water hygiene. *E. coli* is capable of developing biofilm to increase its survivability in adverse situations.

Bacteriophages are viruses which infect bacteria, and research regarding them is a field showing rapid growth and possesses great potential.

In regions characterized by moderate climates, there is a significant level of exploitation of lake and river ecosystems for both recreational and daily household purposes. Multiple studies have indicated that anthropogenic activities are the primary source of contamination in various ecosystems[1]. The issue of water contamination is a significant environmental concern in numerous countries worldwide[2]. The escalation of water contamination has been observed as a consequence of prolonged periods of development, mostly attributed to the discharge of agricultural runoff and the release of harmful industrial effluents[3]. In suburban areas, numerous industries emerge, resulting in the production of a significant volume of sewage annually. In addition to the aforementioned factors, secondary water pollution might arise as a consequence of inadequate sewage treatment practices[4].

For the given study, 7 water samples were collected from the summer season (March 22 - June 30) from Gulshan lake with the objective of obtaining biofilm degrading bacteriophages.

1.1 Background

Bacteriophages, commonly referred to as phages, are a class of viral agents that specifically target and infect bacterial cells[5]. The aforementioned organisms represent the largest population and exhibit the greatest genetic diversity among all living entities on Earth. Every bacterium harbors a range of 5 to 10 viruses[6]. Bacteriophages are widely prevalent in the natural environment and can be observed in several ecological niches that facilitate bacterial proliferation. The current understanding in the scientific community is that phages have a notable impact on the cycling of organic matter and the diversity of bacteria in the biosphere.

Additionally, they are believed to contribute to the maintenance of bacterial equilibrium within ecosystems[7]. It is widely believed that on a daily basis, bacteriophages are responsible for lysing approximately 10-20% of the bacterial community present in marine environments.

Phages, similar to other viruses, are obligatory intracellular parasites that depend on their host bacterial cell throughout their life cycle due to their absence of cell structure and enzyme system necessary for replication[8]. One of the processes through which bacteria can resist antibiotics is the development of biofilms, which can boost their virulence and make them more dangerous to humans and other organisms[9]. In that regard, Bacteriophages have also displayed the capacity to degrade bacterial biofilm and thereby reverting them into their planktonic state[10].

Hence, it is imperative to systematically observe and analyze the behavior of bacteriophages across several parameters, such as temperature, pH, and salt content. Based on the aforementioned data, it is possible to make predictions regarding the optimal circumstances for the degradation of bacterial biofilms.

1.1.1 Life Cycle of Bacteriophage

The life cycle of bacteriophages is divided into two cycles:

i) Lytic Cycle

ii) Lysogenic Cycle

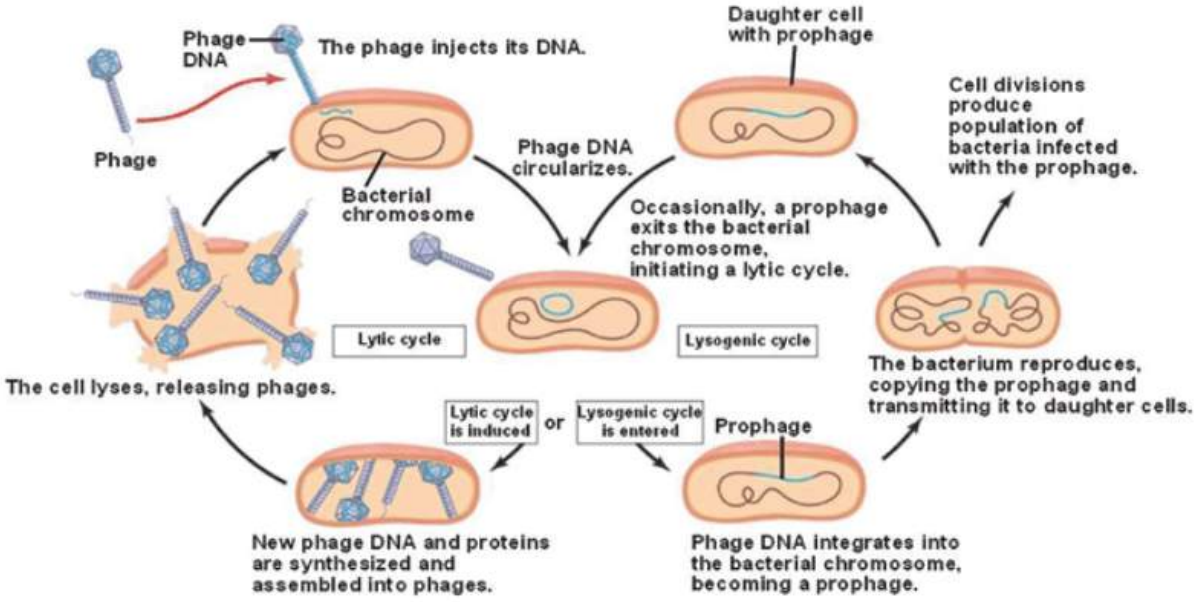


Fig 1.1.1: Life cycle of bacteriophage[11]

1.1.2 Lytic Cycle

Bacterial cells undergo lysis and subsequent destruction by lytic phages following the replication of the virion. The phage offspring are capable of promptly locating novel hosts for infection subsequent to the destruction of the host cell. T4, a bacteriophage that infects Escherichia coli in the gastrointestinal tract of humans, serves as an illustrative instance of a lytic bacteriophage. The utilization of lytic phages in phage therapy is more advantageous. In situations where extracellular phage concentrations are elevated, certain lytic phages may undergo a phenomenon referred to as lysis inhibition, wherein mature phage progeny may not immediately undergo cell lysis.

1.1.3 Lysogenic cycle

In contrast, the lysogenic cycle does not immediately lead to lysis of the host cell. Temperate bacteriophages are a class of bacteriophages that possess the capacity to enter into a lysogenic life cycle. The viral genome has the ability to integrate with the host DNA and undergo replication without causing harm, coexisting with the host genome. Alternatively, it may also establish itself as a plasmid within the host. The viral particles remain in a state of dormancy within the host until the host's conditions deteriorate, potentially as a result of a decrease in available food resources. It is at this juncture that the viral particles' internal phages, referred to as prophages, get activated. At this juncture, they initiate the reproductive process, resulting in the lysis of the host cell. The replication of the virus occurs in the progeny of all cells due to the lysogenic cycle, which enables the host cell to persist and multiply. Bacteriophage lysis occurs as a consequence of the buildup of phage-encoded lysozyme during late stages of protein synthesis.

1.1.4 Mechanism

Bacteriophages kill bacteria by causing them to burst or lyse. This happens when the virus interacts with the bacteria. A virus infects bacteria by introducing its genes (DNA or RNA) into the living thing.

Inside the bacteria, the phage virus duplicates (copies) itself. This allows each bacterium to produce up to 1000 more viruses. Finally, the bacteria are ruptured by the virus, allowing fresh bacteriophages to appear.

Only inside of a bacteria can bacteriophages proliferate and thrive. Until all of the bacteria have been lysed (killed), they will not cease to replicate. Like other viruses, bacteriophages can hibernate (go dormant) until fresh bacteria develop.

1.1.5 Bacteriophage isolation

Bacteriophages used for the experiment were given by M.Sc in Biotechnology student Afroza Khanam Anika, who had obtained said samples from Gulshan lake. The bacteriophages were isolated from the water samples and preserved in SM buffer for further use.

1.1.6 Bacteriophage enrichment

Enrichment of bacteriophages serves to increase the number of phages capable of infecting the intended host. This is achieved by removing endogenous bacteria from the sample and inoculating it with bacterial culture media and a growing culture of the desired host. The enriched bacteriophages were stored in LB broth at 4°C until further use.

1.1.7 Literature Review

Bacteriophages are capable of degrading bacterial biofilms which could be the major cause behind the seasonal outbreaks of diseases. The data presented in the given study provide evidence to support the assertion that bacteriophages specific for *Vibrio cholerae* have the capacity to impact the seasonal patterns of cholera outbreaks and perhaps contribute to the establishment of novel pandemic serogroups or clones of *Vibrio cholerae*.[10] Therefore, characterization of newly identified bacteriophages is imperative for comparison to the available data.

This characterization is an elaboration of previously conducted research in the respective fields of biofilm degrading bacteriophages. Afroza Khanam Anika, M.Sc in Biotechnology, collected water samples during the summer season (March 22 - June 30) from Gulshan lake, from where she isolated three bacteriophages which were capable of degrading bacterial biofilms in her thesis. Furthermore, Dr. Iftekhar Bin Naser identified a species of bacteriophage specific for *Vibrio cholerae* capable of degrading biofilm which was used by Afroza Khanam Anika as a basis for her thesis. Learning through further research about the newly identified bacteriophages is the key to discovering potential benefits in the field of phage therapy.

According to the research conducted by Dr. Iftekhar Bin Naser, toxic *Vibrio cholerae* live in aquatic ecosystems associated with biofilms embedded in exopolysaccharide matrices. The biofilm-associated cells often become conditionally viable environmental cells (CVEC), a dormant state which resists cultivation on standard bacteriological media. Various methods can spontaneously revive these cells into the active planktonic form, grow, and create cholera

epidemics. The given study examined the effects of environmental bacteriophages on pathogen prevalence and dispersion in biofilm and planktonic states.[10] However, he and his team of professionals was able to identify only *Vibrio* phage, JSF7 to be the only biofilm degrading bacteriophage.

Afroza Khanam Anika on the other hand, found three biofilm degrading bacteriophages during her thesis. Her investigation was not repeated or peer reviewed, which can be a sign of bias in her studies. As such, the purpose of this investigation is to provide characteristic data for reliable comparison in further research studies based on the original research of Dr. Iftekhar Bin Naser.

Chapter 2: Materials and Methods

2.1 Standard laboratory practices

❖ Excess agar should not be prepared without first consulting the superintendent or mentor.

❖ The remaining agar after plating agar into Petri plates should not be discarded. Existing Petri plates must be filled with remaining agar. If it is not possible to pour the remaining agar into existing Petri plates, the container should be securely sealed and refrigerated for future use.

❖ Culture plates and fresh plates must not be retained for more than two weeks in the refrigerator.

❖ Plates of culture cannot be stored in the incubator for longer than two days.

❖ Prepared and cultured media must be recorded.

❖ Media plates and containers must be properly labeled with the following information: name, media name, and preparation date.

❖ After weighing the reagents,

a. the scale, spatula, and adjacent areas must be cleaned prior to and after use.
b. The reagent must remain in its designated location.
c. The reagent container's cap must be closed; otherwise, the reagent will solidify when exposed to oxygen.

❖ Before and after work, the surface and adjacent area of the laminar flow hood must be cleaned.

❖ While performing laboratory work, one should don a lab coat.

❖ Avoid wearing lab coats outside of the laboratory.

❖ After completing a task, the burner must be turned off.

❖ If anything malfunctions during research, lab attendants must be notified immediately.

❖ One must exercise caution when using a spirit lamp.

❖ Following completion of work, the micropipette volume parameter must be adjusted.

❖ Dry waste should not be placed in the moist waste bin.

❖ Hair must be secured prior to entering the laboratory.

❖ Laboratory assistants should be notified of any damaged glassware or plasticware.

❖ Proper initial treatment must be administered after any wound or burn; otherwise, laboratory microorganisms will cause infection.

❖ Ethanol containers should not be stored near a flame or burner. Before and after work, an open workbench should be cleansed with a disinfectant (ethanol, Hexisol).

2.2 Materials

2.2.1 Materials for media preparation

- Distilled water
- Luria Broth Medium
- Luria Bertani Agar
- Sodium Chloride
- Yeast Extract
- Tryptone
- Bacterial Agar

2.2.2 Materials for buffer preparation

- Sodium Chloride
- Anhydrous Magnesium Sulfate
- Gelatine
- Hydrochloric Acid
- Sodium Hydroxide
- Tris-HCl

2.2.3 Apparatus

- Inoculating loop
- Inoculating needle
- Conical flask

- Syringe
- 0.22 micron filter
- Beaker
- Test tube
- Glass vial
- Microcentrifuge tube
- Aluminium foil paper
- Weighing Machine
- Centrifuge Machine
- Vortex Machine
- Micropipette
- Micropipette tips
- Falcon tubes
- Water bath
- Spirit Lamp
- Sterilizing fluid(Ethanol, Hexisol, Isopropanol)
- Laminar hood
- Incubator
- Shaker incubator
- Sterile petri plates
- p^H meter
- Autoclave/Sterilizer

2.3 Preparation of media, reagents and solutions

2.3.1 Saline Preparation

The composition of saline is 0.9% sodium chloride (NaCl) and water.

1) Two hundred milliliters of distilled water were measured and placed in a Duran bottle.
2) The reagent was measured by means of an analytical balance. 1.8 grams of salt reagent powder was extracted and measured on foil paper.
3) By shaking the vial in a circular motion, the reagent was mixed with the distilled water. until it dissolved.
4) Subsequently, it was autoclaved for two hours at 121 degrees Celsius and 121 pounds per square inch (PSI).
5) After two hours of autoclaving, the LB was removed from the machine and placed outside.
6) It must be cooled before use.

2.3.2 Luria Broth preparation

Luria-Bertani (LB) medium is a nutritionally abundant liquid medium utilized for the cultivation of bacteria. The composition of LB medium includes Tryptone, NaCl, and Yeast Extract.

1. A conical flask was filled with 500 ml of distilled water after being measured.
2. An analytical balance machine was used to measure the reagent. On a piece of foil paper, 10 gm of LB reagent powder were measured out.
3. Reagent was dissolved by shaking the conical flask in a circular motion while adding it to the distilled water.
4. The conical flask received a thorough swirl while being kept on a flame.
5. It was sterilized in an autoclave machine at 121 °C and 15 PSI for two hours after a brief boil.
6. The LB was removed from the autoclave after two hours and left outside to cool before use.

2.3.3 Luria Bertani agar preparation

For streaking of the inoculated stock bacteria, the use of Luria Agar (LA) media is necessary. LA media is a non-selective powdered media mainly used to routinely culture members of the Enterobacteriaceae family and for coliphage plaque assays. The medium is nutritionally rich for the growth of pure cultures of strains. Readymade LA media was used in this case which contains a mixture of tryptone, yeast extract, sodium chloride and 1.5% agar.

The media was prepared beforehand to minimize delay. To prepare 400 ml media, 16 gm of the powder was measured in an electronic balance machine and then suspended in 400 ml of distilled water. The mixture was then heated to boiling on a Bunsen burner to dissolve the medium

completely. It was sterilized by autoclaving for 2 hours. After cooling to 45-50°C, the media was poured into sterile petri dishes inside a laminar hood and allowed to solidify for a while. Since the media will be used for streaking, large plates were used. 16 large plates were prepared each time and the required amount was used and the rest of the plates were kept in the 4-degree Celsius fresh refrigerator.

2.3.4 Soft agar preparation

Soft agar is an essential media for performing spot tests or plaque assay. 100ml of soft agar was prepared beforehand each time. To prepare 100ml of soft agar, 1gm of NaCl and Tryptone, 0.5gm of yeast extract and 0.6gm bacteriological agar was measured in the weighing machine, and then suspended in 100 ml of distilled water. The mixture was then heated to boiling on a Bunsen burner to dissolve the medium completely. It was then poured into clean test tubes in 6 ml aliquots. Finally, the test tubes containing soft agar were sterilized by autoclaving for 2 hours. The required amount was used, and the rest of the plates were kept in the 4 degree Celsius fresh refrigerator.

2.3.5 Salt Magnesium buffer preparation

For 50ml SM buffer, 0.2 gm NaCl, 0.1 gm MgSO4, 1M 2.5 ml Tris-HCl and 0.5 gm gelatin is required. To prepare the SM buffer, at first Tris-HCl needs to be prepared (approximately 30 ml). From that, 2.5 ml Tris-HCl had been used for the SM buffer. For Tris-HCl preparation, Tris base needs to be prepared and pH needs to be checked and lowered to 8 from 10 by adding drops of HCl. Since 1 ml Tris-HCl is 121.14 g/mol, 3.63 gm Tris was added in 30 ml distilled water to prepare the base. pH value was kept at 8 before autoclaving as after autoclave, it will lower to 7.5. Now, 0.2 gm NaCl, 0.1 gm MgSO4, and 0.5 gm gelatin was measured in the weighing balance and 47.5 ml distilled water was added to it. Then, from the Tris-HCl, 2.5 ml was added and this is how the 50 ml SM buffer was prepared. Finally, autoclave the Tris-HCl and SM buffer and store at room temperature.

2.4 Enrichment of isolated phage:

The isolated phages go through the same enrichment procedure as the bacteriophages in the sample. 50 microliters of isolated phage that had been earlier stored in SM buffer at 4°C were added and further incubated in the shaker incubator for 4 hours after the host bacterial cells (3 ml of LB) had been cultured there for 1.5 hours. The mixture was incubated, and then centrifuged at 13,000 rpm for 10 minutes to separate the enhanced phages suspended in the supernatant from the bacterial cells as pellets. The host bacteria name and the associated phage (provided in brackets) were pre-labeled on the eppendorf tubes, such as "E.coli 15C (10a)" etc. After being filtered using a 0.22-micron syringe filter, the supernatant of each falcon tube was then collected in the fresh eppendorf tubes in the appropriate manner. To avoid contaminating the 0.22 micron filter, caution must be exercised.

To enhance the volume of the phage in case it were to become completely consumed, the enrichment procedure for the isolated phage was repeated. Additionally, for better results, double layer assay requires an enhanced phage solution for subsequent steps of estimating the multiplicity of infection. For future use, the enhanced phage stock was always kept at 4°C.

2.5 Spot test of isolated phage:

The spot test was repeated using the purified phages on the bacterial strains that exhibited observable plaques. Each of these strains was inoculated separately in 3 mL of fresh Luria-Bertani (LB) medium. Subsequently, the vials containing Luria-Bertani (LB) medium were placed in a shaker incubator for a duration of 1.5 hours. During this period, there was a significant presence of huge Los Angeles plates.

The samples were collected in a systematic manner and afterwards identified by assigning them to certain bacterial strains. The aliquots of soft agar that were stored in the refrigerator were heated to their melting point through boiling and afterwards maintained at a precise temperature of 55°C.

Following the designated incubation period, a portion of soft agar was extracted from the water bath. Subsequently, a volume of 200 µl from a culture containing young individual bacteria was promptly introduced into the agar, following a 15-second interval. Subsequently, the solution was adequately homogenized. The suitable LA plate was selected, and the contents of the tube were carefully poured onto the surface of the LA agar to establish a uniform bacterial lawn. The plate was gently swirled in order to evenly spread the molten agar across its surface. Subsequently, the

plate was set aside to allow for the solidification of the bacterial lawn. The aforementioned procedure was subsequently replicated for the remaining plates.

Once the soft agar layer had undergone desiccation, the plates were appropriately labeled with numerical identifiers corresponding to the bacteriophage samples. A volume of 15 µl of the pure phage suspensions, which were stored in SM buffer, was applied onto the top agar layer. The application was performed on indicated labels, and the plate was thereafter left to dry for a duration of 30 to 40 minutes. Following the evaporation of the droplets, the plates were subsequently incubated at a temperature of 37°C for the duration of the night. The following day, an assessment was conducted to determine the presence of distinct regions of transparency within the samples.

For the experiment, the following host bacteria and their specific phages were utilized,

Host bacterial strain	**Specific Phage**
Escherichia coli 9A	ϕ9A
Vibrio cholerae 13B	ϕ13B

2.6 Double Layer Assay

The Double Layer Assay, commonly referred to as the plaque assay, is a frequently employed methodology in phage investigation that facilitates the isolation and characterization of bacteriophage populations at a state of purity. The aforementioned strategy is commonly recognized as the conventional methodology for assessing phage concentration. The determination of bacteriophage concentration can also be achieved through the utilization of phage titration methodology. The quantification of the phage titer, denoted as plaque forming units (pfu), was conducted using the DLA method. Furthermore, the quantification of viable bacteriophages plays a pivotal role in establishing the phage titer, which is essential for accurately and consistently evaluating their effectiveness in future research initiatives. The classic phage assay (PA) is based on the fundamental premise of the interaction between a single lytic phage molecule and a permissive bacteria. This interaction leads to the lysis of the host bacterium and the subsequent release of freshly produced phage progeny. The resumption of host bacterial cell development occurs when a mixture of phage and their host bacteria is placed into liquefied soft agar and later transferred onto nutrient agar, hence promoting bacterial proliferation. When the bacteriophage is not present, the agar plate demonstrates the formation of a lawn in the soft agar overlay, and the bacteria transition into the stationary phase of their growth curve. Once again, the locations in which bacteriophages are present display a clearly discernible transparent region within the soft agar overlay, which is characterized by a multiplicity of phages being released. This region is perceptible to the unaided human eye. The term used to denote the circular region or clean zone is commonly known as "plaque." Plaques are counted, and the abundance of phages, often known as the titer, is usually measured as the number of plaque-forming units (PFU) per milliliter (mL) of the sample under evaluation.

Phage titer (PFU/ml) = Number of plaques (PFU) / (Dilution factor x Volume of phage added to plate)

In order to assess the susceptibility of different bacterial strains to their respective phages, nascent bacterial cells are cultivated in a shaker incubator at a temperature of 37 degrees Celsius and a speed of 160 rpm for a duration of 1.5 hours, using fresh LB medium. This phenomenon is commonly referred to as youth culture.

To initiate the Double Layer Assay (DLA), the first step involves cultivating a youthful population of the host bacteria, from which the required plaques are to be enumerated. Subsequently, the phages corresponding to the host bacteria are treated to a series of dilutions in saline solution, ranging from 10-6 to, in certain instances, 10-8.

Dilution refers to the process of reducing the concentration of bacteriophages prior to inoculation by taking 100 microliters of bacteriophage stock and adding it to 900 microliters of saline (or SM Buffer of varying p^H to assess the effect of p^H on bacteriophage activity). When checking the effect of temperature on bacteriophage activity, the microcentrifuge tubes containing the diluted bacteriophage solution were placed in the water bath at the designated temperature for 2 hours prior to carrying out the double layer assay.

Following the dilution of the phage, a volume of 100 microliters of the phage solution is extracted and combined with 300 microliters of a young culture of host bacteria. This mixture is then added to 6 ml of molten soft agar contained within a sterile test tube. The temperature of the

molten soft agar is meticulously regulated at 55 degrees Celsius to prevent premature solidification.

The components, including the phage solution, a young culture of host bacteria, and molten soft agar, are thoroughly combined and afterwards placed onto Luria-Bertani agar plates. It is important to exercise caution throughout the pouring process in order to prevent the creation of bubbles, as the presence of bubbles in the agar plate may lead to misinterpretation as plaques. The plates are allowed to undergo solidification and thereafter placed in an incubator set at a temperature of 37 degrees Celsius for an overnight duration. Following an incubation period of many hours, the plates are meticulously examined to assess the presence of plaque formation. Following the acquisition of plaques, the calculation of PFU/ml is performed. Typically, plaques within the range of 30-300 are regarded as typical and are included in the count when plaque formation occurs.

Chapter 3: Results

3.1 Effect of Temperature on Bacteriophage activity

The following figures show the effect of temperature on bacteriophage activity when DLA was carried out at 25℃, 35℃ and 45℃.

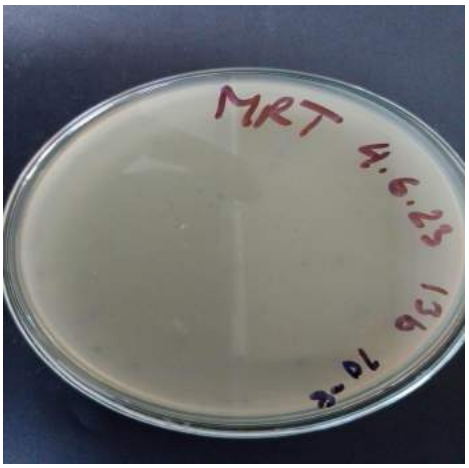


Fig 3.1 (a): ϕ13B At 25℃, 30 plaques

Fig 3.1 (b): ϕ9A At 25℃, 206 plaques

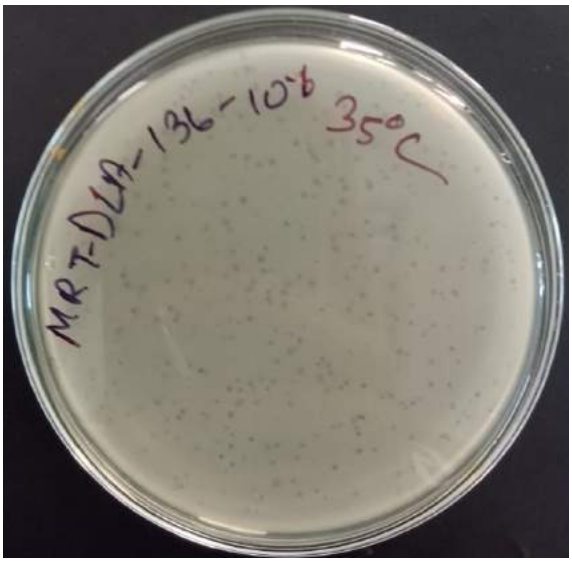


Fig 3.1 (c): ϕ13B At 35℃, 276 plaques

Fig 3.1 (d): ϕ9A At 35℃, 288 plaques

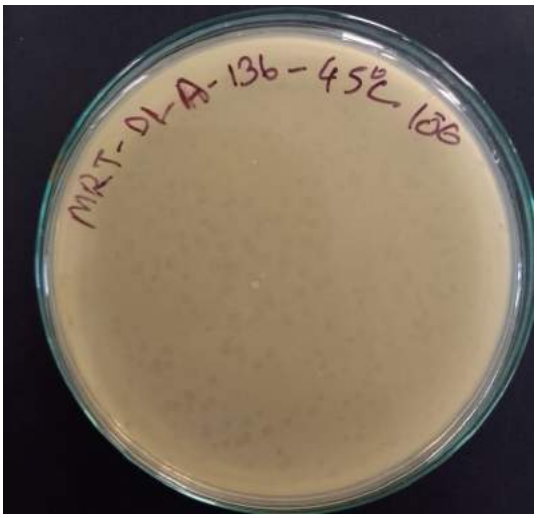


Fig 3.1 (e): ϕ13B At 45℃, 264 plaques

Fig 3.1 (f): ϕ9A At 45℃, 4 plaques

DLA was also carried out at 65℃, but no plaques were obtained at any dilution of the bacteriophages between 10^{-5} to 10^{-8}.

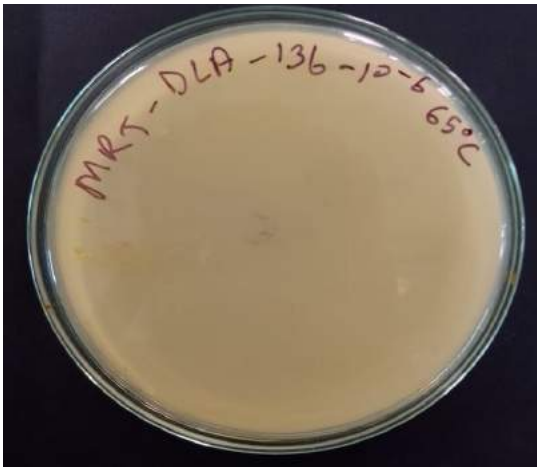


Fig 3.1 (g): ϕ13B At 65℃, No plaques

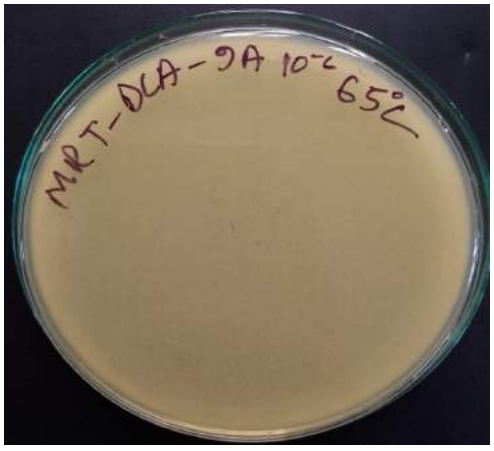
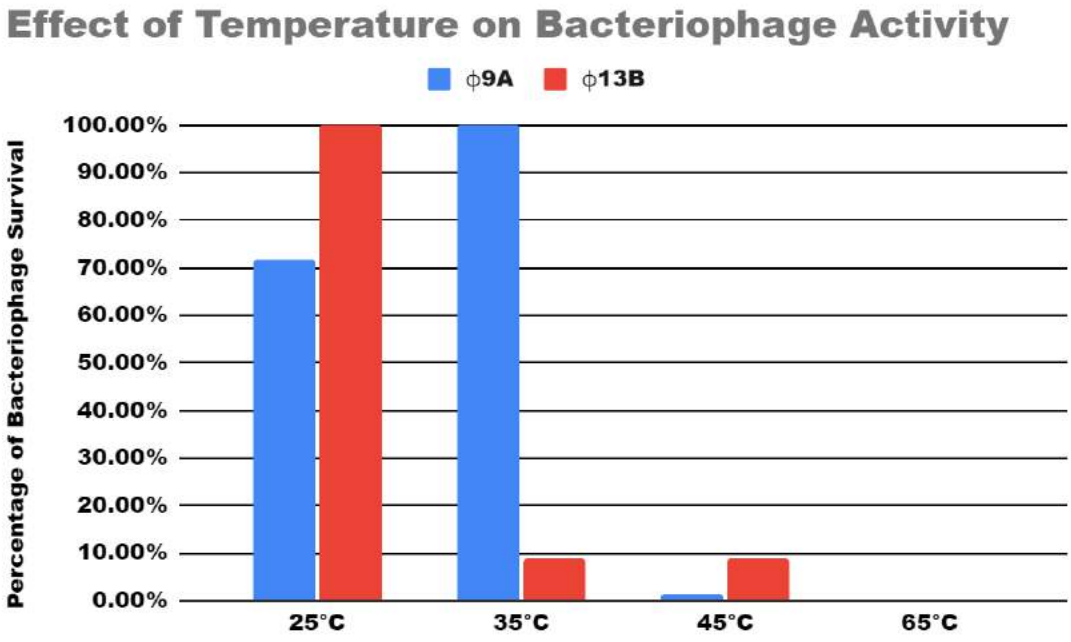


Fig 3.1 (h): ϕ9A At 65℃, No plaques

Bacteriophage	Average number of Plaques	Dilution Factor	Phage Count (pfu/mL)
25℃			
9A	206	10^{-6}	2.06×10^{9}
13B	30	10^{-8}	3.0×10^{10}
35℃			
9A	288	10^{-6}	2.88×10^{9}
13B	276	10^{-6}	2.76×10^{9}
45℃			
9A	4	10^{-6}	4×10^{7}
13B	264	10^{-6}	2.64×10^{9}
65℃			
9A	No plaques	10^{-6}	N/A
13B	No plaques	10^{-6}	N/A



3.2 Effect of pH on Bacteriophage activity:

The following figures show the effect of pH on bacteriophage activity when DLA was carried out at pH 3, pH 5, pH 7 and pH 9.

Fig 3.2 (a): ϕ13B At pH 3.0, 9 plaques

Fig 3.2 (b): ϕ9A At pH 3.0, 56 plaques

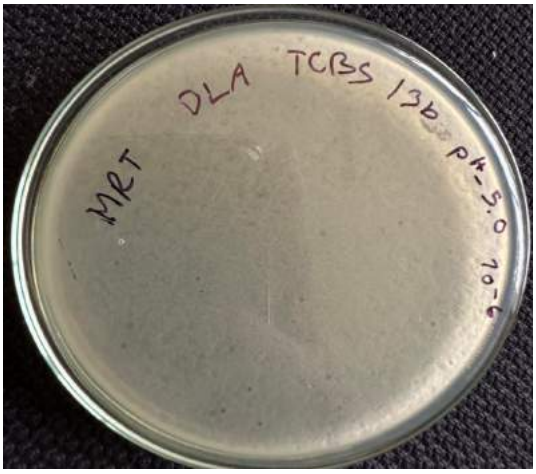


Fig 3.2 (c): ϕ13B At pH 5.0, 23 plaques

Fig 3.2 (d): ϕ9A At pH 5.0, 83 plaques

Fig 3.2 (e): ϕ13B At pH 7.0, 25 plaques

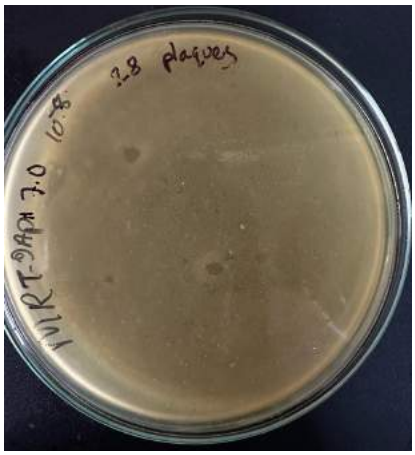


Fig 3.2 (f): ϕ9A At pH 7.0, 28 plaques

Fig 3.2 (g): ϕ13B At pH 9.0, 13 plaques

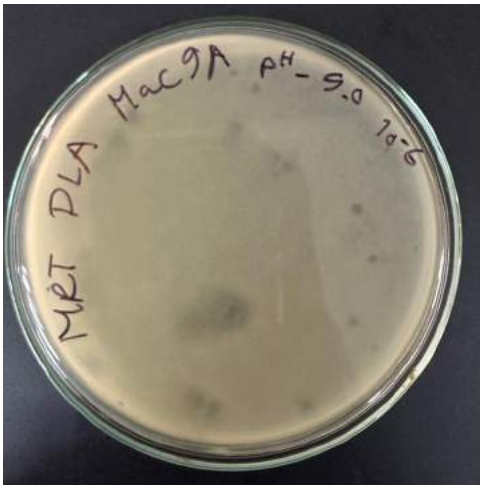
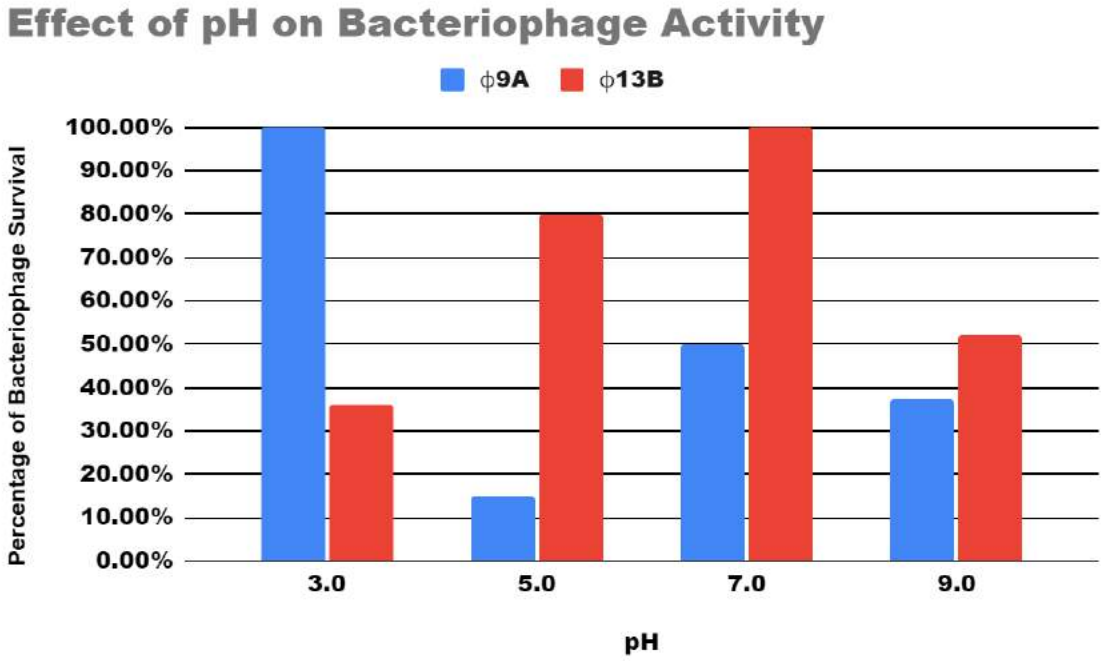


Fig 3.2 (h): ϕ9A At pH 9.0, 21 plaques

Bacteriophage	Average number of Plaques	Dilution Factor	Phage Count (pfu/mL)
p^H 3.0			
9A	56	10^{-6}	5.6×10^{8}
13B	9	10^{-6}	9×10^{7}
p^H 5.0			
9A	83	10^{-5}	8.3×10^{7}
13B	20	10^{-6}	2.0×10^{8}
p^H 7.0			
9A	28	10^{-6}	2.8×10^{8}
13B	25	10^{-6}	2.5×10^{8}
p^H 9.0			
9A	21	10^{-6}	2.1×10^{8}
13B	13	10^{-6}	1.3×10^{8}



3.3 Effect of Salinity on Bacteriophage activity:

The following figures show the effect of salinity on bacteriophage activity when DLA was carried out at salinity 3%, 5%, 7% and 10%.

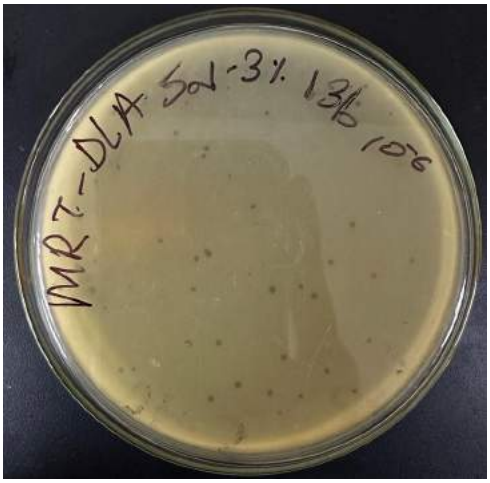


Fig 3.3 (a): ϕ13B At 3% salinity, 57 plaques

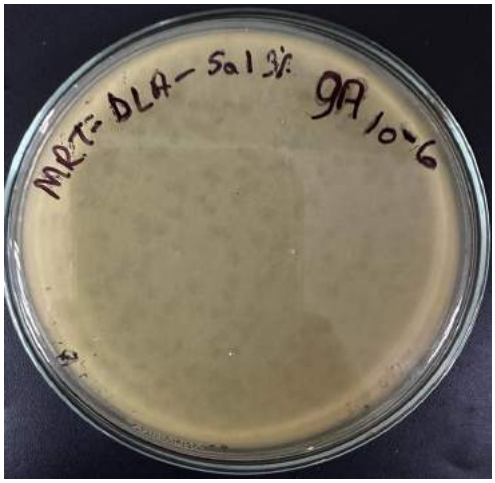


Fig 3.3 (b): ϕ9A At 3% salinity, 128 plaques

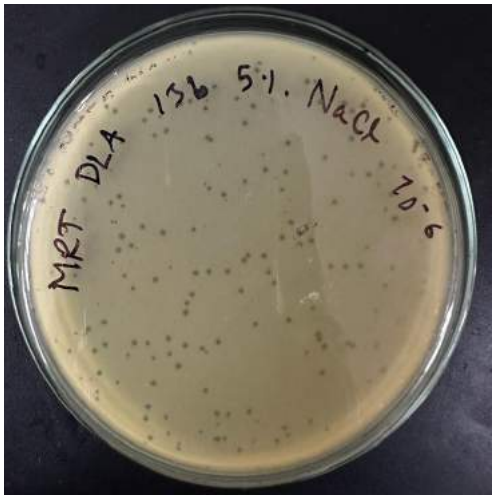


Fig 3.3 (c): ϕ13B At 5% salinity, 204 plaques

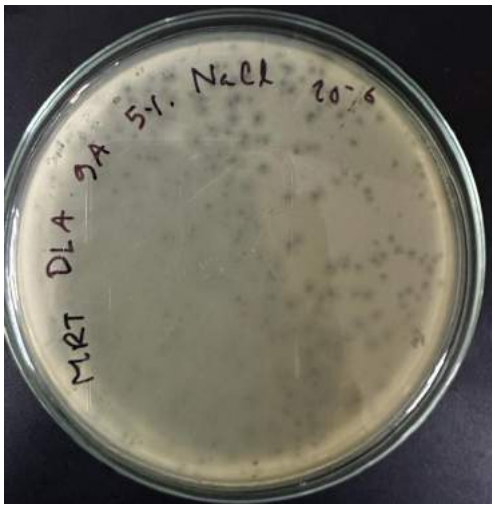


Fig 3.3 (d): ϕ9A At 5% salinity, 162 plaques

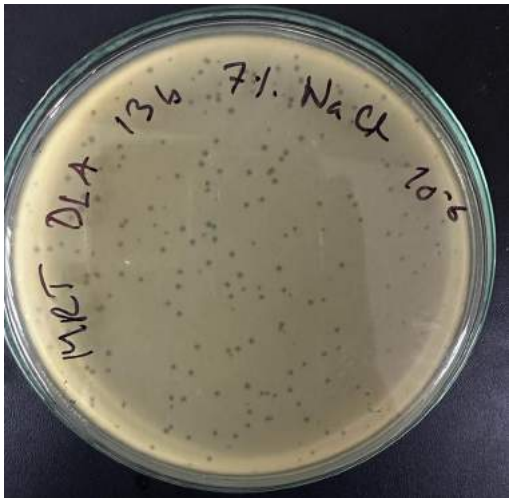


Fig 3.3 (e): ϕ13B At 7% salinity, 234 plaques

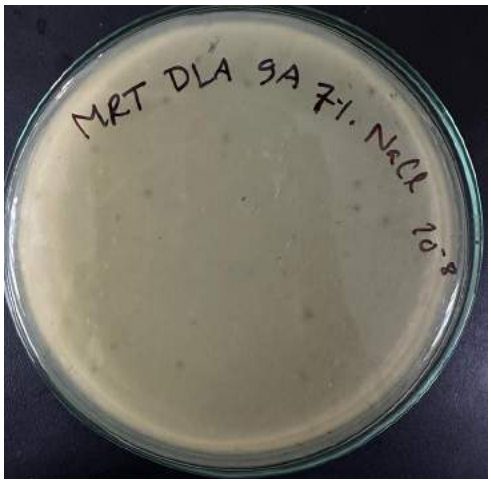


Fig 3.3 (f): ϕ9A At 7% salinity, 29 plaques

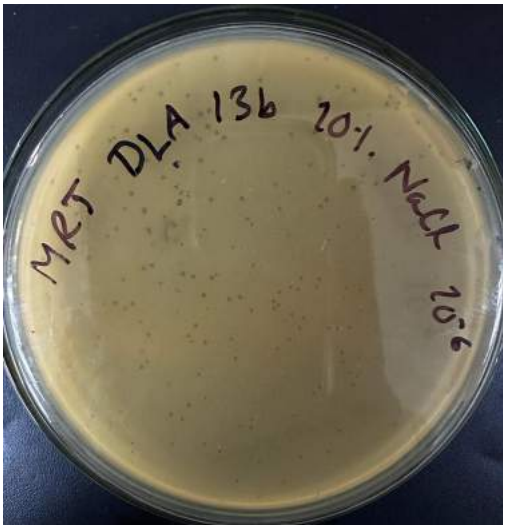


Fig 3.3 (g): ϕ13B At 10% salinity, 202 plaques

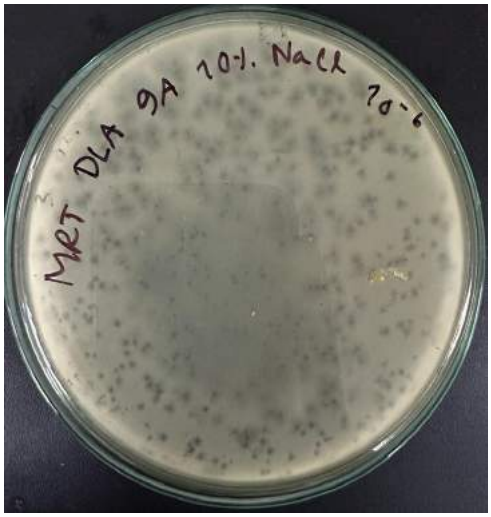
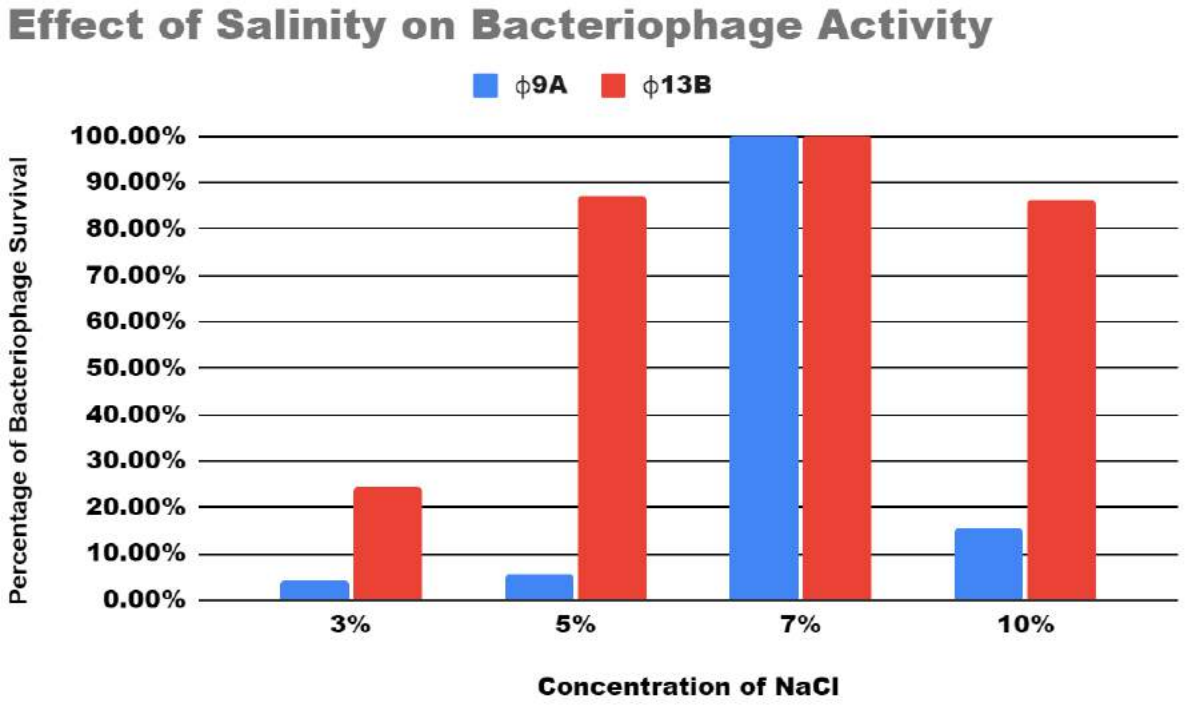


Fig 3.3 (h): ϕ9A At 10% salinity, 444 plaques

Bacteriophage	Average number of Plaques	Dilution Factor	Phage Count (pfu/mL)
3% Salinity			
9A	128	10^{-6}	1.28×10^{9}
13B	57	10^{-6}	5.7×10^{8}
5% Salinity			
9A	162	10^{-6}	1.62×10^{9}
13B	204	10^{-6}	2.04×10^{9}
7% Salinity			
9A	29	10^{-8}	2.9×10^{10}
13B	234	10^{-6}	2.34×10^{9}
10% Salinity			
9A	444	10^{-6}	4.44×10^{9}
13B	202	10^{-6}	2.02×10^{9}



Chapter 4: Discussion

4.1 Observed Activity of Bacteriophages

Both ϕ9A and ϕ13B have their own active temperature ranges that differ from one another. The peak activity of ϕ13B occurs at 25°C and remains elevated up to 45°C. When temperatures rise above 35°C, the effectiveness of ϕ9A begins to deteriorate. Based on the results, it appears that temperatures above 35°C may impede the growth of ϕ9A, as its activity decreases above that point. At temperatures of 65°C, neither bacteriophage can survive. Denaturation of bacteriophage enzymes at temperatures of 65°C and above may render them incapable of replicating inside their bacterial hosts.

Activity of ϕ9A fluctuates along with increasing p^H, being the most active at a p^H of 3.0. An acidic environment may allow the active site of ϕ9A reverse transcriptase to bind with the circular DNA of *E. Coli* with greater affinity. Conversely, ϕ13B remains the most active at a p^H of 7.0. Acidic or alkaline environments may adversely affect the survival of ϕ13B, which may explain the reduction of activity following an increase or decrease in p^H from 7.0.

Both ϕ9A and ϕ13B show optimum activity at a NaCl concentration of 7%. Research indicates that the effectiveness of phage therapy in aquaculture increases along with an increased salt concentration[13], which may explain the increase in phage activity from 3% salinity up to 7% salinity, however, it does not explain the subsequent decrease in bacteriophage activity when the salt concentration is increased to 10%.

4.2 Limitations and Recommendations

During the thesis, the major challenge that we faced was the lack of equipment provided. Equipment such as test tubes, petri dishes, glass vials, etc. were the key equipment for our daily work. However, due to shortage of these supplies the duration of our thesis was longer than expected. There were regular fungal contaminations inside the laminar air flow cabinets which contaminated the culture plates and affected the end results.

The Double Layer Assay (DLA) results which were found as plaques often overlapped one another which caused difficulty during PFU counting. There were faulty equipment such as the pH meter, auto-clave machine, weighing machine, water-bath, etc. To elaborate, the auto-clave machine had to be used for 2 and half hours instead of 15 minutes which drastically increased the duration of a single day's work, overall decreasing efficiency.

The overcrowded lab is the sole reason behind the shortage of equipment. If the university authorities could look into the matter, and rather than increasing funding, if they could focus more on building space to accommodate the large number of students, then the chaos within the lab would subsequently decrease. The laboratory contamination can only be prevented by more regular decontamination activities by the laboratory heads. In order to get accurate results for DLA, we had to repeat the process several times. Faulty equipment should be removed and replaced as soon as possible which would make conducting experiments much more efficient.

Chapter 5: Conclusion

The activity ranges of ϕ9A and ϕ13B vary at different temperatures. The optimal activity of ϕ13B occurs at 25°C, and it remains active up to 45°C. ϕ9A is only active at temperatures below 35°C, and its activity decreases as the temperature rises. At 65°C, neither bacteriophage is active.

In general, it can be observed that the optimal activity of ϕ9A is at pH 3.0 and that of ϕ13B is between pH 5.0 and pH 7.0 (slightly acidic to neutral). The optimal pH range for bacteriophage activity is typically between 5.0 and 9.0[12]. An experiment previously conducted shows the phage was subjected to a wide range of pH and temperature conditions. In comparison to pH 6, the phage was significantly more active at pH 7 and 8[14]. In contrast to other bacteriophages, ϕ9A exhibits increased activity at an acidic pH of 3.0, a result that is unique.

At a salinity of 7%, both ϕ13B and ϕ9A demonstrated their highest levels of activity. ϕ9A displayed an excessive number of plaque forming units at this percentage concentration of salinity, whereas ϕ13B demonstrated a marginally increased level of activity in comparison to 5% and 10%. The findings are consistent with those of earlier studies involving bacteriophage activity.

5.1 Future Prospects:

With the increase of antibiotic resistance in pathogenic bacteria, the use of bacteriophages for the elimination of pathogenic bacteria shows much potential. *E. coli* and *Vibrio* are the causative organisms behind major diseases, and their growing resistance to antibiotics warrants the establishment of an alternative to prevent their spread among underdeveloped and developing countries. There is therapeutic promise in the use of a combination of phages to eliminate bacteria that are difficult to eradicate in the food business. Disinfectants based on phages and phage-lysing proteins that can eliminate biofilms hold great potential[15].

The agricultural and petroleum industries could benefit from the employment of phages as biocontrol agents. In addition, phages are employed as DNA and protein vaccine delivery systems, pathogen detection systems, and as a system for displaying a wide variety of proteins and antibodies. Since bacteriophages can be easily manipulated, they may find applications in biotechnology, scientific study, and medical treatment.[16]

The mechanism behind the function of bacteriophages and their genome are yet to be fully understood. Further research is needed to expand the scope of the application of bacteriophages, and overcoming the boundaries set by bacteriophages on the control over biofilm is a necessity. Enzymes present within bacteriophages which are yet to be isolated have the potential to be drugs capable of dealing with bacterial biofilm. If the correct steps are taken, then bacteriophages can be established as a green alternative to antibiotic drugs for the prevention and control of biofilms. Restriction Fragment Length Polymorphism(RFLP) and nonspecific degradation experiments can assist in this regard.

References

1. Goel, V., Bell, G. J., Sridhar, S., Islam, M. S., Yunus, M., Ali, M. T., ... & Emch, M. (2020). Considering Alternate Pathways of Drinking-Water Contamination: Evidence of Risk Substitution from Arsenic Mitigation Programs in Rural Bangladesh. *International Journal of Environmental Research and Public Health*, *17*(15), 5372.

2. Xu, T., Coco, G., & Neale, M. (2020). A predictive model of recreational water quality based on adaptive synthetic sampling algorithms and machine learning. *Water research*, *177*, 115788.

3. Hanna, N., Purohit, M., Diwan, V., Chandran, S. P., Riggi, E., Parashar, V., ... & Lundborg, C. S. (2020). Monitoring of water quality, antibiotic residues, and antibiotic-resistant escherichia coli in the kshipra river in india over a 3-year period. *International Journal of Environmental Research and Public Health*, *17*(21), 7706.

4. Shi, Z., Qin, S., Zhang, C., Chiu, Y. H., & Zhang, L. (2020, April). The impacts of water pollution emissions on public health in 30 provinces of China. In *Healthcare* (Vol. 8, No. 2, p. 119). MDPI.

5. Abedon, S. T. (2012). Bacterial 'immunity' against bacteriophages. *Bacteriophage*, *2*(1), 50-54.

6. Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS microbiology reviews*, *28*(2), 127-181.

7. Bondy-Denomy, J., Qian, J., Westra, E. R., Buckling, A., Guttman, D. S., Davidson, A. R., & Maxwell, K. L. (2016). Prophages mediate defense against phage infection through diverse mechanisms. *The ISME journal*, *10*(12), 2854-2866.

8. Carlton, R. M. (1999). Phage therapy: past history and future prospects. *ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS-ENGLISH EDITION-*, *47*, 267-274.

9. Liu, S., Lu, H., Zhang, S., Shi, Y., & Chen, Q. (2022). Phages against pathogenic bacterial biofilms and biofilm-based infections: a review. *Pharmaceutics*, *14*(2), 427.

10. Naser, I. B., Hoque, M. M., Abdullah, A., Bari, S. N., Ghosh, A. N., & Faruque, S. M. (2017). Environmental bacteriophages active on biofilms and planktonic forms of toxigenic Vibrio cholerae: Potential relevance in cholera epidemiology. *PLoS One*, *12*(7), e0180838.

11. Pcsclasses. (2023, April 29). *The viruses- classification, Structure and diseases - biology*. PCSSTUDIES. https://pcsstudies.com/the-viruses-classification-structure-and-diseases/

12. Jończyk, E., Kłak, M., Międzybrodzki, R., & Górski, A. (2011). The influence of external factors on bacteriophages. *Folia microbiologica*, *56*, 191-200.

13. Silva, Y. J., Costa, L., Pereira, C., Cunha, Â., Calado, R., Gomes, N. C., & Almeida, A. (2014). Influence of environmental variables in the efficiency of phage therapy in aquaculture. *Microbial biotechnology*, *7*(5), 401-413.

14. Pradeep, A. N., Ramasamy, S., Veniemilda, J. K., & Kumar, C. V. (2022). Effect of PH & Temperature Variations on Phage Stability-A Crucial Prerequisite for Successful Phage Therapy. *Int. J. Pharm. Sci. Res*, *13*, 5178-5182.

15. Tian, F., Li, J., Nazir, A., & Tong, Y. (2021). Bacteriophage–a promising alternative measure for bacterial biofilm control. *Infection and drug resistance*, 205-217.

16. Haq, I. U., Chaudhry, W. N., Akhtar, M. N., Andleeb, S., & Qadri, I. (2012). Bacteriophages and their implications on future biotechnology: a review. *Virology journal*, *9*(1), 1-8.