PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST *Pseudomonas aeruginosa* CAUSING URINARY TRACT INFECTION; AN APPROACH TO BIOFILM REDUCTION

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Recommendation

This is to certify that **Mr. Sudip Timilsina** has successfully completed his dissertation work entitled **"PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST Pseudomonas aeruginosa CAUSING URINARY TRACT INFECTION APPLICATIONS; AN APPROACH TO BIOFILM REDUCTION"** under my supervision.

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here are 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 "**PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST Pseudomonas aeruginosa CAUSING URINARY TRACT INFECTION APPLICATIONS; AN APPROACH TO BIOFILM REDUCTION"** presented to evaluation committee by **Mr. Sudip Timilsina** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……. **…….**

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Declaration by the candidate

Date: ……..

I hereby declare that the thesis entitled "**PHYSIOCHEMICAL AND GENOMIC CHARACTERIZATION OF BACTERIOPHAGE AGAINST *Pseudomonas aeruginosa* CAUSING URINARY TRACT INFECTION; AN APPROACH TO BIOFILM REDUCTION**" submitted to Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu for partial fulfillment of the requirement for the degree of M.Sc. in Biotechnology is a genuine work performed by me, **Sudip Timilsina** (T.U. Registration No: 5-2-453-68-2012) under the guidance and supervision of **Prof. Dr. Rajani Malla**. No copies of this work have been published or presented previously anywhere or in any form.

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ACRONYMS

DLAA	: Double Layer Agar Assay
DNA	: Deoxyribonucleic Acid
dsDNA :	:Double Stranded DNA
ICTV	: International Committee on Taxonomy of Viruses
LB	: Luria Bertani
MDR	: Multi Drug Resistance
ml	: microliter
OD	: Optical Density
PBS	: Phosphate Buffer Saline
PCR	: Polymerase Chain Reaction
rpm	: revolution per minute
TAE	: Tris Acetate EDTA
TSA	: Tryptic Soy Agar
UTI	: Urinary Tract Infection
WHO	: World Health Organization
NCBI	: National Center for Biotechnology Information
ORF	: Open Reading Frame
PFU	: Plaque Forming Unit
SM buffer	: Sodium Magnesium Buffer
SDS-Page	: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
ssDNA	: Single Stranded Deoxyribonucleic Acid
ssRNA	: Single Stranded Ribonucleic Acid
µg	: microgram
µl	: microliter
AST	: Antibiotic Sensitivity Test

BLAST	: Basic Local Alignment Search Tool
bp	: Base Pair
GC content	: Guanine-Cytosine content
mRNA	: messenger RNA
CLSI	: Clinical and Laboratory Standards Institute
MIC	: Minimum Inhibitory Concentration
KDa	: Kilo Dalton
CTAB	: Cetyltrimethylammonium bromide
SDS	: Sodium Dodecyl Sulphate
NaCl	: Sodium Chloride
ELISA	: Enzyme Linked Immunosorbent Assay
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
ATP	: Adenosine Triphosphate
CDC	: Center for Disease Control
UV	: Ultra Violet
FDA	: Food and Drug Administration
EDTA	: Ethylenediamine tetraacetic acid

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is resistance to most of the antibiotics. This makes treatment of *Pseudomonas aeruginosa* difficult. The problem is further compounded by its ability to form biofilm. The aim of this study was to isolate lytic phage against antibiotic resistant *Pseudomonas aeruginosa* and to use it in the reduction of *Pseudomonas aeruginosa* biofilm.

Methodology: Bacteriophage isolation was done by Double Layer Agar Assay method. Burst size and latent period of the phage was determined by one step growth curve experiment. Phage stability was also analyzed against different temperature and pH range. Phage cocktail was used to disrupt biofilm. The synergistic effect of phage and antibiotic in reducing biofilm was also analyzed. Effect of various external factors in phage stability was examined. Whole genome sequencing of phage DNA was done.

Result: Lytic bacteriophage against *Pseudomonas aeruginosa* was isolated. The latent period of the phage TU_pse1B was 30 minute and burst size was 27 virion per bacterium. The optimum temperature for the phage TU_pse1B was 37^{o}C and optimum pH was 9. Three distinct bands of phage proteins of size 35, 40 and 100KDa were observed after performing SDS PAGE. Phage DNA size was determined to be larger than 10 Kb from agarose gel electrophoresis. Whole genome sequencing of phage revealed its size to be 43,428 nucleotides (43 Kb) in length and the GC content of 62.16%. Calcium ion increased the phage adsorption. Phage showed stability against SDS and osmotic shock whereas it was susceptible to ethanol, acetone and CTAB. Phage TU_pse1B reduced the biofilm by 60.99% whereas Phage TU_pse1Bi reduced the biofilm by 60.37%. Synergism of phage and antibiotic was observed in reducing biofilm. Phage plus antibiotic reduced biofilm by further 26.67% than phage alone.

Conclusion: Phage TU_pse1B showed good stability to various physiochemical factors as well as it was efficient in reducing *Pseudomonas aeruginosa* biofilm. So, this phage can be a good candidate for controlling antibiotic resistant *Pseudomonas aeruginosa*.

Keywords: Antibiotic resistance, Biofilm, SDS, Phage cocktail, AST, Burst size.

1. INTRODUCTION

1.1 Background

1.1.1 Discovery of Bacteriophage

One of the most significant developments in microbiology was the discovery of bacterial viruses, sometimes known as bacteriophages or "phages." Bacteriophages were found by Felixd'Herelle in France (1917) and Frederick William Twort (1915) in England almost simultaneously. However, British bacteriologist Ernst Hankin reported the first observation of their lytic activity much earlier, in 1896. D'Herelle proposed that his unique creatures were viruses and coined the terms "bacteriophage" and "bacteria eater." He proposed that they were in the form of particles and multiplied inside of bacterial cells. Other researchers disagreed with this theory and thought of phages as transmissible autolysis, genes, or enzymes (H.-W. Ackermann, 2012).

Bacteriophages, also known as phages, are bacterial viruses that enter bacterial cells and, in the case of lytic phages, disturb the metabolism of the infected bacteria, resulting in the bacterium lysing. British bacteriologist Ernest Hankin noted in 1896 that the Ganges and Jumna rivers in India had pronounced antibacterial activity (against Vibrio cholerae), and he proposed that an unknown substance (which passed through fine porcelain filters and was heat labile) was responsible for this phenomenon and for preventing the spread of cholera epidemics. When working with *Bacillus subtilis* two years later, the Russian bacteriologist Gamaleya noticed analogous phenomena, and it is believed that several other researchers' results are also connected to the bacteriophage phenomenon. But until Frederick Twort, an English bacteriologist with medical training reintroduced the topic nearly 20 years after Hankin's observation by describing a similar phenomenon and advancing the theory that it may have been caused by, among other things, a virus, none of these researchers went any further with their findings. Twort did not follow this discovery, however, due to a number of factors, including financial difficulties, and it took another two years for Felix d'Herelle, a French-Canadian microbiologist at the Institut Pasteur in Paris, to "officially" discover bacteriophages. The bacteriophage phenomenon was reportedly first noticed by d'Herelle in 1910. D'Herelle had less uncertainty regarding the nature of the event than Hankin and Twort did, and he claimed that it was brought on by a virus that could parasitize bacteria. D'Herelle also suggested the name "bacteriophage." The word "bacteria" and the Greek verb "phagein" (to eat or devour) were combined to create this term, which was intended to signify that phages "consume" or "devour" bacteria. In the meantime, d'Herelle avidly continued investigations of bacteriophages in contrast to Twort and vigorously pushed the notion that phages were live viruses, not "enzymes," as many of his contemporaries believed (Sulakvelidze et al., 2001).

D'Herelle felt confident that the event he observed was caused by a virus that could parasitize bacteria, in contrast to Twort, who seemed to support the idea that lysis was governed by an enzyme released by the bacteria itself. He had to wait until the newly developed electron microscope, which was invented in 1939, verified the phage's viral nature. D'Herelle also pioneered the concept of "phage therapy," seeking the use of phages in human medicine as therapeutic and preventative measures, utilizing phage selectivity for pathogenic bacteria and researching phage safety for human host cells. D'Hérelle established the Bacteriophage Laboratory in France and started making the first phage cocktails for sale. At the same time, bacteriophages were employed in the US for medical treatment. The era of antibiotics began with the discovery of penicillin in 1940, and phage therapy was discontinued in North America and Western European countries (Essa et al., 2020).

1.1.2 Current scenario

A serious issue in contemporary medicine is the advent of pathogenic germs that are resistant to the majority, if not all, of the antimicrobial medicines that are now on the market. The possibility of returning to the "pre-antibiotics" age has increased, and modern medicine and biotechnology now place a high priority on the creation of alternative anti-infection treatments (Sulakvelidze et al., 2001).Given that germs are becoming increasingly resistant to antibiotics, we may soon be in a post-antibiotic era and will need to develop alternate methods of treating microbial infections (Tynecki et al., 2020).

The World Health Organization (WHO) has identified antibiotic resistance as a global hazard, naming it one of the biggest challenges of the twenty-first century. This has led to the creation of a Global Action Plan to address the issue. The entire globe is looking for strategies to prolong the usefulness of antibiotics. Phage therapy may be able to address the problem faced by bacterial infections that are multidrug resistant (Manohar et al., 2019).Phages are once again being examined as potential therapeutic agents as a result of the impending global dilemma of antibiotic resistance (Ofir & Sorek, 2018).

The World Health Organization (WHO) declared a state of emergency worldwide in 2012 as a result of the widespread spread of multidrug-resistant (MDR) bacteria, warning that an era in which antibiotics are no longer effective against bacterial illnesses may be approaching. In order to direct and encourage the development of novel antibiotics, WHO issued a list of microorganisms in 2017.Among the various strategies offered, the use of clinical goods containing bacteriophages was taken into consideration (Essa et al., 2020). Antibiotic resistance is one of the top three dangers to public health in the twenty-first century, according to the World Health Organization (WHO). Therefore, it seems conceivable that the end of the era of antibiotics is gradually coming and that humans will soon have to adapt to a world without powerful antimicrobial medications. When compared to antibiotics, phages have a number of benefits, such as high

specificity, low dosage, low production costs, excellent safety, and anti-biofilm activity (Li et al., 2021).

In 2017, the World Health Organization released a list of 12 bacterial species that were classified as critical, high, and medium priority pathogens based on their level of resistance and the availability of therapies. The current rate of antibiotic discovery and development greatly outpaces that of antibiotic resistance development, which poses a threat to global public health. Antimicrobial resistance may cause up to 10 million deaths annually by the year 2050, according to estimates. Even though this figure is debatable, it nonetheless illustrates the serious issue we have with the therapeutic options for multi-drug resistant (MDR) bacterial infections. Phages are possible antibacterial treatment agents against such MDR infections since they evolved along with bacteria (Furfaro et al., 2018).

The current medical treatment of common diseases is seriously threatened by antibiotic resistance genes that code for bacterial resistance to popular antibiotics including tetracycline, aminoglycosides, chloramphenicol, and -lactams. These genes currently appear to be prevalent in the environment. Since many antibiotics are becoming less effective at treating common infections, especially the challenging-to-treat nosocomial illnesses, the emergence of antibiotic resistance genes poses a special threat. Antibiotic resistance has been deemed a hazard to world health by regulatory bodies including the WHO and the Centers for Disease Control (CDC). According to the CDC, antibiotic-resistant infections cause at least 23000 deaths year and 2 million illnesses, with many more people dying from conditions made worse by antibiotic-resistant infections. However, there has been less commercial interest in the study and production of new chemicals as a result of the speed at which bacteria develop antibiotic resistance. Antibiotic resistance was rated "the greatest and most urgent global issue" by the United Nations General Assembly, which met on September 21, 2016. One of the more common recommendations in the search for substitute methods of bacterial infection prevention and management is to reconsider the use of phage therapy (Lin et al., 2017).

1.1.3 Phage as an alternative

Phage therapy has been around for a while, but due to germs that are resistant to several medications, it is making a comeback to kill pathogens as chemical drugs lose their effectiveness. Since antibiotic resistance in bacteria has become a very severe issue for human health, there is a greater need than ever for an alternative therapy against infectious diseases over a century after the discovery of phages and the first clinical trials. Once more, phage therapy is receiving a lot of attention globally (Manohar et al., 2019).

The Western world's interest in bacteriophage (phage) investigations has been revived as a result of rising reports of antimicrobial resistance and the lack of new antibiotic discoveries and development. In phage therapy, lytic phages are primarily used to

destroy the bacterial hosts that they are intended for while leaving human cells unharmed and minimizing the wider impact on commensal bacteria that is frequently caused by antibiotic use. Rapid advancements in phage therapy have led to several clinical trials and stories of life-saving therapeutic application (Furfaro et al., 2018).

As a result of the advent of multidrug resistant pathogens, the idea of employing bacterial viruses therapeutically against bacterial diseases has lately acquired popularity; however, the method has been around for about a century. After pharmaceutical antibiotics were developed in the 1940s, phage therapy was largely disregarded by the majority of western medicine. The former Soviet Union and Eastern Europe are the exception to this rule, where clinical phage therapy has been extensively used to treat infections that are resistant to antibiotics and are brought on by a variety of infectious bacteria, including *Staphylococcus, Pseudomonas, Klebsiella,* and *E. coli* (Lin et al., 2017).

Phage therapy proponents point out that phages have a number of significant advantages over antibiotics, including host-specificity, self-amplification, biofilm destruction, and low toxicity to humans. The science of phage biology is just now gaining maturity due to the advent of analytical methods capable of analyzing very small biological entities (about 25-200 nm in length), including as next generation sequencing and electron microscopy. As seen by a wave of recent human clinical trials and animal studies, these technological breakthroughs have ushered in resurgence in phage therapy research (Lin et al., 2017)

The advancement of next-generation phage technology is anticipated to be accelerated by our improved understanding of phage biology and evolution, as well as of defense and counter-defensive processes (Ofir & Sorek, 2018).

1.1.4 Bacteriophage, structure and morphology

The most prevalent creatures on the planet are bacteriophages. These bacterial viruses are made up of genetic material that is encapsulated by a protein coat and can take the form of DNA or RNA. The fiber-filled tail of the capsid, which is attached to it, is employed to adhere to receptors on the surface of bacterial cells. All phages but filamentous phages have polyhedral capsids (Haq et al., 2012). Bacteriophages were formerly thought to be simple nucleoprotein particles; however, it has now been shown that their structure is incredibly complex. The tadpole-shaped T-group phages (T2, T4, and T6) have a bi-pyramidal hexagonal prism head and a cylindrical tail that serves as the phage's organ of attachment to its host (Brenner et al., 1959).

The genetic material of phages is made up of single-stranded (ss) or double-stranded (ds) DNA or RNA. Phage MS2 has a 3.5 kb ssRNA genome, while Bacillus phage G has a 500 kb dsDNA genome. Phage genomes can also contain mutated nucleotides as a defense against restriction enzymes. Phage morphology can be polyhedral, filamentous, pleomorphic, or tailed (96% of phages), and some include lipid or lipoprotein envelopes. The order Caudovirales (ds DNA genome with a tailed morphology) contains the majority

of characterized phages, which are divided into three families: Myoviridae with contractile tails (such as phage T4), Siphoviridae with non-contractile tails (such as phage), and Podoviridae with very short tails (such as phage T7) (Essa et al., 2020).

In a tailed proteinaceous capsid, the linear, double-stranded DNA (dsDNA) genomes of the vast majority of isolated phages (>95%) found to date are housed. Non-tailed capsids containing dsDNA, single-stranded DNA (ssDNA), or RNA genomes can be found in other types of phages (Ofir & Sorek, 2018). The sizes of phage genomes range greatly, from *Leuconostoc* phage (2,435 bp) to *Pseudomonas* phage (3, 16,674 base pair). The distribution of genome sizes is not uniform, and they can be divided into three different ranges. The largest is a surge of genomes between 30 and 50 kbp, which accounts for almost 50% of all phages. The smaller than 10kbp group makes up a second group (approximately 20% of the total), and the 100-200kbp intervals make up the third group (6% of the total). The most common morphological types of bacteriophages are dsDNA-containing tail phages. The bulk of these, if not all, have genomes greater than 15kbp, and very few of them have genomes smaller than 10kbp. This is in line with the discovery that the genes responsible for virion shape and assembly typically occupy at least 15kbp of genomic space. Genomes greater than 20kbp are found in the majority of phages with Siphoviral morphologies. In contrast, Myoviruses make up the majority of phages with bigger genomes (>125 kbp) (with contractile tails). Uncertainty exists over why there aren't any larger Siphoviruses (Hatfull, 2008).

The most well-known member of Myoviridae, the most intricate family of tailed phages, is Bacteriophage T4. The head, tail, and long tail fibers are the three separate routes that make up T4 assembly. Double stranded DNA genome of 172 kbp is enclosed in the head. The contractile sheath encircles the tail, which terminates in a hexagonal baseplate. The host cell's identification sensors are located on six long tail fibers that are attached to the baseplate's edge. During infection, the baseplate and sheath both experience significant conformational changes. One of the seven *Escherichia coli* phages is called Bacteriophage T4 (T1–T7, T for type). In 1944, Delbruck and colleagues proposed T4 as models for investigation by the phage community. Because Bacteriophage T4 includes a contractile tail, it is categorized as a member of the Caudovirales order's Myoviridae family. Before being combined to form a mature phage, T4's head, tail, and long tail fibers (LTFs) are built separately. T4 has 289 open reading frames in its 168 kbp dsDNA genome. The prolate head of the mature phage, which encapsidates the genome, is 1150 Å-long and 850 Å-wide. At one end of the head, a unique portal vertex is connected to a contractile tail that is 925 Å lengths and 240 Å diameters. The distal end of the contractile tail is connected to a hexagonal baseplate that is 520 Å in diameter and 270 Å high. Six 1450 Å-long LTFs are fastened to the hexagonal baseplate's edge. The sensors that can identify receptor molecules on the host are called LTFs. Under the baseplate, there are six short tail fibers (STFs) that, upon host recognition, unfold. These STFs bind to the host cell in an irreversible manner after unfolding, which improves the

effectiveness of infection. By enabling the tail tube to pierce the outer host cell membrane before phage DNA is delivered into the host cell, the contractile tail increases the effectiveness of infection. An ATP-dependent packaging machine first assembles the head as an empty capsid, which is then filled with DNA. The head is prolate, which means that it has a cylindrical middle and two icosahedral ends. A helical sheath encircles the tail tube, which makes up the tail. The dome-shaped baseplate is joined to the tube and sheath at the end that faces away from the head. Under the baseplate are six STFs that have been folded. The baseplate experiences a significant conformational change during the infection process that relaxes the high-energy dome-shaped structure (270 Å-high and 520 Å-diameter) to a low-energy star-shaped structure. The dome of the infectious virus surrounds the cell-puncturing device because it terminates the distal end of the tail tube. The phage tail tube is mechanically inserted into the periplasmic space using this device, which also has three lysozyme domains to break down the peptidoglycan layer there. When the virus infects a host cell, this conformation of the sheath collapses into the low energy state. The baseplate transforms from a dome-shaped structure to a star-shaped structure during infection. The sheath contracts as a result of releasing energy. In order to force the sharp point of the tail into the outer cell membrane like a drill, the phage head rotates around the tail axis (Yap & Rossmann, 2014).

1.1.5 Bacteriophage life cycle

Phages can spread through two different life cycles—lytic and lysogenic—and infect bacteria. The term "lytic life cycle" refers to the phage's vegetative growth in which it kills its hosts. However, certain phages, referred to as temperate phages, have the ability to develop vegetatively and integrate their genome into the chromosome of the host, replicating with the host for a number of generations. If exposure to extreme conditions, such as UV radiations, occurs, the prophage will evade capture by lysing bacteria (Haq et al., 2012). The cellular apparatus of the host is needed for bacterial phage to proliferate. Phage nucleic acids are then delivered into the infected cell once the phage particle attaches to its host cell by specifically recognizing a receptor on the host surface. When the phage enters the bacterium, it seizes control of the bacterial cell, cellular components, and defensive mechanisms (Ofir & Sorek, 2018).

Phages are incapable of reproducing on their own (they are hence non-living), and their survival eventually depends on a bacterial host. The majority of the time, phages attach to specific receptors on the surface of the host cell, inject their genetic material into the host cell, and either integrate this material into the bacterial genome (so-called "temperate" phages) and reproduce vertically from mother to daughter cell, or they hijack the bacterial replication machinery to produce the next generation of phage progeny and lyse the cell (so-called "lytic" phages). The majority of phages can only infect bacteria that have their complementary receptor, which effectively limits the spectrum of hosts that they can infect. Phages differ in their host specificity; some are

strain-specific while others have shown the ability to infect a variety of bacterial strains and even species (Lin et al., 2017).

The great majority of phages can be classified as lytic or lysogenic depending on how they spread. The lytic cycle is carried out by lytic phages, which are also known as virulent phages. During this cycle, the virus adheres to the surface of the target bacteria, injects its genome into the host cytoplasm, and stimulates the bacterial molecular system to produce virions that will eventually destroy the bacterial cell and release themselves into the environment. As long as the target bacteria are present, this cycle will continue, generating hundreds of new virions in a matter of minutes or hours. Lysogenic phages, also known as temperate phages, are viruses that may switch from the lytic cycle to the lysogenic cycle. In the lysogenic cycle, phages assume the quiescent condition of a prophage and integrate their genome into the host nucleic acid (or finally persist as a plasmid), ensuring viral reproduction in the bacterial cells. The prophage will emerge from its dormant stage and start the lytic cycle in response to particular stimuli. Transduction may take place during viral genome excision, leading to horizontal gene transfer throughout the bacterial community (for example that of resistance determinants). Temperate phages are ineffective for phage therapy because of this. Although it is theoretically possible for obligatory lytic phages to undergo transduction, the likelihood is extremely low given the quick bacterial host death and concomitant phage population growth. In addition to these two types, a third life cycle, in which bacteriophages destroy their host without lysing host cell, was seen in filamentous phages (Essa et al., 2020).

Lysogeny is a relatively common alternative life cycle that temperate phages can use, in which they become latent by integrating into their host genome or by creating an episome inside the host cell, rather than replicating and lysing their host. Following lysogenization, these phage genomes replicate alongside the host genome and can eventually begin their lytic cycle in response to particular triggers, most frequently host stress. Temperate phages have a variety of effects on bacterial communities, including novel gene transfer, changes in gene expression, protection from other phage infections, and induction-dependent host population eradication. Recently, these phenomena were examined. Since the very beginning of molecular biology, the lysis-lysogeny decision (or "molecular switch") has served as a paradigm for molecular decision-making processes. The *E. coli* phage lambda was used to conduct extensive research on this subject, and it was discovered that it involves a complex network of transcriptional activators and repressors, as well as RNA degradation, transcription anti termination, and proteolysis. To arrive at its judgment, the network combines data on the metabolic status of the cell and the number of infected phages (Ofir & Sorek, 2018).

Phages are infectious particles that contain nucleic acid and protein, at a minimum. A cycle of phage generation is started when a phage enters a bacterial cell that is open to it, or at least its nucleic acid does. In this cycle, the cell is reprogrammed to function as a

phage factory, diverting the biosynthetic machinery's components (including ribosomes and ATP producers) from their usual roles in bacterial development. Phage-specified proteins, which are translated from phage mRNA produced after infection, start the numerous processes for reprogramming. The temporal schedule is structured and governed. Replication of nucleic acids typically happens first, then the production of the phage particle's structural proteins (Campbell, 2003).

At least five phases are involved in a phage cycle: adsorption, nucleic acid injection, virions' assembly, release, and subsequent transmission. One of the most important stages in the life cycle of a phage is adsorption, which requires the precise recognition of host surface proteins, lipopolysaccharides, or other molecules (teichoic acids, fimbriae, or flagella) on the bacterial cell membrane. The ability of the phage to successfully recognize bacterial surface receptors results in long-lasting phage adherence, which permits penetration of the bacterial cell membrane and injection of the viral genetic material. Phage-encoded enzymes, such as the virion-associated peptidoglycan lysozyme, which hydrolyzes 1,4-ß-linkages between N-acetylmuramic acid and N-acetyl-D glucosamine residues of the cell wall peptidoglycan backbone, are necessary for cell envelope penetration. The phage genome can either stay free-standing in a plasmid-like form (episome, in pseudolysogeny), depending on the phage type and physiological state of the bacterial cell, or it can be incorporated into the host genome (prophage, in lysogeny). Pseudolysogeny affects both lytic and temperate phages and is associated with starvation stress on the bacterial host. The growth cycle of the phage is interrupted after entry into the host cell because the genetic material is still dormant and exists as a circular episome. Lysogens and phage-carrying cells are two subpopulations of bacteria that can result from infection with temperate phages, whereas the growth cycle of lytic phages is simply interrupted. In other words, lysogens and a steady proportion of productive phage-carrying cells are present in bacterial populations exposed to temperate phages. Phage-carrying cells, unlike lysogens, segregate the phage genetic material asymmetrically during bacterial cell division, resulting in infected and non-infected cell lineages. A temperate phage can remain latent as a prophage reproducing together with the host genome while being incorporated into the host chromosome. The temperate phage occasionally, and mostly under stressful circumstances, enters its lytic phase. Both lytic and temperate phages share the same lytic phase of the phage life cycle. Phage infection triggers the reprogramming of host genetic processes upon activation of the lytic cycle, leading to fast viral genome replication and production of structural and useful phage proteins. After the host cell is lysed, phage virion particles are put together, loaded with viral nucleic acid, and discharged into the extracellular environment. Certain lysis proteins, like as endolysins (cell wall peptidoglycan hydrolases), holins (plasma membrane disruption), and spanins (cell wall disruption), are required for the breaking of bacterial membrane and cell walls (destabilization of the Gram-negative bacterial outer membrane). According to recent studies, particular signal peptides encoded by the phage are required for the start of the lytic or lysogenic cycles

in temperate phages. This groundbreaking discovery proves that phages have a molecular communication system (reminiscent of bacterial quorum sensing) (Olszak et al., 2017).

1.1.6 Bacteriophage classification

The most prevalent biological entities currently understood are phages, which are characterized as viruses that infect bacteria. The International Committee on Taxonomy of Viruses (ICTV) and the ICTV's Bacterial and Archaeal Subcommittee (BAVS), which concentrates on phages, maintain the taxonomic classification of viruses and the naming of virus taxa. The system is based on an assessment of a number of phage characteristics, such as the molecular make-up of the virus genome (ss/ds, DNA, or RNA), the structure of the virus capsid and whether or not it is enveloped, the host range, pathogenicity, and sequence similarity. Based on these various characteristics, the ICTV created a very significant and well-known virus taxonomy (Chibani et al., 2019).

There are different morphologies of phages, including phages with filamentous structures, phages with lipid-containing envelopes, and phages with lipid-containing particle shells. They have a genome, either DNA or RNA, which can be single or double stranded that contains data on the proteins that make up the particles as well as other proteins that are in charge of switching cell molecular metabolism in favor of viruses. As a result, the data on the self-assembly process is contained in the genome. The phage capsid contains the genome, which can be single or multipartite. The size of a viral genome correlates strongly with the size of its capsid, which in turn shows the complexity of the organization of the virus. The tailed phages (order Caudovirales) which are divided into three suborders based on the length of their non-contractile tails (Siphoviridae, Podoviridae, and Myoviridae), are the most extensively researched group of phages. Phages can come in a variety of sizes and shapes. The largest and most well-studied group of tailed phages is that which has a dsDNA genome. The three main parts of a tailed phage are a capsid, which contains the genome, a tail, which acts as a pipe during infection to ensure the transfer of the genome into the host cell, and an adsorption apparatus, which is a special adhesive system that can recognize the host cell and penetrate its wall (White and Orlova, 2020).

All tailed phages have a hollow, helical tail made up of subunits and a head. Its function is to introduce DNA into a bacterium. Icosahedral or a more or less extended version of this structure is the head or capsid. Fixation mechanisms like base plates, fibers, or spikes are present in the majority of tails. Myovirus tails (which make up 24.5% of tailed phages) are contractile and comprise an axial needle encased in a contractile sheath that is separated from the head by a neck (H.-W. Ackermann, 2009). While podovirus (14%) tails are short and typically 10–20 nm long, siphovirus (61%) tails are lengthy, flexible or rigid tubes. Fixation mechanisms like base plates, fibers, or spikes are present in the majority of tails. Phages can be aggressive or mild. All are released by the infected bacterium's burst. Icosahedral or quasi-icosahedral entities make up polyhedral bacterial

viruses. They are supposed to consist of seven families of viruses, four of which include lipids, and two of which contain RNA. They are said to have cubic symmetry (H.-W. Ackermann, 2009). A neck, a contractile sheath, and a central tube make up a tail. Around 1300 observations, or 25% of all tailed phages, are associated with myoviruses, which tend to be larger than other groups and contain some of the largest and most well evolved tailed phages. Siphoviridae tails are straightforward, noncontractile tubes that might be flexible or rigid. The majority (over 3,200 observations, 61%) of tailed phages are siphoviruses. In the family Podoviridae, tails are short and noncontractile. Rather than myoviruses, podoviruses may be more closely connected to siphoviruses (14.5 percent, roughly 750 observations). Phages are viruses having single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), and single-stranded RNA (ssRNA) genomes (dsRNA; very rare). The majority of viruses (96%) have tails, whereas the remaining virions are "cubic (7 families)," "filamentous (3 families)," or "pleomorphic (7 families)" (around 200 samples, less than 4%). The terms "cubic" and "icosahedral" refer to shapes with cubic symmetry (H.-W. Ackermann, 2009). Lipids are a component of some kinds' envelopes or interior parts. These are generally susceptible to ether and chloroform. Order Caudovirales Tailed Phages (dsDNA) DsDNA is present in the heads of icosahedral or elongated tail phages. Helix-shaped tails are typically equipped with fixing mechanisms (baseplates, spikes, fibers). The envelope is absent. Particles adhere to their hosts and cause external infection. Phage DNA enters already-formed capsids as the progeny phages are put together through intricate routes. Of all viruses, tail phages are the most common and widespread. They also exhibit the widest range of size, shape, DNA content, genome structure, protein content, antigenic potential, and biological characteristics (H.-W. Ackermann, 2009).

1.2 Current studies

Globally, the spread of multidrug-resistant bacterial strains has posed a serious risk to the public's health when it comes to the regular management of infectious diseases. Since the previous few decades, the development of new antibiotics has slowed down despite the global upsurge in such resistance bacteria (Bhetwal et al., 2017). Therefore, it calls for constant efforts to create a promising replacement for treating infectious diseases. Bacteriophages, which have antibacterial characteristics and reproduce themselves during infection, are recently gaining popularity. Bacteriophages selectively infect harmful bacteria, including multidrug resistant pathogens, and have a different mode of action from antibacterial regimens. Additionally, they do not exhibit negative effects when applied to the human body and are ecologically safe and effective in lower dosages (Bhetwal et al., 2017). Recent antibiotic crisis has reignited interest in the widespread use of phages. Pathogenic bacterial strains in Nepal are continuously becoming more resistant to antibiotics. Despite the growing threat of antimicrobial resistance in our nation, little focus is placed on its management, and novel solutions have not yet been explored. Along with that, research into lytic phages in our sacred rivers may be a potential solution to combat the effects of antibiotic resistance because our country has abundant rivers and abundant water resources (Bhetwal et al., 2017).

1.3 Research Hypothesis

The research aimed to assess the efficacy of bacteriophage to reduce biofilm synergistically with antibiotics.

Null Hypothesis (H_o): Bacteriophage can reduce biofilm in synergism with antibiotic.

Alternative Hypothesis (H_1): Bacteriophage cannot reduce biofilm in synergism with antibiotic.

1.4 Objectives

1.4.1 General Objective

Study the synergism of phage and antibiotic in reducing biofilm.

1.4.2 Specific objectives

i) Investigate the stability of phage against different external factors (ethanol, CTAB, acetone, SDS and osmotic shock).

ii) Perform whole genome sequencing of phage genome and perform its bioinformatics analysis.

iii) Study the effect of Calcium ion on phage adsorption.

1.5 Rationale

Pseudomonas aeruginosa is an opportunistic pathogen. It is a leading cause of nosocomial infections. It causes several diseases such as infection of respiratory tract, bacteremia, septicemia, burn and wound infections. It is the third most common cause of urinary tract infection. It is capable of extensive colonization and can aggregate into biofilms. *Pseudomonas aeruginosa* can form biofilm in medical equipments, hospitals causing cross infections in hospitals and clinics. Biofilm promotes resistance to antibiotics and it limits antibiotic dispersion. *Pseudomonas aeruginosa* are resistant to most of the commonly used antibiotics such as aminoglycosides, penicillins, cephalosporins. Due to antibiotic resistance and capacity to produce biofilm, *Pseudomonas aeruginosa* is very difficult to treat. So, use of bacteriophage against multi drug resistant *Pseudomonas aeruginosa* can be an alternative. Phages are host specific and they can efficiently reduce host bacterial population. They are safe, have low production cost. They do not affect commensal bacteria and human cells. They can co-evolve with phage. However before its application, phage needs to be characterized as well as its genome should be checked if it has harmful genes in its genome.

The main purpose of the study was to isolate and characterize lytic bacteriophage against *Pseudomonas aeruginosa*. The synergism of phage and antibiotic in reducing biofilm was assessed. The stability of phage against different external factors was also analyzed.

2. LITERATURE REVIEW

2.1 Bacteriophage

The most numerous and astonishingly diversified group of organisms on Earth are phages. The estimated total number of phages on earth is between 10^{30} and 10^{32}, and remarkably high concentrations of phages were found in samples from unpolluted lake water (Nabergoj et al., 2018). Bacteriophages which are around ten times more widespread than bacteria are among the most diverse and numerous organisms in the biosphere. Because of the two separate infection cycles—lytic and lysogenic—phages may have diverse connections with their bacterial hosts (Liao et al., 2019). Phages are prokaryotic viruses that can be found practically anywhere, including in harsh conditions and in almost every nook and cranny of the human body. In the biosphere, they vastly outnumber bacteria. Endolysins, often referred to as phage lysozymes, lysins, or muralytic/mureolytic enzymes, are peptidoglycan hydrolases that are encoded by bacteriophages and produced at the conclusion of the bacterial cell's multiplication cycle. These enzymes attack one of the peptidoglycan's four main linkages in order to compromise the integrity of the cell wall. This results in bacteriolysis and the subsequent release of the bacteriophage progeny because they reduce the mechanical strength and resistance of the bacterial cell wall, which is necessary to withstand the internal cytoplasmic turgor (osmotic) pressure (Jonczyk-Matysiak et al., 2019). Given that there are an estimated 10^{31} different types of phages on Earth, bacteria are the most prevalent form of life. It exceeds the quantity of bacterial cells by ten times. This makes it astonishing how little we actually know about the diversity of bacteriophages. Despite the fact that 1,910 whole bacteriophage genome sequences (as well as 77 genomes of viruses infecting archaea) have been deposited in the NCBI database, this amount is small when compared to the 67,806 full genome sequences of bacteria (as of 2016). Nevertheless, a great diversity of bacteriophages, which are still partially understood, can be inferred from the information now available (Jurczak-Kurek et al., 2016).

Bacteriophage genomes may also include the newest genetic material known to biology, with up to 80% of their encoded genes having no known protein relationships and unknown biological functions. Bacteriophages continuously infecting bacteria results in a strong selection for phage resistance, necessitating the evolution of phage that overcome resistance. Unsurprisingly, there are numerous host-mediated defense mechanisms, including restriction-modification, CRISPR, tRNA cleavage, and toxin-antitoxin systems, as well as frequently occurring mechanisms encoded by phages for producing genomic diversity. Phages can carry genes that offer protection from other viruses in addition to genes that interfere with host defense mechanisms including anti-restriction and RNA repair enzymes. Therefore, it is not surprising that phages occasionally encode their own immunity, toxin-antitoxin, and restriction systems using genes from other bacteriophages. Although we anticipate that many more phage genes will take part in these dances for survival, the answers to these queries will need to be

found through additional analysis of bacteriophage genomes and their biology (Hatfull & Hendrix, 2011).

Since they are straightforward to culture and have simple genomes, bacteriophages such and T4 have long been used as model systems in genetic research. Since the wild-type phage is a temperate phage, it can engage in either lytic or lysogenic life cycles. The host's proteins are used by the phage to transcribe and translate the phage genes required for replication and the creation of new phages during the lytic life cycle. New phage bodies containing the new phage genomes eventually erupt from the infected cell, killing it, and spread infection to surrounding cells. Since they lyse or destroy every bacterial cell they infect, lytic phages produce visible plaques on a bacterial lawn. In contrast, the phage genome merges with the host genome during the lysogenic life cycle. Since temperate phages do not completely lyse the bacteria they infect, they leave turbid plaques on a bacterial lawn. The lytic life cycle of bacteriophages is suppressed in favor of the lysogenic life cycle by the CI repressor. The single nucleotide polymorphism (SNP), a T to C transition, is the only difference in the genomes of the mycobacteriophages that produce clear and turbid plaques. A missense mutation is created, changing Isoleucine (Ile) to Threonine (Thr) (Gudlavalleti et al., 2020).

Bacteriophages are plentiful, varied, and economical. As there are 10^{31} phage particles on Earth, they are easily accessible from the environment and can spread naturally when host bacteria are present. Lytic phages do not damage humans since they only infect particular bacteria and do not affect eukaryotic cells. Due to a number of benefits, the use of lytic bacteriophages has thus been emphasized as a unique technique to manage dangerous bacteria (Park et al., 2021). Due to their inherent antibacterial characteristics, bacteriophages, also known as phages, are regarded as one of the most promising antibiotic alternatives. High-specificity bacteria-infecting viruses have utility in a variety of industries, including agriculture, food, and human or animal therapy (Nobrega et al., 2016). Phages may precisely target particular bacteria, unlike antibiotics. Phages can be used to carefully manage dangerous germs and prevent negative consequences because they only kill one species or strain. It calls for a deep comprehension of how a phage and a host interact as well as a large collection of potential phages. A phage's ability to recognize and attach to a receptor on the surface of a bacterium characterizes its specificity. One of the Gram-negative bacteria's surface features, the O antigen, is thought to be the phage receptor. The phages may identify and take up lipopolysaccharides (LPSs) or outer membrane proteins (OMPs) on the surface of Gram-negative bacteria. Since bacteria are becoming more resistant to antibiotics, phage therapy has become more popular (Yuan et al., 2021).

Bacteriophages have a fatal effect on bacteria. They contain genetic material that is encapsulated by a protein sheath and can take the form of DNA or RNA (single or double stranded). A resurgence of interest in phages as a medicinal agent followed the rise of multidrug resistant bacteria. Phages differ from antibiotics and other antimicrobial drugs

in a number of ways, including host specificity, the absence of side effects, and the ability to multiply in the presence of their hosts. Recently, the Food and Drug Administration of America authorized several phages as acceptable for use in food products to prevent Listeria infections. Phage therapy is now being tested on animals to treat a variety of bacterial illnesses. In mouse models and in guinea pigs, phages have been successfully employed to treat infections caused by *P. aeruginosa* and *Escherichia coli.* In the environment, there are roughly 10^{30} bacteriophages, but only about 300 of them have been described. Phage therapy in the days before antibiotics failed because uncharacterized phages were utilized. Therefore, it's crucial to isolate and characterize phages before using them. Phages are typically found in environments where their host cells naturally occur, such as seawater, sewage water, sludge ponds, etc. Because of fecal and hospital drainage water contamination, sewage generally has a wide variety of bacteria (Piracha et al., 2014). Bacteriophages are excellent candidates to be employed as antimicrobial agents due to their frequency and widespread abundance over the planet. Phages can destroy particular bacterial strains and have extremely targeted killing mechanisms. The necessity to discover new phages with the capacity to control and eradicate multi-drug resistant *P. aeruginosa* strains has arisen as a result of the high specificity of the phage-host connection (Adnan et al., 2020). A phage self-replicates during the lytic cycle of infection by injecting its genetic material into the target bacteria and using the host's cellular machinery to create new phages. Via cell lysis, the phage progeny is freed from the host, and the cycle is then restarted. Primary infection refers to the original phage infection of bacteria, while secondary infection refers to the infection of bacteria by the offspring released from lysed cells. Phages can shield the host organism from bacterial invasion for days while also preventing bacterial development. The development of phages as preventative medicine may be advantageous to medical professionals, immune compromised patients, and loved ones of patients with lung infections (Chang et al., 2018).

Bacteriophages spread through the lytic or lysogenic cycles of the infection of particular bacteria. Their capacity to destroy germs makes them potentially effective against the global problem of antibiotic-resistant bacteria (World Health Organization 2018). Research on the possible application of phages for the treatment of human bacterial illnesses is growing for this reason. For lytic phages, the normal phage multiplication cycle includes phage adsorption to the bacterial cells, genetic material injection, and the creation and release of new phages. The adsorption constant, latent time, and burst size are referred to as bacteriophage growth parameters and can be used to explain this process. After mixing the proper quantity of bacteria and phages, the adsorption constant—which indicates the rate of phage adsorption to the bacterial cells—is typically calculated by observing a reduction in phage concentration over time. A one-step growth experiment evaluating the rise in phage concentration over time is commonly used to measure the latent period, which represents the interval between

infection and phage release, and burst size, which represents the number of phages released per bacterial cell (Sivec & Podgornik, 2020).

Plaque assays are frequently used to find new phage strains in a variety of environments, including sewage, water bodies, liquefied soil, and body fluids. Small zones of lysis are evident as the bacterial lawn expands. Each PFU originates from a single virion infection that is followed by the lysis of nearby cells by a phage. Techniques for agar Petri plating are frequently employed to count the amount of phage particles present in a preparation. The main objective of sequencing and annotation for phages that will be used for expanded-access phage therapy is to make sure that no damage is likely to be introduced by the phage preparation, and the main concern is the transfer of genetic material between bacteria by transduction. Toxins, genes associated with antibiotic resistance, and virulence factors are the three components that pose the greatest threat to transmission. Cholera, diphtheria, scarlet fever, shigella, and botulinum toxin genes are all encoded by phages that have been incorporated into the genomes of their host bacteria. The manifestation of one of those toxins could result in higher morbidity and mortality if a phage used in expanded-access phage therapy were to transmit it. Similar to the previous example, the introduction of antibiotic resistance genes—whether already present on phage genomes or transferred between bacterial hosts via specialized or generalized transduction—could render conventional antibiotic therapy useless. Phages are linked to the horizontal transfer of virulence genes in addition to toxin and antibiotic resistance genes (Luong et al., 2020).

Typically, it is believed that a single viral particle is the cause of each plaque on a plate. Simply put, cycles of infection of the implanted host cells by the countless viral progeny dispersing in all directions from the initial center of infection culminate in the typical circular plaque appearance. The easiest technique to determine whether a phage is present is via the development of a plaque. Plaque counting is still the simplest and most popular technique for counting infectious phages in a sample. The transition of the host's physiology from the initial exponential expansion to the eventual immobile stasis is one of the most significant alterations throughout this typical incubation period. Most phages, with a few notable exceptions, are unable to maintain productive infections when they infect stationary phase cells. As a result, the amount of time available for productive infections would be a limiting factor for the plaque size. The extracellular phase for virion diffusion/adsorption and the intracellular phase for progeny formation are the two distinct phases of a phage's life cycle on an agar plate. Given all other factors being equal, a longer extracellular phase would enable the virion to disseminate further. A longer intracellular phase, on the other hand, would result in more progeny that might be spread (Gallet et al., 2011).

Temperate phages are known to facilitate the exchange of bacterial sequences between strains, which inevitably results in the spread of genes encoding toxins or antibiotic resistance. Therefore, it is recommended to only use virulent (strictly lytic) phages for

therapeutic purposes. Phages have a number of benefits over antibiotics. First off, because they are obligate bacterial viruses, they have a tendency to be exclusive to the bacteria they infect and only have a limited ability to eliminate certain pathogenic strains. Contrary to broad-spectrum antibiotics, this prevents incidental damage to human and animal healthy commensal microbiome (Forti et al., 2018). A phage self-replicates during the lytic cycle of infection by injecting its genetic material into the target bacteria and using the host's cellular machinery to create new phages. Via cell lysis, the phage progeny is freed from the host, and the cycle is then restarted. Primary infection refers to the original phage infection of bacteria, while secondary infection refers to the infection of bacteria by the offspring released from lysed cells. Phages can shield the host organism from bacterial invasion for days while also preventing bacterial development. The development of phages as preventative medicine may be advantageous to medical professionals, immunocompromised patients, and loved ones of patients with lung infections (Chang et al., 2018).

For three main reasons, lytic phages are preferred to temperate phages during phage therapy. I) Temperate phages have the potential to carry toxin genes that act as virulence factors for lysogenized bacteria, increasing the risk of a more dangerous pathogen developing in patients receiving treatment. II) A large number of temperate phages have the ability to transfer genes from one bacterium to another, potentially enhancing the virulence of the recipient bacterium. III) Lysogenized bacteria typically develop immunity to the lysogenizing phage and other phages with comparable repression systems inflicting lytic infections on them. Clear plaques are thought to be a sign of a lytic phage for the majority of phages (DNA genomes, tailed), but turbid plaques (particularly those with turbid cores) may be a sign of a temperate phage. As a result, phages can be examined for the presence of integration/excision genes using PCR, nucleic acid hybridization, whole-phage genome sequencing, or another method. The favored method is moving toward whole phage genome sequencing, particularly as the cost of genome sequencing has decreased recently. Finding obligate lytic phages that specifically target a certain bacterial species is not difficult. To improve the use of temperate phages (together with lytic phages) in phage treatment, some groups are considering the idea of genetically altering them to convey antibiotic sensitizing genes, for instance (Hyman, 2019).

Because of fecal and hospital waste contamination, waste water in general may contain a wide range of bacteria. Bacteriophages from the Myoviridae, Sipho-viridae, Micro-viridae, and Podo-viridae families, which target bacteria in the Enterobacteriaceae family, particularly *Escherichia*, *Pseudomonas*, and *Morganellacea*, are found in wastewater. Lytic bacteriophages from these sources can be used for therapeutic purposes in clinical settings as well as to control pathogenic bacteria in wastewater treatment in an energy-efficient, environmentally beneficial, and self-sustaining manner. Phages typically have less of a tendency to bind with receptors on the surface of

different types of bacterial cells and more of a tendency to bind to the precise receptor present on one type of bacterial cell. Phage tolerance levels for their thermal stability and pH range widely, according to several research findings (Adnan et al., 2020). Sewage water that has been exposed to human waste is thought to be a great source of phages that can fight different pathogenic bacteria that are common in a certain community. The potential use of phages recovered from sewage and other sources in the treatment of specific illnesses, such as *P. aeruginosa* infections, that were otherwise resistant to conventional antibiotics has been established by researchers. Infections that are resistant to therapy and the eradication of biofilms can both be successfully treated with phages. Therefore, phage therapy has become a promising antibiotic substitute, particularly for bacteria that build biofilms and have multiple drug resistance (Sharma et al., 2021). Without a host, bacteriophages can be kept in storage indefinitely without degrading. It is similar to nucleoproteins, a class of compounds to which it appears to belong, in that it may be concentrated, purified, and handled in general. Bacteriophage growth begins with its adhesion to susceptible bacteria. After the phage particle adheres to a receptive bacterium, multiplication takes place. The size of individual bursts fluctuates greatly, more so than one might anticipate given the size variations among bacteria in a culture. They might range from a few particles to 200 or more (Ellis & Delbrck, 1939).

2.2 *Pseudomonas aeruginosa*, an opportunistic pathogen

Pseudomonas aeruginosa is a rod-shaped, gram-negative opportunistic bacterium that is frequently linked to nosocomial infections across the globe. Previous research against pathogenic *P. aeruginosa* and the biofilm has revealed several drug resistances, rendering the use of antibiotics pointless. It ranks second among Gram negative bacteria and accounts for 16.1% of all nosocomial infections, according to the United States National Nosocomial Infection Surveillance System. Adults who are healthy rarely contract the infection, but those with weakened immune systems are its primary target. From self-limiting folliculitis to life-threatening bacteremia, wound-related morbidity, septicemia, skin infections, endocarditis, and device-related infections, the infection can affect anyone. It is the third most common cause of 12% of urinary tract infections, upper and lower respiratory tract infections, and cystic fibrosis that occur in hospitals. 18% of all isolates of *P. aeruginosa* were multidrug resistant, according to research by Souli and colleagues. Its great resistance is caused by a mechanism of chromosome-encoded efflux and low outer membrane permeability, rendering the use of antibiotics useless. Most antimicrobial agents become less effective because the biofilm promotes resistance and limits antibiotic dispersion. By way of plasmids, transposons, and bacteriophages, it is also able to take on new resistance genes from other organisms (Piracha et al., 2014).

The pathogen *Pseudomonas aeruginosa* is opportunistic. It is a natural component of human skin's flora. It is widespread and occurs in a wide variety of environmental

contexts. It is possible to separate it from different living sources, including people, animals, and plants. The third most common cause of nosocomial UTIs is thought to be *P. aeruginosa* (UTIs). *P. aeruginosa* worsens the prognosis of patients with lower and upper respiratory tract infections, such as cystic fibrosis, which increases the risk of death, particularly in individuals with impaired immune system (Jamal et al., 2017). *P. aeruginosa* is most frequently found in sewage water, soil, farms, slaughterhouses, and hospitals. *P. aeruginosa*, which causes sepsis in immunosuppressed patients, persistent lung infections, urinary tract infections, and ventilator-associated serious pneumonia, is a major cause of death by nosocomial infections, particularly in patients with severe wounds. Due to its several mechanisms of antibiotic resistance and capacity to produce antibiotic-resistant biofilms, *P. aeruginosa* treatment is highly difficult*. P. aeruginosa* aggregations can form biofilms via producing extracellular matrix, rendering them nearly impossible to treat or eradicate with antibiotics. *P. aeruginosa* is a perfect pathogen for nosocomial transmission through contaminated surfaces, invasive ventilation equipment, urinary catheterization, nasogastric feeding systems, etc. thanks to its several modes of motility, quorum-sensing, ability to form resistant biofilms, etc. (Sharma et al., 2021).

Pseudomonas aeruginosa is one of the main culprits behind drug-resistant nosocomial infections in underdeveloped nations. This bacterium has chromosomally encoded efflux pumps, poor outer-membrane permeability, and a high propensity for biofilm formation, all of which are mechanisms for conferring resistance. *P. aeruginosa* has been linked to extremely high fatality rates in immune-compromised patients who develop acute pneumonia. In people with cystic fibrosis, it causes persistent inflammation and may even cause the lungs to completely stop working. Biofilms are also created by *Pseudomonas* on the surfaces of medical equipment. Because biofilm is resistant to the majority of antibacterial medications and occasionally compromises the immune system, it is difficult to treat. The National Institutes of Health (NIH) estimates that bacterial biofilms are responsible for about 65% of fully developed microbial infections and 80% of all chronic illnesses. One of the leading causes of hospital acquired infections, primarily in underdeveloped nations, is *P. aeruginosa*. Up to 80% of human infections are caused by *P. aeruginosa,* which also has a high capacity for creating biofilms that are resistant to routinely used antibiotics (Adnan et al., 2020). The opportunistic pathogen *Pseudomonas aeruginosa* is one of the main bacteria recovered from people with cystic fibrosis (CF) and mostly affects the airways of immunocompromised patients. The spread of *P. aeruginosa* isolates that are multidrug resistant (MDR) is what's causing antibiotic use to become more and more ineffective (Forti et al., 2018).

Multidrug-resistant (MDR) *P. aeruginosa* is a significant cause of death in cystic fibrosis patients, particularly in youngsters who take preventive antibiotic therapy. One of the leading causes of persistent lung infection in people with cystic fibrosis is *Pseudomonas aeruginosa*. In addition, *P. aeruginosa* also causes pneumonia, bacteremia, and urinary

tract infections in immunocompromised patients. Only a few types of antibiotics, including carbapenem, aminoglycoside, quinolone, and polymyxin B, are effective against *P. aeruginosa* because of its inherent resistance mechanism. By acquiring plasmids encoding resistance genes or by chromosomal mutation to increase the expression of resistance mechanisms, further resistance to these antibiotics can be created (Ong et al., 2020). *P. aeruginosa*-specific bacteriophages may prove to be a potential treatment strategy for managing infections caused by this bacterium (Piracha et al., 2014).

A particularly alluring possibility for phage and antibiotic therapy is *Pseudomonas aeruginosa. P. aeruginosa* is a major cause of morbidity and mortality in burn patients, immune-compromised patients, and individuals with the skin ulcers that typically afflict diabetics, in addition to being the primary cause of death in many cystic fibrosis patients. Many antibiotics are naturally resistant to *P. aeruginosa*, and many others have developed resistance to it. Studies showed that phage and antibiotic combinations were effective against biofilms and planktonic cultures of *P. aeruginosa* (Chaudhry et al., 2017).

2.3 Antibiotic resistance in bacteria

Antibiotic resistance is a significant global public health issue. Antibiotics that are sold commercially are losing their effectiveness as resistance rates increase. As a result, many bacterial infections in the intestine are becoming more persistent or virulent. Such resistance phenotypes are typically related to antibiotic usage, which has increased susceptibility to obstruct infection therapy and subtly raises mortality rates. For both wealthy and developing nations, the problem of antibiotic use and resistance is serious (Abdelsattar et al., 2019).

Bacteria have developed a variety of escape mechanisms, making them immune to the majority of antibiotics due to indiscriminate use of antibiotics. Antibiotic resistance is expected to cause 10 million deaths annually by the year 2050. Antibiotic-resistant infections are more likely to occur in patients with chronic diseases than in healthy individuals. An important global worry in recent years has been the rise in morbidity and mortality brought on by infections with multidrug-resistant bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae*, etc. The WHO has identified three of these pathogens—carbapenem-resistant *A. baumannii, P. aeruginosa*, and Enterobacteriaceae—as "Priority 1 Critical Pathogens" for which new antibacterial drug R&D is urgently needed (Sharma et al., 2021).

Opportunistic pathogen (OP) infections, such those caused by *Pseudomonas aeruginosa*, are on the rise and can cost the US up to $45 billion in direct medical expenses each year. Antibiotic resistance is rising among opportunistic bacteria that cause hospital-acquired infections (HAIs). In the United States, 32,600 hospital acquired infections caused by *P. aeruginosa* alone resulted in 2,700 fatalities in 2017. (Stachler et al., 2021).

As antibiotics lose their effectiveness, it is become harder to treat an increasing range of infections. Antibiotic resistance thus causes increased mortality, longer hospital stays, and higher medical expenses. Antimicrobial resistance was identified as a worldwide health security problem by the WHO in 2014. Phages may be a fascinating antibiotic alternative in light of the rising antibiotic dilemma (Nabergoj et al., 2018). Bacteriophages are extremely host-specific because only certain receptors on the bacterial surface will connect to them. To protect the microbiota, they only influence the targeted bacteria without affecting other, untargeted microorganisms. Other benefits of bacteriophages include their capacity for self-replication and their ability to kill quickly (Yan et al., 2020). Bacteriophages represent the last option in the post-antibiotic era to save patients with bacterial illness from which no classes of antibiotics are now successful. Antibiotic resistance is one of the top 10 threats to world health in 2019, according to the World Health Organization. In this catastrophic crisis, phages provide a possible alternative to antibiotics, and thorough understanding of phage biology is urgently required. They should be completely characterized, particularly in the case of those that are utilized for medicinal purposes (Jonczyk-Matysiak et al., 2019).

2.4 Phage therapy

The Centers for Disease Control and Prevention (CDC) and the World Health Organization report that the number of deaths caused by bacteria that are resistant to antibiotics is at an all-time high. Every year, more than 2.8 million multidrug-resistant (MDR) illnesses develop in the US, and as a result, more than 35,000 people pass away. Since antibiotics are becoming less effective, it is vital to find new ways to treat infections. Otherwise, MDR infections would overtake other causes of death in the world by 2050 (Luong et al., 2020).

Problems caused by bacteria that are resistant to the majority of current antibiotics are getting worse. As a result, there is interest in the potential for bacteriophage treatment, the medicinal use of a particular type of virus that only targets bacteria. When phage therapy was initially created at the turn of the century, it showed great promise but also generated a lot of debate. Over the past 50 years, Eastern Europe has continued to conduct extensive clinical research and use phage therapy. Contrarily, since the development of antibiotics in the 1940s, it has been used far less frequently in the West and other nations (Tanji et al., 2004). In the West, phages were superseded by antibiotics in the 1940s, but in the Soviet Union, phage preparations were still used extensively to treat enteric disorders, nosocomial infections, burns, and wound infections (Chibani-Chennoufi et al., 2004). Phage therapy was created concurrently with antibiotics during the first half of the 20th century. It was not widely developed in Western nations, and little is known about the PK/PD (pharmacokinetics/pharmacodynamics) of phages, drug interactions, in vivo efficacy, and the establishment of resistance (Oechslin et al., 2016). The co-discoverer of phages, Felix d'Herelle, supported the notion of using phages' lytic action on bacteria for medical

treatment. Only in the Soviet Union did phage therapy start to be used often to treat skin and intestinal illnesses. Phage therapy is currently experiencing resurgence in attention. No notable adverse effects were seen during the oral phage trial, and the medicines were well tolerated. (Bruttin & Brüssow, 2005).

After years of neglect in the west, phage therapy is once more a subject deserving of consideration. Recent years have seen an increase in research using phages as bacterial infection control organisms, mostly as a result of the emergence of bacterial resistance to a variety of antimicrobial treatments (Sillankorva et al., 2008). The resurgence of phage therapy has given rise to hope and caused a paradigm shift in the research and development of a new class of antibiotics in the context of the rise in antibiotic resistance among dangerous microorganisms. Utilizing phages' lytic activity against particular bacteria has shown to be a successful strategy, despite some drawbacks. In comparison to traditional chemical-based antibacterial agents, phages are thought to be safer for humans and more environmentally friendly (Sharma et al., 2021). Phage therapy provides many benefits, such as specificity (targeted) or the capacity to self-dose at the site of the infection. Recent research has shown that phages are natural, safe, and have no significant negative consequences. Additionally, phages cannot harm the liver or kidney, which is crucial for people with impaired immune systems. Chronic infections brought on by multidrug-resistant (MDR) strains can be treated with personalized phage therapy and on-demand phage production (Jonczyk-Matysiak et al., 2019).

Phage therapy is a viable therapeutic approach because of the multidrug-resistant (MDR) bacterial species' worrisome spread. The results of recent, effective compassionate treatments in Europe and the US support it. Phage cocktail could treat bacteremia in wax moth (Galleria mellonella) larvae and treat acute respiratory infection in mice. Numerous studies have shown that some phages can restrict a pathogenic bacterium's growth both in vitro and in vivo. Phages have been successfully utilized in a number of studies to treat experimental bacterial infections, supporting their usage as first-line therapy, particularly for illnesses brought on by MDR pathogens. Recently, reports of phage therapies in Europe and the United States confirmed their effectiveness and safety (Forti et al., 2018).

The US Food and Medication Administration (FDA) or the European Medicines Agency have approved the emergency treatment of an increasing number of patients even though phages still do not have drug approval in Western medicine. Future clinical trials and present antibiotic failures are being bridged by phage therapy. (Luong et al., 2020). In the United States and Belgium, phage therapy centers are opening. In Europe, phage therapy has been used as an experimental treatment (Poland), a compassionate treatment (e.g., France), or both (e.g., Belgium, which recently established a "magistral approach" where phage production can be done in a pharmacy under the guidance of a

doctor's prescription). In order to use phages as an emergency investigational novel drug in the USA, FDA approval is typically required (Jonczyk-Matysiak et al., 2019).

The prevention and treatment of bacterial infections, especially those brought on by germs resistant to antibiotics, may be revolutionized by inhaled phage therapy. As a potential treatment for bacterial infections, including those brought on by multidrug-resistant (MDR) bacteria, bacteriophage (phage) therapy is regaining popularity. Phages are naturally occurring anti-bacterial with low inherent toxicity, effective against MDR bacterial infections, self-replicating agents, able to co-evolve with bacteria, highly specific, avoiding disruption of non-targeted bacteria, and (vi) able to penetrate biofilms, a problematic state of bacteria in, for example, cystic fibrosis (CF) patients. These are just a few of the key advantages of phage therapy over conventional antibiotic treatment (Chang et al., 2018). Bacteriophages have been shown to diminish *E. coli* O157:H7 in the gastrointestinal tracts of mice and sheep as well as on the surface of the meat in studies. According to studies, phage application might lower the chicken mortality rate on infected farms. It is possible to treat pathogenic bacteria in the food chain with bacteriophages, including zoonotic pathogens that infest the intestines of farm animals (Abdelsattar et al., 2019). Phage therapy has been tested in the veterinary field against a number of zoonotic pathogens with the goal of preventing the spread of sickness to humans and minimizing financial loss. *Escherichia coli* infections in calves, piglets, lambs, and poultry, *Campylobacter* infections in poultry, *Salmonella* infections in poultry and pigs, *Clostridium perfringens* infections in poultry, and *Pseudomonas aeruginosa* infections in dogs are just a few of the documented successful uses. Currently, a few phage products are being sold to combat *Salmonella* infections and *Clostridium perfringens* infections in poultry feed (Nobrega et al., 2016).

Several farm animals with *E. coli* infections responded favorably to phage treatment (calves, piglets, lambs, and chickens). The coliphage T4 family is a prime option for phage therapy of *E. coli* infections. The selected T4-like phages endured adult mice's gastrointestinal transit. More specifically, the orally administered phage titer and the fecal phage count closely matched. In the stomachs of adult mice, T4-like phages weren't significantly inactivated, though. Unprotected T4-like phage can thus pass through the whole gastrointestinal tract without significantly losing its ability to infect. Strong evidence that pathogenic *E. coli* is sensitive to orally administered phages comes from the success of phages in curing experimental *E. coli* diarrhea in mice, calves, piglets, and lambs (Chibani-Chennoufi et al., 2004).

Invivo *P. aeruginosa* infection control has been achieved using phages in animal models like zebrafish, mice, and murine mice. It has been reported that using a phage cocktail to treat P. aeruginosa infections in zebrafish is effective. Phage cocktail's therapeutic potential against *P. aeruginosa* was shown to produce notable decreases in both planktonic cultures and biofilms. The same phage cocktail was similarly effective in treating bacteremia in wax moth larvae as well as acute respiratory infection in mice.

When used against the host biofilm and bacterial planktonic cells, phage MA-1 demonstrated a powerful lytic activity. However, a cocktail (combination of two or more phages) will be a viable choice to control bacterial infections and contamination in order to ensure complete eradication and prevent potential bacterial resistance to phages (Adnan et al., 2020). An acute *P. aeruginosa* lung infection mouse model was used by Pabary et al. to evaluate the effectiveness of a phage cocktail. After receiving simultaneous dose, all mice showed a full bacterial clearance. 86% and 71% of mice who received delayed and preventive treatments, respectively, showed evidence of bacterial clearance. Overall, inhaled phage therapy has demonstrated to be quite effective in treating both acute and persistent lung infections (Chang et al., 2018). Human phage therapy's effectiveness and safety has been the subject of clinical trials. Numerous studies have demonstrated the therapeutic potential and safety of phages both in vitro and in vivo (Park et al., 2021). Phage cocktail can also be used on people. On healthy adult volunteers, a high oral dose of the phage cocktail and a placebo were tried. The makeup of the fecal microbiota in stool was not affected by the phage cocktail, and no negative effects on the functions of the liver, kidney, and blood were noted (Yang et al., 2017).

One of the most important aspects affecting the outcome of a phage therapy is a well-characterized phage. A phage is an ideal candidate for safe phage therapy if it lacks integrase, toxin-related genes, and virulence genes (Ong et al., 2020). The genetic makeup of the phage should be examined to see if it contains genes that give antibiotic resistance, lysogeny (such as integrases and transposons), virulence, and toxicity. Phages having a high adsorption rate, large burst size, and brief latent duration are favored for medicinal usage (Jonczyk-Matysiak et al., 2019). Phages that have a lytic life cycle, large burst sizes, and improved thermal stability have offered themselves as strong prospects for therapeutic use (Piracha et al., 2014). Few numbers of traits are typically screened for a phage to be applied in phage therapy: i) the capacity to eradicate target bacteria in a culture ii) some other indicator of phage virulence Transduction potential, iii) obligate lytic growth or lack of lysogenic capacity, iv) toxin gene screening, and v) host range (Hyman, 2019).

Phage therapy application depends critically on the genetic backgrounds and bacterial phage classification (Yan et al., 2020). There are many ways to give phages. However, they can also be used as spray, creams, tablets, gels, powders, suppositories, bandages, and ocular drops. Liquid application is the most common form in which they are used. Phages used as medicines can be given by a variety of ways, including topically, orally, rectally, parenterally, and intravenously. Phage administration via oral therapy appears to be a simple and immunogenic method (Jonczyk-Matysiak et al., 2019).

2.5 Phage to treat biofilms

A biofilm is described as a collection of microorganisms that are attached to a surface and encased in a matrix made primarily of polysaccharides. Organisms that are

connected with biofilms differ significantly from populations of suspended cells. Antibiotics, disinfectants, and host immune system clearance are all things that bacteria in biofilms exhibit high levels of resistance to (Stepanovic et al., 2007). Bacteria that are connected with biofilms are shielded from removal and inactivation procedures like disinfection. The use of bacteriophages to treat infections that are resistant to antibiotics appears promising. Phages are used in the medical field to treat infections that are resistant to antibiotics, such as those found on implant-associated biofilms and in cases of compassionate care. To stop illnesses from opportunistic pathogens, phage therapy can be employed in the built environment and drinking water infrastructures. Phages can be used in conjunction with chemical disinfectants to better remove bacteria that has been spotted onto surfaces and wet biofilms while halting the establishment of dry biofilms (Stachler et al., 2021).

Due to its distinctive structure, biofilm can offer bacteria a potent protection system. Disinfectants cannot get rid of biofilm. It has been shown that phages can harm biofilm by destroying its structural elements. Bacteriophages thus provide extremely particular choices for the management of bacterial infections that are resistant to antibiotic treatment. Antimicrobial medications cannot effectively treat biofilms. Bacteriophage can even break down *P. aeruginosa* biofilm that has been present for 20 days. It can also break down alginate polymers through enzymatic processes. Bacteriophages are often used to destroy biofilm on the surface of medical devices. When P. mirabilis and *E. coli* were treated with phage as opposed to left untreated, there was a roughly 90% reduction in biofilm. A phage cocktail (combination of phages) may be utilized to remove biofilms more completely and effectively (Adnan et al., 2020). Blood cultures may have a harder time diagnosing infections caused by organisms that create slime than infections caused by organisms that do not produce slime because the daughter organisms are less likely to split out from the parent colony and seed the blood stream (Christensen et al., 1985).

2.6 Phage plus antibiotic synergism

There has been a lot of interest in researching the use of antibiotics in conjunction with phage therapy. Combining phages with antibiotics improves biofilm clearance compared to either treatment used alone, stops the emergence of resistance, and makes antibiotics more effective at lower concentrations (Stachler et al., 2021). Phage treatment worked quite well in conjunction with antibiotics. Phage-resistant mutants were prevented from regrowing when combined with low doses of ciprofloxacin or meropenem, indicating the potential efficacy of in vivo therapy. The combination of phages and ciprofloxacin showed a significantly synergistic effect from a therapeutic standpoint. Antibiotic-induced bacterial elongation is thought to be the mechanism of PAS and may make it easier for phages to approach their bacterial target. Phage would work best when paired with synergistic antibiotics (Oechslin et al., 2016).

Phage treatment can exert far more evolutionary selective pressure than antibiotic treatment alone. Even when several medicines targeting various components are used in combination, *P. aeruginosa* frequently develops resistance to them. Phage cocktail plus either antibiotic (CIP or MEM) had a greater inhibitory effect on resistant *P. aeruginosa* development than phage cocktail or antibiotic alone. In addition to the antibiotic, other phages in the cocktail may exert strong selective pressure on *P. aeruginosa*. The combination of an antibiotic with a phage cocktail is one method for treating pseudomonal infection. Antibiotics combined with a phage mixture offer crucial clinical application insight (Ong et al., 2020). Using phages and six antibiotics from four distinct pharmacological classes, the synergy between phages and antibiotics was evaluated both in vitro and in vivo. Meropenem, a -lactam antibiotic, significantly increased the plaque size of the phage. The expansion of the plaque shows that phages combined with antibiotics are more effective at killing bacteria. Phages may have easier access to receptors on elongated bacterial cells by utilizing antibiotics at sub-inhibitory concentrations. Verma et al. evaluated the effectiveness of ciprofloxacin and phage on immature (12-hour-old) K. pneumonia biofilms. Bacterial burden in the biofilm was reduced by ciprofloxacin or phage monotherapy. In both planktonic cells and biofilm, combination therapy dramatically decreased the generation of mutants that were phage or antibiotic resistant. When a 48-hour-old *P. aeruginosa* biofilm was treated with a combination of phage and tobramycin, Coulter et al. discovered that the emergence of antibiotic- and phage-resistant cells was reduced by 60% and 99%, respectively (Chang et al., 2018). Antibiotic resistance and bacterial fitness are altered by phage. For instance, *P. aeruginosa's* sensitivity to antibiotics was boosted when treated with its phage (OMKO1). The mechanism of this synergistic bactericidal effect is explained by the host's evolution. Bacteria may experience selective pressure from phage infection, which increases their susceptibility to gene mutation. Under this selective pressure, some of the host bacteria's crucial components related to bacterial toxicity, medication sensitivity, and growth factors are lost or down regulated. According to studies, phage-resistant strains are less toxic, more antibiotic-sensitive, and develop more slowly than wild strains (Li et al., 2021).

The interaction between nebulized phages and antibiotics demonstrated that synergy was strain- and antibiotic-dependent against *Pseudomonas aeruginosa*. When the podophage PEV20 and one of the three antibiotics (ciprofloxacin, amikacin, or colistinwere combined, synergy was shown in one clinical strain. In order to prevent bacterial growth synergistically, pre-treating either planktonic or biofilm-growing cells with phage followed by treatment with any of the five antibiotics tested (rifampin, daptomycin, fosfomycin, ciprofloxacin, or vancomycin) was more efficient than co-treatment. Phage reduced the titer of metabolically-inactive cells, such as stationary phase cells or persisters generated either by ciprofloxacin, and destroyed the exopolysaccharide component of an MRSA strain biofilm. Because phages are effective at dispersing biofilms, they may work in synergy with some antibiotics. However, the

sequence in which they are administered is crucial, as doing so may have the opposite of the anticipated synergistic effects. Bacterial populations went down a path of decreased virulence due to mutations brought on by phage infection (Segall et al., 2019).

A promising method to lower the dosage of antibiotics and prevent the emergence of antibiotic resistance during therapy is the use of phages in conjunction with antibiotics. Scientists have suggested that using both of these antibacterial agents in combination may be more effective than using each one alone, as opposed to switching from antibiotics to phages. A joint strategy may have benefits like improved bacterial suppression, more powerful penetration into biofilms, and a decreased ability for bacteria to acquire phage and/or antibiotic resistance. However, when phages are coupled with particular antibiotics, synergistic bactericidal effects are produced that are effective. The type of antibiotics or phages employed determines how they interact with one another (Li et al., 2021).

Biofilms serve as a risky holding area for bacteria that are tenacious and have developed high antibiotic resistance. As a result, biofilms are harder to get rid of than planktonic bacteria that are just given antibiotic therapy. Biofilms are therefore taken into consideration as a possible non-antibiotic treatment. The combined effect of phages and antibiotics for eradicating bacteria in biofilms has been the subject of numerous investigations. An earlier study investigated the effectiveness of phage alone or in combination with several antibiotic classes for the removal of *S. aureus* biofilms in a rat model. Even though it did not completely eliminate the biofilm, treatment with phage alone greatly reduced residual bacteria. However, concurrent phage and antibiotic treatment greatly reduced EPS and eliminated the *S. aureus* biofilm (Li et al., 2021).

Phage-antibiotic synergy (PAS) is the phenomena wherein sub-lethal dosages of particular antibiotics can significantly boost the creation of pathogenic phage by the host bacterium. T4 plaque size and T4 concentration increased with increasing sub-lethal cefotaxime concentrations. An increased burst size and shorter latent period were also produced by applying PAS to the T4 one-step growth curve. Compared to cefotaxime therapy alone, the combination of T4 bacteriophage with cefotaxime considerably improved the removal of bacterial biofilms. The minimal biofilm eradication concentration value of cefotaxime against E. coli ATCC 11303 biofilms was reduced from 256 to 128 and 32 g/ml, respectively, by the addition of medium (10^4 PFU/mL) and high (10^7 PFU/mL) phage titres. This study reveals that bacteriophage and conventional antibiotics can work in cooperation to dramatically enhance in vitro biofilm management (Ryan et al., 2012).

Biofilm-embedded bacteria are notoriously resistant to treatment with antibiotics or bacteriophage when compared to planktonic cells. Phages and antibiotics by themselves typically only had marginal benefits in killing the germs. However, certain phage-drug combination significantly lowered bacterial densities compared to the best single

therapy. Combination therapy was especially successful in eliminating *Pseudomonas* biofilms. Additionally, phages were able to control the rate of ascent of minorities of bacteria that were resistant to the antibiotic being used to treat them. Bacteriophages are anticipated to be more efficient at eliminating bacteria in biofilms than antibiotics because: i) they create polysaccharide depolymerase enzymes that can damage biofilms' extracellular matrix while antibiotics cannot. (ii) By lysing the bacteria on the outside of biofilms, lytic phages expose cells within these formations to exogenous nutrients, increasing their metabolic activity and making them more vulnerable to antibiotic death (Chaudhry et al., 2017).

2.7 Phage cocktail

Phage preparations can either be made up of a single phage or a cocktail of different phages that act as the active agents. The use of a single phage may be advantageous because it may be challenging to produce preparations made up of multiple phages. However, using a phage mixture might be more efficient in battling infections brought on by germs that are resistant to antibiotics. Application of a cocktail (phages using various receptors to adsorb bacteria) may help prevent the occurrence of cross-resistance to the phages used. Synergy occurs when two or more phages that infects the same bacterial strain work together to kill more germs than they would alone (Jonczyk-Matysiak et al., 2019). Phage cocktails are more effective than a single phage in vitro or as a preventive treatment in medical devices, according to studies on the effects of phage, virus cocktails, and antibiotics on *P. aeruginosa* growth. This is because each phage asserts different selection pressures. Due to various phage infection processes, our findings demonstrate that a cocktail of two phages was able to inhibit *P. aeruginosa* development for a longer period of time than a single phage. The absence of cross-resistance in phage resistant strains provided support for this. When compared to resistance to a single phage, a host that developed resistance to both phages might incur a larger fitness penalty (Ong et al., 2020).

Phage mixture had the power to infect and eradicate mucoid strains isolated from CF patients with a chronic infection. The use of a phage cocktail to treat a *P. aeruginosa* biofilm revealed that phages can infiltrate the biofilm, kill the biomass, and get to the bacteria buried inside. In this way, the single-impact phage was significantly enhanced by the usage of the phage cocktail. The cocktail demonstrated the advantage of being more quickly effective at reducing the bacterial burden as compared to single phage. In mice, it was discovered that the cocktail eliminated biofilms more quickly than the individual phages did. This implies that utilizing numerous phages has a synergistic effect. We can speculate that the cocktail inhibits the development of phage-resistant bacteria (Forti et al., 2018). If only one phage strain is employed against specific bacteria, the most important issue for phage therapy is the formation of phage-resistant *E. coli* strains, which happens regularly. In contrast, using a phage cocktail to combine several different phages prevents or delays the emergence of phage-resistant cells. The

majority of phage-resistant cells modify the elements of their outer membrane that are responsible for particular phage binding (phage receptors. Phage resistant mutants emerged as a result of receptor loss and change, as evidenced by the phages' decreased ability to bind to the mutant *E. coli* cells. A two-phage cocktail significantly postponed the emergence of phage-resistant cells. Therefore, a cocktail of more than two phages exploiting several cell receptors ought to postpone and possibly even prevent the formation of phage-resistant cells (Tanji et al., 2004).

The physiological cost of the changes that conferred resistance to the phage cocktail was high. Mutant cells motility was flawed. LPS in the mutant was shortened. In addition to serving as a phage receptor, LPS can be altered to confer phage resistance. LPS is a key component of *P. aeruginosa* virulence, and its mutation can lead to diminished virulence. Therefore, while these changes confer phage resistance, they may potentially reduce animal fitness while maintaining normal growth in less demanding in vitro circumstances. Phage cocktails cover more strains than single phages, but they also run a higher risk of inter-phage interference and undesired gene transfer (Oechslin et al., 2016).

2.8 Characterization of phage

A bacteriophage must possess a number of qualities in addition to the ability to eradicate the target bacterial pathogen in order to be effective in phage therapy. These include advantageous traits like a sizable host range and the absence of undesirable traits like toxin gene carrying and the capacity to generate a lysogen. The usefulness of a phage for phage therapy depends on a number of factors, including host range. It is preferable to have a host range restricted to a single species since this stops the phage from eradicating other species and preserves the remainder of the host's microbiome. However, a phage that infects most or all strains is advantageous since it makes it possible to treat many bacterial infections caused by that species. This is comparable to using broad-spectrum antibiotics before identifying the infection or conducting an antibiotic sensitivity test. Using phages with a wider host range in phage therapy should prevent more treatment failures brought on by inappropriate host and phage pairings. So, a wider host range is preferred in terms of strains within target species. There are some mixed infections (polymicrobial). Therapeutic phage mixes are frequently used to address this (cocktails). A phage should, ideally, not infect other species because doing so could kill nonpathogenic members of the natural flora and could reduce the phage's effectiveness in attacking the target bacteria. (Hyman, 2019).

The majority of phage studies lacked crucial characterization of the phage, which is necessary to determining whether or not the phages are suitable for therapeutic and other purposes. A number of researchers have lately concentrated their efforts on the isolation, characterization, and use of lytic phages against *P. aeruginosa* infections due to the inherent benefits of phages over chemical antibacterial. Burst size describes the typical amount of new phage particles produced from each infected cell following a lytic

cycle, while latency time describes the interval between virion attachment to the host bacteria and release of new phage particles. Short latency and big burst size are thus regarded as defining characteristics of an effective lytic phage and demonstrate a phage's aptitude for therapeutic uses. Phage stability at different temperatures, pH conditions, and storage procedure tolerance are additional crucial factors for their practical employment in a variety of contexts. The candidate phage must endure a variety of pH levels depending on the route of administration in clinical applications, but in an environmental use, it must tolerate a wide range of pH and temperature settings (Sharma et al., 2021).

The one step growth curve is one of their most famous bacteriophage studies. The average number of phage offspring discharged from infected cells following a single infection was demonstrated in this experiment. The average burst size is another name for this figure. A single strain of *E. coli* might produce burst sizes of 20 to over 1000 pfu per cell when infected with a single kind of bacteriophage. *E. coli* cells that develop more quickly are bigger and have proportionally more surface area, which means they have more surface receptors for phage to attach to and be adsorbed. Additionally, due to the difference in cell volume, larger cells contain a higher percentage of the equipment used in protein synthesis than smaller cells. This might make it possible for a larger number of virus particles to reproduce, increasing the burst size (Choi et al., 2010).

The least amount of time between a virus's adsorption and the release of its progeny is known as the minimum latent period of intracellular virus proliferation. Usually, the one-step growth experiment is used to determine it. The minimal latent period of a virus-host cell system is astonishingly stable under constant environmental circumstances. The temperature, numerous metabolic toxins, the physiological state and nutritional milieu of the host cell, as well as the hereditary characteristics of the virus and host cell, all have an impact on the latent period. Damage to the phage particle before infection, such as that brought on by ultraviolet and ionizing radiation, may potentially have an impact on the latent period (Adams & Wassermann, 1956).

Thermal treatments will cause morphological changes in heat-sensitive phage particles. These modifications include the release of phage DNA from viral capsids, phage dissection into head and tail structures, and phage tail aggregation. Numerous researches have looked at the *L. lactis* phages' responsiveness to heat treatment using specific temperature/time combinations. This research showed that a number of phages can tolerate the pasteurization process. Additionally, certain viral groups with very strong heat resistance have developed in recent years. For instance, after heating lactococcal phage viral particles at 90 °C for 20 min or 97 °C for 5 min, infectious viral particles were still present (Geagea et al., 2018). There have only been a few investigations on the thermostability of naturally occurring and recombinant phage. Early research on filamentous phages showed that temperatures below 70 °C do not damage virions, and that heating to 80 °C for 10 minutes does not impair the virion's

infectious potential. The thermostability of virions at 70–80 °C has also been demonstrated by other filamentous phages. It has been demonstrated that filamentous bacteriophages from extreme thermophiles like *Thermus thermophilus* are stable at temperatures as high as 130 °C. Actually, a second extremely thermostable phage was discovered after sewage was heated to 90 °C for 10 minutes to destroy other phages (Brigati & Petrenko, 2005)

2.9 Effect of external factors on bacteriophage

External elements like temperature, pH, water activity, and salt concentration can have an impact on the lytic activity and stability of phages (Park et al., 2021). The key criteria limiting bacterial persistence are temperature and acidity of the environment, which both serve to limit phage activity. To prepare and get high titer phages for therapy, the right circumstances, growth medium composition, and strain selection are essential (Jonczyk-Matysiak et al., 2019). The concentration of bacteria and the physiological state of the bacteria have already been mentioned as having a substantial impact on phage propagation. Hadas and colleagues demonstrated that the host's growing parameters affect the phage T4's ability to spread. It was discovered that the latent period and burst size of phage T4 are dependent on the pace of bacterial growth. The eclipse and latent period are both reduced by an increase in bacterial growth rate, but burst size is increased. The findings unmistakably demonstrated that the three phage growth parameters—adsorption constant, latent period, and burst size—that collectively define the rate of bacteriophage population increase are significantly influenced by the rate of bacterial growth (Nabergoj et al., 2018).

2.10 Bacteriophage encapsulation

Since the low pH values dramatically reduce phage titer and proliferation, the acidity of the gastric environment in the majority of animals is a key concern when phages are administered orally. Animal body temperature, which ranges from 38 °C for dogs to 42 °C for chickens, is another factor that may influence the effectiveness of a treatment. This is because temperatures above the optimum lengthen the phage's latent period while temperatures below the optimum inhibit phage penetration and subsequent proliferation. Phages may also be degraded by gastro intestinal tract (GIT) enzymes such pepsin in gastric fluid and amylase, lipase, and protease in pancreatic fluid. Orally given phages have difficulties, so it is preferable to offer additional protection to improve their survival in the GIT. To increase viral stability, phages have been enclosed in matrices made from natural biopolymers. With the help of this technology, phage survival has been improved in the GIT of cattle, pigs, and poultry (Nobrega et al., 2016).

2.11 Bacteriophage purification

Phages are a particular class of virus that can only grow inside of a bacterial cell. The separation of phages from bacterial cell detritus, such as endotoxins (such as lipopolysaccharides, or LPS), peptidoglycan, exotoxins, flagella, nucleic acids, and other substances, is thus a significant challenge in the development of medical phages. These

severe contaminants could cause possibly fatal inflammation, sepsis, and septic shock in people if they are not sufficiently eliminated. Endotoxin content has up till now been the main safety issue with phage products for human use. It has been difficult to effectively remove endotoxins from phage products for use in human phage therapy; this is because doing so frequently necessitates large product dilution, which lowers the amount of phage's active ingredient. When creating therapeutic phages in bacterial systems, endotoxin reduction is crucial. Furthermore, bacterial contamination in phage preparations may cause inflammatory reactions in cells (Luong et al., 2020).

Lipopolysaccharide (LPS) and lipoteichoic acid, respectively, are common endotoxins found in phage lysates made by Gram-negative and Gram-positive bacteria. In order to get rid of any bacterial waste, endotoxins, and undesirable elements like growing media, phage purification is a crucial step. Phages may be inactivated during this procedure, which would reduce their titer (Chang et al., 2018). Purification is required to get rid of contaminants such lipopolysaccharides (endotoxin), flagella, proteins, and peptidoglycan fragments that come from the bacterial host. Elimination of endotoxins is crucial for preventing anaphylactic reactions. Bacteriophages can be purified using a variety of methods, such as chromatography, ultracentrifugation (differential centrifugation, density gradient centrifugation), polyethylene glycol (PEG) precipitation, or organic solvent clarification. These methods allow the samples to be prepared to an extremely high level of purity. There may be a considerable danger of inactivating phages during the purifying process. Lysate purification from bacteria should be carried out using a filtration approach through 0.22 m pore filters in the case of lipid encased phages that are susceptible to chloroform (Jonczyk-Matysiak et al., 2019).

2.12 Bacteriophage storage

The stability of phage particles should be guaranteed, and phage storage should guard against phage activity loss. Disaccharides including lactose, sucrose, and trehalose should be added to boost the survival of phages after freezing and lyophilization. Better phage survival after lyophilization and after rehydration is also made possible by the inclusion of proteins (casein and lactoferrin), peptides, and monosodium glutamate to the amino acid formulation. Additionally useful as an addition is PEG 6000. It is important to regulate and keep an eye on the temperature, humidity, and lighting levels. During storage, phage lysate must be guarded from contamination and evaporation. Because phages are protein structures and a high temperature promotes denaturation of their protein coat, phage inactivation may take place at a high temperature. Methods of encapsulation can be employed to keep the bacteriophages active and enable release at the intended spot. Additionally, it offers defense against deactivation brought on by the stomach's acidic pH, digestive system materials, and released enzymes. As a result, they can be utilized as an alternative to antibiotics and as an ingredient in drinking water and animal feed (Jonczyk-Matysiak et al., 2019).

2.13 Phage applications apart from phage therapy

The benefits of phages' antimicrobial effects have long been understood, and bacteriophage therapy has been employed in a variety of settings, including both human and veterinary medicine. Phages can also be utilized in clinical diagnostics, as delivery systems for vaccines, or as possible carriers of therapeutic genes, in addition to bacteriophage therapy. The availability of phages also makes it possible to use phage display as a method for studying protein-protein, protein-peptide, and protein-DNA interactions (Nabergoj et al., 2018). In numerous contexts, including agriculture, the food industry, and environmental applications, phages are already used to manage and prevent bacterial infections (Sivec & Podgornik, 2020).

Disinfection is one of the finest methods for stopping the spread of these infections. The system may later become populated by more resilient pathogen subpopulations, though. Therefore, there is a strong need for new techniques for pathogen management in the built environment. Bacteriophages have drawn more attention recently as potential therapy options for the crisis of antibiotic resistance. Phages are utilized as bio control agents in a variety of fields, including medicine, agriculture, aquaculture, and the food industry, as preventative health measures. Phages are therefore promising disease control agents in the built environment. To remove more surface-associated bacteria, phage and chemical disinfectants can be combined. It is possible to achieve the same level of inactivation as using the highest tested phage concentrations alone by combining lower phage concentrations with chemical disinfectants. This supports the possible use of phage-based treatment alternatives as a means for reducing or getting rid of harmful bacteria in the built environment, especially those that are found in biofilms and are more resilient to conventional removal techniques. Combining chemical disinfection with phage treatment may be able to remove more bacteria than each method by alone (Stachler et al., 2021).

Results from decontamination trials demonstrated the phage's notable effectiveness in cleaning solid surfaces. Phage treatment can efficiently cleanse polluted surfaces that cannot be treated to more traditional decontamination techniques like UV exposure, autoclaving, etc. Previous research has also shown that *P. aeruginosa* biofilm formation on catheters and endotracheal tubes may be effectively controlled by phages, as well as biofilm formation in mono and mixed cultures utilizing various in vitro and in vivo methods. Additionally, the use of phage produced an efficient decontamination, indicating that it is a promising decontaminating agent for *P. aeruginosa* (Sharma et al., 2021).

Because phages are host-specific, they have little effect on the natural microbiological characteristics of food products, making them ideal bio control agents in the food business. In fact, the SalmoFresh® and SalmonelexTM commercial phage products were given the GRAS designation by the US FDA (generally recognized as safe). To increase food safety, numerous researches have sought to use phages on a variety of foods,

including raw meat, chicken, and milk. As a result, phage-based agents might be used as secure bio-control agents and might not provide a risk to the safety of food (Park et al., 2021). A phage preparation against *Listeria monocytogenes* was approved by the US Food and Drug Administration in 2006 with the status generally recognized as safe (GRAS) (FDA). Apart from their therapeutic use, genetically modified phages have potential uses, primarily in medicine and biotechnology, for the delivery of DNA and protein vaccines, as gene delivery vehicles, for targeted drug delivery (used in cancer treatment), as tissue specific peptides, as a display system for screening and isolation of peptides used in drug discovery, for the detection and identification of pathogenic bacterial strains, as well as to prevent resurgence. They can be applied as biocontrol agents in agriculture, food production, water treatment, membrane filtration, and material engineering (Jonczyk-Matysiak et al., 2019).

Escherichia coli (STEC), a well-known foodborne pathogen that can result in serious disease, produce the shiga toxin. Interest in identifying and characterizing various lytic phages is developing in order to use phages as an alternative to antibiotics in managing bacterial diseases due to worries about antibiotic resistance as well as the benefits of host specificity of lytic phages against the bacterial hosts. Knowing the genome sequence of lytic phages is essential for ensuring that no lysogenic factors, virulence-related genes, or antibiotic-resistance genes are encoded in bio control applications. Phage stability is important and strongly related to how well infections can be controlled when applied. These results support the phage as a viable bio control agent to stop Shiga toxin-producing *Escherichia coli* from spreading in the pre-harvest environment (Liao et al., 2019). The most effective, potent, and widely used treatment against pathogenic *E. coli* that causes severe diarrhea in hospitals and animal farms is antibiotics. But abuse of antibiotics over the past 50 years has resulted in a significant increase in drug-resistant bacteria. Antibiotics also destroy the healthy gut flora and the delicate balance of the intestinal micro biome. Phages can be used in animal agriculture to reduce infections brought on by pathogenic *E. coli*. These outcomes also supported the use of phage to reduce the number of harmful *E. coli* in the environment. To decrease *E. coli* contamination and improve food safety, a phage cocktail can be administered to food. Phage surface application is a workable method of food preservation that might possibly be used on other meats (Yang et al., 2017). T7-group bacteriophages may be an effective sanitizing agent for regulating situations where *P. fluorescens* may be a health risk. The research with *P. fluorescens* biofilms treated to phage suggest that a variety of lytic *P. fluorescens* phages may be easily extracted from the environment and that their usage as alternative sanitation agents will most likely be possible for application in the food and dairy industries. The results of this investigation support the idea that the phage IBB-PF7A can be a useful choice for phage-based sanitation in food processing environments, avoiding product deterioration brought on by strains of *P. fluorescens* that produce extracellular enzyme (Sillankorva et al., 2008).

Phages are a potentially effective method for reducing foodborne microorganisms. In a range of food matrices, such as milk and lettuce, phage indicated potential efficacy as a biological control agent against *Salmonella*. The usage of antibiotics to lessen the impact of salmonellosis on agricultural output has led to the establishment of antibiotic-resistant bacteria. In these situations, phage-based bio control against bacterial diseases in the food supply chain can be a tempting alternative. Food and Drug Administration (FDA) and Food Safety and Inspection Service of the United States Department of Agriculture have approved the Phage application (USDA). These can be utilized as antibacterial agents in processed foods and raw materials for foods (Yan et al., 2020).

The advancement of molecular biology and biotechnology has been greatly aided by these viruses. Studies on bacteriophages led to the discoveries of (among other things) that DNA is a genetic material, that the genetic code is based on nucleotide triplets, and that gene expression is carried out by mRNA molecules to realize their significance in understanding the molecular bases of biological processes. Additionally, the development of genetic engineering and biotechnology was greatly aided by bacteriophages. In fact, the initial cloning vectors were based on bacteriophages, and many of the genes and regulatory sequences utilized in systems for regulated gene expression and genetic recombination come from bacteriophage genomes (Jurczak-Kurek et al., 2016).

2.14 Next Generation Sequencing (NGS)

First-generation technologies: Sanger sequencing, developed in 1977, is a member of the first generation. It uses four fluorochromes to mark dideoxynucleotide triphosphates (ddNTPs), which is based on the DNA chain termination principle. After gel electrophoresis is used to separate the tagged fragments, fluorescence detection is used to identify the base. This technique produces lengthy contiguous sequence reads (> 500 nucleotides), and it is being utilized to verify the outcomes of NGS investigations. Only a few thousand nucleotides can be sequenced in a week due to the lengthy process (Wadapurkar & Vyas, 2018). The Sanger method was the leading technology for genome sequencing for approximately 30 years. This technique produces expensive, low-throughput long reads (800–1000 bp) (El-Metwally et al., 2013).

The terms "second generation" and "next generation sequencing" (NGS) apply to a broad range of techniques in which numerous sequencing operations take place simultaneously, producing enormous amounts of sequencing data. Whole genome sequencing can be done more affordably and with fewer errors using NGS techniques. With the help of second-generation sequencing, sometimes referred to as high-throughput sequencing, thousands or millions of short sequence reads can be produced quickly, accurately, and in a matter of hours. In contrast to Sanger sequencing, which uses dye terminator science to produce a single lengthy read (often > 1 kb), NGS approaches generate a large number of small reads in the range of 50–250 bp using

reversible sequencing chemistry. First, DNA molecules are split into smaller fragments at random places using restriction enzymes or mechanical pressures. The sequencing process then proceeds in three broad steps. The final stage involves sequencing these fragments and mapping them back to a known reference sequence from a sequencing library made up of such known insert size fragments. Shotgun sequencing is the name given to this technique. The sequence is derived from the tiny sequenced fragments that were assembled into contigs (groupings of overlapping, continuous fragments). This is done where there is no known reference sequence for the given organism. By sequencing a massive number of small DNA fragments in parallel, NGS equipment provide better throughput data at an extremely fast rate (Wadapurkar & Vyas, 2018).

Since the development of parallel sequencing technology, often known as next-generation sequencing, the area of biological research has undergone fast change (NGS). These sequencers deliver short, high-throughput reads at a reasonable price. Numerous biological research fields including genomics, transcriptomics, metagenomics, proteogenomics, gene expression analysis, and noncoding RNA, are being advanced by NGS (El-Metwally et al., 2013).

NGS platforms have been developed by Biotechnologies/SOLiD, Roche 454 and Illumina (Wadapurkar & Vyas, 2018). The Illumina HiSeq/MiSeq, Life Technologies SOLiD, Roche 454 and Ion Torrent are currently available NGS platforms. The first commercial NGS technology, Roche 454, was released in 2005. The most affordable sequencing technology is Illumina HiSeq/MiSeq, which was created in 2006 and costs $0.02 per million bases (Wadapurkar & Vyas, 2018). The cost of sequencing has significantly lowered with the advancement of next-generation sequencing (NGS) technologies. The price of a DNA sequence per megabase has dropped from around $10,000 to less than $0.1 over the previous seventeen years. Illumina platforms dominate the sequencing market out of all the platforms (Liu et al., 2019).

By sequencing a massive number of small DNA fragments simultaneously, NGS equipment provides better throughput data at an incredible speed. The Illumina platform can generate a lot of precise sequencing reads (Wadapurkar & Vyas, 2018).

The majority of the data generated, however, is skewed by frequent sequencing errors and genetic repetitions. They produce short reads (usually 100 to 150 bp for Illumina) , but with greater error rates (El-Metwally et al., 2013). Reads generated via sequencing have mistake because of the technology's nature. Nearly 1% is the average mistake rate of the Illumina Miseq system, which is far greater than the 0.001% error rate of conventional Sanger sequencing systems (Liu et al., 2019).

Quality control (QC) procedures like read filtration are crucial for producing reliable conclusions from downstream analyses. Additionally, adapter contamination, which can result in NGS alignment issues and an increase in the frequency of misaligned reads, necessitates adapter trimming in the majority of cases. Several tools with various

features, including FastQC, Seqtk, and PIQA, have been created to date to deal with FASTQ files generated by Illumina sequencing FASTQ or FASTA files (Liu et al., 2019).

In order to make sure that the input read files are free of contamination and sequencing artifacts, such as adapters, necessary quality control (QC) processes must be taken. Quality trimming may be suggested in some circumstances since it could dramatically cut the mistake rate (Prjibelski et al., 2020).In the quality control step, problems such base calling mistakes and poor quality readings are evaluated. By taking into account the errors when calculating quality score, the FASTQC tool performs quality control (Wadapurkar & Vyas, 2018).

For downstream data analysis to produce high-quality and high-confidence variations, quality control and preprocessing of sequencing data are essential. Overrepresented sequences, base content biases, and adapter contamination can all harm data. Even worse, mistakes are always made throughout the library preparation and sequencing processes, which can lead to erroneous representations of the original nucleic acid sequences. A Java-based quality control tool called FASTQC provides per-base and per-read quality profiling (Chen et al., 2018). When calculating quality scores, the FASTQC program evaluates the quality while taking into account errors (Wadapurkar & Vyas, 2018).Fastp, an extremely quick utility for read filtering, base correction, and quality checking on FASTQ data. Fastp has the ability to automatically find adapter sequences in Illumina data that are single-end or paired-end. Fastp is significantly faster than its competitors because it was created in C/C++ as opposed to the aforementioned tools, which were created in Java or Python. Furthermore, compared to traditional methods, fastp can produce even more accurate clean data by removing sequencing errors. Fastp offers the best data filtering of all the tested solutions and is faster than its rivals. It is quick and effective in filtering and quality-controling FastQ files (Chen et al., 2018).

Starting with a collection of short reads, the next-generation genome assembly process may or may not include errors, depending on the experimental sequencing techniques used. A computer tool called an assembler joins these reads to create longer contiguous reads known as contigs. Contigs from these contigs are combined to create scaffolds, which are longer contigs. The comparative technique and the de novo approach are the two methods used for genome assembly. A reference genome from the same organism or a closely related species is utilized as a road map during comparative assembly, sometimes referred to as reference-based assembly. The genome is not assembled using a map or other assistance during de novo assembly. De novo assembly is therefore employed to recreate genomes that differ from previously sequenced genomes (El-Metwally et al., 2013).

Software for de-novo sequence assembly is called SPAdes. It seeks to construct accurate and continuous sequences from short reads (often referred to as contigs and scaffolds). SPAdes were initially intended to assemble bacterial genomes using short Illumina reads.

Short reads are used by SPAdes to build a de Bruijn graph at the beginning of assembly. Additionally, the created graph goes through a simplification process that involves removing incorrect edges (Prjibelski et al., 2020). SPAdes accept input in the FASTA and FASTQ formats as paired-end reads, mate-pairs, and single (unpaired) reads. The first generation of SPAdes was developed in 2011 by a team of young Russian researchers. The name of the assembler comes from St. Petersburg, where the facility is located. The first objective was to create a mechanism for assembling a novel type of data—single-cell bacterial sequencing (Prjibelski et al., 2020).

VirFinder is a machine learning approach for virus contig identification that is based on k-mer frequency. Gene-based similarity searches are completely disregarded. Instead, VirFinder exploits the empirical finding that viruses and hosts have distinctly different k-mer signatures to identify viral sequences. VirFinder outperformed VirSorter, the most advanced gene-based virus classification tool, in terms of accurately detecting real viral contigs. The most recent and effective tool for finding viral sequences is called VirSorter. Similarity searches to accessible viral datasets are crucial to VirSorter's operation. To make a prediction, VirSorter, which is still gene-based, needs at least three anticipated genes to be present within a contig. As a result, several shorter contigs are excluded. Since many novel viruses' genes have not been described or are not well represented in reference databases, VirSorter may miss many of them. K-mers approaches can perform better for short sequences because they don't require gene discovery or gene similarity comparisons. Using k-mer patterns further eliminates the need for signature genes or alignment to well-known viruses. When detecting prokaryotic viral sequences from genomic data, VirFinder uses a k-mer-based software. VirFinder predicts viral sequences based on sequence signatures using machine learning. Particularly for small (1000 bp) contigs, VirFinder outperforms VirSorter in accurately detecting novel viruses (Ren et al., 2017).

VirFinder is a machine learning technique based on k-mer frequency features to identify viral sequences. The fact that VirFinder uses k-mer frequencies to describe sequences allows it to anticipate contigs from both coding and non-coding areas. In addition, VirFinder is independent of gene discovery and homology-based searches, allowing it to forecast small viral contigs with few or even only partial genes (Ren et al., 2020). The majority of the available tools, including Phage Finder, Prophage Finder, Prophinder, and PHAST tools, detect phage sequences by homology searches against recognized phage sequences in the databases of the day. Only a small number of viral genomes have been deposited in the present databases, despite the fact that there are thought to be 1031 viral particles invading microbial populations. Because of this, many phages that are undiscovered or uncultured may be overlooked by the present technologies. Recently, VirSorter and VirFinder were created in order to forecast unknown phages from metagenomic data. To find viral genes, VirSorter has used two reference libraries of viral protein sequences. In order to extract viral sequences from metagenomic samples, a k-

mer-based machine learning model was developed. VirFinder hypothesize that viruses have variable k-mer frequencies (Zheng et al., 2019).

The process of locating and marking each relevant feature on a genomic sequence is known as genome annotation. The locations of projected coding regions and potential products should be included. Prokka is a piece of software used to annotate genomic sequences. Preassembled genomic DNA sequences in FASTA format are what Prokka anticipates. The ideal input is whole sequences without gaps, but it is anticipated that the average input will be a collection of scaffold sequences generated by de novo assembly tools. To locate the coordinates of genomic features within contigs, Prokka uses external feature prediction tools. Genes that code for proteins are annotated in two stages. Prodigal finds the positions of potential genes but does not provide information on the potential gene product. A large database of known sequences is often compared at the protein sequence level in order to anticipate what a gene code for traditionally. The best significant match is then transferred as the annotation for the gene. This strategy is employed by Prokka, but in a hierarchical fashion, starting with a smaller reliable database, progressing to a medium-sized yet domain-specific database, and lastly to curated models of protein families. The result is labeled as "hypothetical protein" if no matches can be found. In the output directory that is chosen, Prokka creates 10 files. Prokka was created to be quick and precise (Seemann, 2014).

2.15 Genome annotation

Gene recognition is a major issue for bioinformatics. The three primary methods for identifying genes are ab initio exon recognition, indirect evidence based on sequence similarities to known genes and proteins, and direct evidence of transcription supplied by ESTs or mRNAs. The latter two strategies serve as the foundation for most theoretical forecasts. But right now, it's still unknown what a DNA sequence's coding potential actually entails (Luo et al., 2003). One of the most frequently observed signs of a gene’s presence are an open reading frame (ORF) that is sufficiently lengthy, particularly in organisms where splicing is uncommon. In order to not overlook potential significant genes, ORFs that are only as long as the shortest typical proteins (let's say, 50–100 amino acids) are frequently recorded. It is well known that only a small percentage of them are actually likely to be protein coding regions. However, it has been challenging to determine the ORF length intelligent cutoffs and to evaluate the likelihood of gene function at each cutoff. There are several computational and experimental techniques that can be used to assist a researcher in determining whether a section of DNA contains a gene. Comparing proteins listed on Swiss-Prot that have undergone experimental verification is one method, while simulating DNA using random models is another (Fickett, 1995).

Open Reading Frames (ORFs), which are separated by start and end codons, are used to encode proteins. Most ORFs are quite brief. Because stop codons tend to be AT-rich, it is widely known that the distribution of overall ORF lengths and GC concentration of a

genome correlate. In viruses, arrangements of overlapping genes have long been recognized. It refers to large overlaps where two genes are integrated in one another or share a DNA locus. In most genomic annotation tools, the majority of shadow is disregarded as false positives. In bacteriophages, several overlapping genes that are embedded have been found. We could demonstrate that shorter than average ORFs can also be significant depending on which alternative frame they occur, in contrast to conventional techniques where only the largest ORFs are considered being candidates for protein coding genes (Mir et al., 2012).

The primary basis for genome annotation is the recognition of similarity between newly discovered genes and proteins and previously annotated sequences. Genes predicted in recently sequenced genomes or metagenomes are translated and compared to reference databases to find homologues, in general. Well-studied species still have sizable percentages of their coding sequences functionally unannotated, despite the exponential growth of sequence databases and the continual addition of annotation data in reference databases. Predicted protein sequences are often categorized as "hypothetical" proteins when they cannot be functionally annotated (Lobb et al., 2020). Particularly when they are unique to a certain lineage or organism, a significant portion of newly found genes have an unidentified functional role. These genes, which are currently classified as "hypothetical," may support crucial biological cell activities and may one day be the subject of medicinal, diagnostic, or pharmacogenomics research. Associating these newly anticipated genes with a biological function that can be verified by experimental screens is a significant problem for the scientific community. We must rely on cutting-edge biotechnological approaches, such as DNA chips and protein-protein interaction screens, as well as computational techniques to assign potential roles to these genes in the absence of sequence or structural homology to known genes. A staggering number of novel genes have been discovered as a result of recent advancements in genomic sequencing, but their biological functions are still unknown. (Karaoz et al., 2004).

Open Reading Frame (ORF) is the segment of DNA between start codon and stop codon. ORF helps us in gene prediction. Long ORF, along with other information is used to identify protein coding region in DNA sequence. However, all of the ORF are not translated so they necessarily code proteins. A gene prediction software typically looks for a start codon and then followed by a DNA sequence long enough to code a protein. So, an ORF should be at least 100 codons in length. Proteins are encoded in Open Reading Frames (ORFs) delimited by a start and stop codon. Although the number and the typical length of ORFs may vary, bacteria share common characteristics of their open reading frame length distribution, which is correlated to their GC-content. Most ORFs are rather short. It is a well-known fact that the distribution of the overall ORF lengths correlates with the GC content of a genome, simply because stop codons being AT-rich. The GC-content of a genome also governs overall codon usage in a genome. s. An ORF is

defined as the longest string of triplets beginning with a start codon. Statistical properties of ORFs are important in the context of shadow genes, a phenomenon generally accepted in viruses and bacteriophages. The term 'shadow gene' refers to extensive overlaps in which two genes share the same DNA locus or are genes even embedded one in the other. Most shadow genes have escaped discovery, as they are dismissed as false positives in most genome annotation programs. This is in sharp contrast to many embedded overlapping genes that have been discovered in bacteriophages (Mir et al., 2012)

The length of an open reading frame (ORF) is one important piece of evidence often used in locating new genes, particularly in organisms where splicing is rare. A sufficiently long open reading frame (ORF) is one of the most commonly noted indications for the existence of a gene, especially in organisms where splicing is rare. Two techniques may be used to evaluate the significance of ORF length as one piece of evidence supporting the identification of putative genes. The first is a comparison between the length distribution of experimentally verified proteins listed in Swiss-Prot, on the one hand, and the length distribution of ORFs in the nucleotide sequence collections, on the other. The second is simulation of DNA under random models to find the frequency with which ORFs of different lengths occur by chance. The probability that an ORF of 100 codons represents a true gene is quite small. This does not, of course, mean that all ORFs of 100 codons are without function. On the contrary, this evidence neither proves nor disproves the hypothesis that an ORF of 100 codons is a gene (Fickett, 1995).

A substantial fraction of hypothetical open reading frames (ORFs) in completely sequenced bacterial genomes are short, suggesting that many are not genes but random stretches of DNA. The density of genes, coupled with the presence of several gene-specific features and the lack of introns, makes the identification of coding sequences in bacterial genomes a relatively straightforward procedure, at least when compared with the task of gene recognition and annotation of genes in eukaryotes. But, despite the numerous shared properties of bacterial genes, the thorough and robust annotation of complete bacterial genomes cannot rely solely upon a simple set of pre-established rules. Among the more troublesome tasks is the verification of very small ORFs. The preponderance of short annotated ORFs is hypothetical, of unknown function, and not likely to be genuine genes. Based on the numbers and size distributions of ORFs with matches in the current databases, it was estimated that, for the majority of species, perhaps 10–30% of recognized ORFs do not actually encode proteins. The reading frame of protein coding regions can usually be deduced from alignments of homologous sequences in closely related organisms. Many short, annotated ORFs are not genuine protein-coding regions (Ochman, 2002)

DNA annotation is the process of finding the locations of genes and coding regions in the genome. It also determines the function of gene. After the process of whole genome sequencing, the next step that is done is DNA annotation. DNA annotation provides

various informations to the newly sequenced genome. It includes gene names, intron and exon, regulatory sequences, repeats and protein products. Genome annotation mainly consists of predicting genes and attaching biological information to these genes. Protein non coding regions are also identified. DNA annotation can be done by searching the homologous genes in databases by using search tools such as BLAST and the information derived from it can be used to annotate genome. So, at first, identification of ORFs, regulatory motifs and coding regions is done. Then it is followed by adding biological information to these elements. DNA annotation takes help of both biological experiments and computer (in silico) analysis. The emergence of high-throughput biology has increased our ability to identify new genes. However, a lot of newly discovered genes have unknown function and such genes are named ‘‘hypothetical”. These proteins might perform important biological functions in cell. Apart from this, these hypothetical genes can also be targets for medical or pharmacogenomics studies. So, one of the main challenges for the researchers is to assign biological functions to these newly predicted genes. Recent development in genomic sequencing technology has generated large number of new genes whose biological functions remains still to be identified. For example, large numbers of prokaryotic genes (35%) are annotated as ‘‘function unknown”. Sequence homology can help us providing clue about the function of these newly discovered genes (Karaoz et al., 2004).

Gene finding in bacterial genome is relatively easy but assigning function to these genes is challenging task. This leads to a vast quantity of hypothetical sequences whose function remains unknown. Genome annotation is based on detecting homology between newly identified genes and sequences that have been previously annotated. Genes predicted in newly sequenced genomes are compared against reference databases to find the homologues and then annotations are provided to these query proteins based on homologues. Early model organisms, such as *Escherichia coli*, *Bacillus subtilis* and *Caulobacter crescentus*, have their annotations experimentally derived and their genome is used as reference for annotation of newly found genes. However, such limited source can lead to result biases in genome annotation. The success rate is greater in species that are phylogenetically closer. However in this digital age, functional annotations can be transferred between sequences through a variety of computational method such as standard approaches (include sequence-to sequence searches such as blast that scan newly identified sequences against models of protein and/or domain families) and profile based methods (such as the National Center for Biotechnology Information's (NCBI’s) Conserved Domain Database) NCBI database is one of the most sensitive approaches for protein classification. NCBI database searches are capable of detecting distant matches to protein. In some case if full protein match cannot be found, domain families are used to find matches. Sequence databases are growing at an exponential rate. However well-studied organisms still have large proportions of their coding sequences (CDSs) functionally unannotated. When predicted protein sequences cannot be functionally annotate, these protein sequences are classified as ‘hypothetical’

proteins. These hypothetical sequences consist of proteins of unknown function. They can also be potential pseudo genes (Lobb et al., 2020).

When two or more genes interact together to produce effect distinct from each other such genes are called complement and two or more of these complements can interact to produce one distinct function. Examples related to intragenic complementation have showed separable functional domains of proteins. It also further suggested that there are interactions among polypeptide chains in (proteins) oligomeric complexes. Examples haves shown that mutation in one gene fails to complement other gene. This leads to the conclusion that product of two or more interacting genes are involved in the same function (Hays et al., 1989).

3. MATERIAL AND METHODOLOGY

This study was performed in Central Department of Biotechnology, Tribhuvan University. Most of the work was wet lab based. Required equipment such as centrifuge, incubator, vortex, PCR machine, media, reagents, glass wares, plastic wares, syringe filters, etc. were made available by the department.

3.1 Bacterial strain collection and preservation

Pseudomonas aeruginosa used in this study was collected from Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu. This bacterium was Urinary Tract Infection (UTI) causing bacteria. It was immediately transported to microbiology laboratory of Central Department of Biotechnology, TU Kirtipur at cold box maintaining 2-8°C.

Glycerol stock of *Pseudomonas aeruginosa* was prepared for preservation and further use. For the preparation of glycerol stock, a pure colony was cultured in LB broth. It was then incubated for 24 hours at 37° C. One milliliter of the bacterial culture was taken in cryovial and it was centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded and pellet was resuspended in 300µl fresh LB broth. Then 700µl 50% autoclaved glycerol was added and incubated at 37° C for 3 hours. After incubation, tubes were cooled at 4°C for overnight. Then further cooled at -20°C for next 24 hours and then finally transferred to -80°C for long term storage.

3.2 Identification of bacteria by biochemical test

Pseudomonas aeruginosa obtained from Teku hospital was subcultured in Nutrient Agar (NA). After this, biochemical tests were performed. The biochemical tests performed includes MRVP (Methyl Red Voges Proskuar), Citrate, Urease, TSI (Triple Sugar Iron) and SIM (Sulphur Indole Motility) test. For these tests the respective media were prepared in test tube and bacteria were cultured in these media. Bacterium was incubated overnight at 37°C. Color change was observed for citrate, urease, MR VP, TSI. TSI was also observed for Hydrogen Sulphide (H_2S) production. SIM test was observed for H_2S production and motility.

3.3 Antibiotic Susceptibility Test

Kirby-Bauer disc diffusion technique was used for testing antibiotic susceptibility pattern of bacteria. Antibiotics used in this study were made available at the laboratory. These antibiotics were classified under sensitive(S), intermediate (I) and resistant (R) according to the Clinics and Laboratory Standard Institute (CLSI) guidelines.

Log Phage bacterial culture was grown and their turbidity was matched with 0.5 Mac-Farland solution. After this the bacterial culture was lawn on Muller Hinton Agar using sterile cotton swab and left for air dried. After air drying, antibiotic discs were placed on

the lawn culture and incubated at 37°C for 24 hours. After incubation, the zone of inhibition of the antibiotics against the bacterial growth was observed and measured using calibrated ruler. Antibiotics used were given in the table below

Table 1: Antibiotics, their codes and concentrations

S.N.	Antibiotics	Code	Conc of disc (mcg)
1	Meropenem	MRP	10
2	Imipenem	IMP	10
3	Nalidixic Acid	NA	30
4	Cefotaxime	CTX	30
5	Piperacillin	PI	100
6	Cephalothin	CEP	30
7	Cefoxitin	CX	30
8	Amikacin	AK	30
9	Gentamicin	GEN	10
10	Ampicillin	AMP	10

3.4 Genomic DNA extraction

Genomic DNA extraction of *Pseudomonas aeruginosa* was performed by CTAB method. In this method, 1 ml of overnight bacterial culture was taken in micro centrifuge tubes tube. The cells were harvested by centrifugation at 13000 rpm for 5 minutes. To the pelleted cells, 567µl of TE buffer was added and mixed gently by vortexing for resuspension. 30µl of 10% SDS was added followed by addition of 3µl of proteinase K to the solution by proper mixing. Then it was incubated at 37° C for 1 hour. After incubation, 100µl of 5M NaCl was added followed by addition of 80µl CTAB/NaCL (0.7 M NaCl, 10% CTAB) and mixed well. Then the solution was incubated at 65°C for 10 minutes. After incubation, equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the solution and it was mixed well by up and down of the tube. Then the mixture was centrifuged at 13000 rpm for 5 minutes. After centrifugation, upper aqueous layer was transferred to a new tube and 600µl isopropanol was added to the solution. It was then gently mixed until the DNA was precipitated. The solution was then centrifuged at 13000 rpm for 5 minutes. The supernatant (isopropanol) was removed after centrifugation. Then 1 ml of 70% ethanol was added to wash the DNA and centrifuged for 5 minutes. Supernatant (ethanol) was discarded after centrifugation and the microcentrifuge tube was left for air dry. DNA was then resuspended with 50µl of TE buffer after air dry and the DNA was stored at 4°C.

3.5 Bacteriophage isolation, manipulation and processing

3.5.1 Water sample collection and processing

Water sample was collected from different rivers and places of Kathmandu valley. Water samples were collected from Balkhu, Kalimati and Balaju. Water sample was collected in

50ml sterile falcon tube and transported to the laboratory within 1-2 hours. After transporting the sample to the laboratory, the water sample was centrifuged at 4000 rpm for 30 minutes to remove cell debris and unwanted particles. The supernatant was transferred to another falcon tube without disturbing the cell debris. It was then filtered through 0.22µm syringe filter and collected in a sterile falcon tube. Now this filtrate is used as phage source. This phage source is considered to be free from bacterial contamination or other debris which has larger than 0.22µm pore size.

3.5.2 Bacteriophage isolation

Double Layer Agar Assay (DLAA) method was used for the isolation of bacteriophage. Double Layer Agar Assay (DLAA) is the standard method for the isolation of phages from water sample, sewage, dairy waste, soil, etc. In this assay, a hard agar plate of Tryptic soya agar (1 or 1.5 % TSA) is used as base layer which support the growth of host bacteria. Soft agar of Tryptic soya broth (TSB, 0.5 % agar) was used as top layer. 1 ml of syringe filtered water sample was taken in sterile falcon tube or test tube and 100 µl of log phase (OD 0.25) bacterial culture was added on it and mixed. This was allowed for attachment for 5 minutes without any disturbance.

After attachment, 3 ml of soft agar (TSB with 0.5 % agar at 50°C) was added to tubes. The mixture is gently mixed avoiding bubble formation and poured over the already prepared hard agar (TSA plate with 1.5% agar). Plates were incubated in the incubator at 37°C for 24 hours. For the negative control, only the bacterial culture was added to semisolid media and poured over TSA plate and incubated at 37°C for 24 hours.

After 24 hours incubation, plates were observed for the presence of plaques and examined for clear and turbid lysis, number of plaques count, shape and size of plaque. Plates with clear lysis were selected for further processing.

Naming of the phage:

For naming of phage, ICTV recommendation was used. Word “phage” or symbol ø is followed by university where research is carried out. Then bacterial code (two uppercase letters if genus and species are known and three lower case letters if only genus is known) along with numerical serial code and finally ending with an uppercase letter code that represents sample collection site.

3.5.3 Bacteriophage purification

Bacteriophage purification was done by continuous streaking method. The clear and separate plaque was selected, and then its center was touched with the help of sterile inoculating loop and streaked continuously over another TSA (1.5%) plate. After streaking, 3 ml of semisolid TSB (0.5 %) was taken in sterile test tubes and 100µl of active log phase host bacteria was added and mixed well. The mixture was then poured into TSA plate slowly. The mixture was allowed to spread across the plate by gently tilting the plate. The plates are now allowed to harden and then incubated at 37°C for 24

hours. After incubation, clear lysis and plaques were observed in the agar plate. Three rounds of streaking were done from single plaque. This is done to obtain pure isolated single plaque morphology.

3.5.4 Bacteriophage stock preparation

Phage stock was prepared by extracting phage from the plates with clear lysis and plaques after three rounds of streaking. 5ml of Sodium Magnesium (SM) buffer was poured on the plate. SM buffer help to absorb and detach the phage particles from the media. The upper layer of top agar is collected in sterile falcon tube by scrapping the agar with help of sterile cotton swabs. The agar mixture was then vortexed vigorously. Then centrifugation is done at 4000 rpm for 30 minutes. The supernatant then collected was filtered through 0.22µm syringe filter to avoid bacterial cell or debris in the solution. The solution obtained after filtration is pure phage stock. It was stored at 4°C for further use and characterization.

3.5.5 Phage titer assay: spot assay

Phage titer assay is the basis for determining the concentration of phage particles in pure phage stock solution. For titer assay, bacterial lawn was prepared by overlaying the mixture of 3 ml semisolid and 100µl of active log phage host bacteria to a TSA plate. Then the plate was allowed to dry. Now phage stock solution was serially diluted from 10^{-1} to 10^{-10} dilutions. For this 100µl of phage stock solution was mixed with 900µl of sterile SM buffer taken in a 1.5 ml microcentrifuge tube. Then 100µl of diluent was withdrawn from the first microcentrifuge tube and it was mixed in second tube containing 900µl of SM buffer. The dilution was done in subsequent tubes up to 10^{-10} with changing the tips in every dilution. After completion of dilution, 5 µl of all respective prepared phage dilutions were transferred aseptically onto the corresponding blocks which was labeled on the bottom of the plate. The droplets were allowed to soak into the agar and then the plate was incubated at 37^{o}C incubator for 24 hours. Clear spot or plaques were observed after incubation.

Spot assay should be done, before determination of phage concentration. Spot assay helps us to know up to which dilution phage could do host cell lysis. If the phage can cause host lysis at higher dilutions, the phage is considered better. This suggests the phage can work effectively even in higher dilutions.

3.5.6 Determination of phage stock concentration

Double Layer Agar Assay (DLAA) was done from 10^{-1} to 10^{-10} for the determination of phage stock concentration. 1 ml of serially diluted phage solution and 100µl of log phase bacteria was mixed and left for attachment for 5 min. Then 3ml of soft agar was added and overlaid onto the TSA plate. Plates were incubated overnight after solidification. After overnight incubation, the dilution from which countable plaques were formed was selected for phage titer determination. Then the total number of plaques forming units

per milliliter of phage solution was calculated by using the following formula. This gives the phage stock concentration.

$$\text{PFU/ml} = \frac{\text{No of phages}}{\text{Volume of Phage x dilution}}$$

Where, PFU/ml = plaque forming unit per milliliter of sample

3.6 Host range analysis

Host range is the ability of a specific phage to infect and lyse closely related other bacterial strain.

Phage TU_pse1B and phage TU_pse1N and cocktail of these two phages were used for host range analysis. Four *Pseudomonas aeruginosa* strains were used for intra host range analysis. They were *P. aeruginosa* (209205), *P. aeruginosa* (6661), *Pseudomonas aeruginosa* 1 (lab strain), *Pseudomonas aeruginosa* 2 (lab strain). Fourteen bacteria of other genus were used for inter host range analysis. They were *Staphylococcus aureus* (8299), *Klebsiella pneumoniae* (6697), *Klebsiella pneumoniae* (5615), *Klebsiella pneumoniae* (A), *Escherichia coli* (2778 2nd), *Escherichia coli* (2278 3rd), *Escherichia coli* (1380), *Escherichia coli* (13164), *Escherichia coli* (1137), *Escherichia coli* (10146), *Citrobacter*, *Acinetobacter*, *Staphylococcus aureus* (lab strain), *Klebsiella pneumonia* (lab strain).

Standard spot assay and DLAA method (Kwon et al., 2008) with minor modification was performed to determine multiple host range. For spot assay, all the bacteria were grown aseptically to the active log phase. Three milliliters of 0.5% soft agar were mixed with 100 µl of each bacterial culture in different sterile test tubes and poured on properly labeled separate fresh Tryptic Soya Agar Plate (TSA) and the plates were left to solidify. Grid for two of the above-mentioned phages as well as for its cocktail and negative control (SM buffer) was made in each plate. Now 5 µl of the phage stock, cocktail and SM buffer was applied to the respective spots marked in the plates and left to dry for 20 minutes. The plates were then incubated for 24 hours at 37^{o}C and then checked for the presence or absence of bacterial lysis and clear zone.

3.7 Characterization of Phage

The isolated phage with good host cell lysis capability was chosen for further characterization. The characterization of bacteriophage was done by growth curve analysis, stability of phage against temperature and pH. The phage isolated from Balkhu river (phage TU_pse1B) against *Pseudomonas aeruginosa* was used for characterization.

3.7.1 One step growth curve experiment

Bacteriophage follows five steps for the completion of its life cycle. Its lifecycle starts with the infection of the host cell and ends with the release of new phage by the lysis of host cell. Bacteriophage generally takes one hour or more for the completion of its life cycle. For bacteriophage growth curve experiment the protocol of Adams and Wassermann, 1956 was adopted with some modifications. Seven sterile micro centrifuge tubes were taken and labeled as 5 min, 10 min, 20 min, 30 min, 40 min, 50 min and 60 min. 1000µl of high phage titer (the phage of the dilution at which countable plaques were observed i.e.,, 10^{-9}) was transferred on each tube. After this 100µl of log phage host *Pseudomonas* (OD 0.25) was mixed in each of those seven tubes. Then those tubes are incubated at 37^{o}C. After 5 minutes, the tube labeled 5 min was taken out. It was then centrifuged at 12000 rpm for 5 minutes. Supernatant was discarded to remove unadsorbed phage. The pellet was resuspended in 100µl of SM buffer. Then 3 ml of soft agar was added to this solution and overlaid on a TSA plate. The same procedure is done for other six different tubes in their respective time. The plates were then incubated at 37^{o}C for 24 hours. After overnight incubation, plaques were counted and expressed as PFU/ml. Then graph of PFU/ml was plotted against time of incubation. This gives the growth curve of the bacteriophage.

3.7.2 Stability of phage against temperature

Temperature stability of phage against temperature was determined by exposing the phage to different temperature. Phage stock was diluted to different dilutions in SM buffer and the dilution at which countable plaques were formed (i.e., 10^{-9}) was taken. 1 ml of phage of this dilution was kept in each of 6 different micro centrifuge tubes labeled 10 min, 20 min, 30 min, 40 min, 50 min and 60 min. Now all of these tubes were incubated at 37^{o}C. After 10 min incubation the tube labeled 10 min was withdrawn and immediately 100µl of log phage *Pseudomonas* (OD 0.25) was mixed phage solution. Now 3 ml of soft agar was mixed with this mixture and poured over TSA plate. The same procedure was done for other tubes at their respective time. Now the plates are incubated overnight. After overnight incubation, plaques were counted and expressed as PFU/ml. Then graph of PFU/ml against different time of incubation was drawn.

The above-mentioned procedure was also done for other temperature i.e., 50^{o}C, 60^{o}C and 70^{o}C.

3.7.3 Stability of phage against pH

The pH stability of phage was determined by treating it with solution of different pH. A pH ranging from 2 to 12 was prepared by adjusting the pH of fresh Luria Bertani (LB) broth. First fresh LB broth was taken in conical flask (120 ml). HCL (1 M) was added to bring the pH of this broth to 2. Now 10 ml of this LB of pH 2 was withdrawn and pipetted into test-tube and labeled as pH 2. After this the pH of the LB was raised to 3 by adding NaOH (1M) dropwise. Again 10 ml of this LB of pH 3 was pipetted into another test-tube.

In this way pH of the LB broth was made up to 12- and 10-ml LB of each pH was taken in test-tube. Finally, we will have 11 test-tubes with pH ranging from 2 to 12.

After preparing LB broth with different pH in different tubes, the tubes were autoclaved. Meanwhile 10^{-9} dilution of phage was prepared. Now 700µl of different pH adjusted autoclaved LB broth was aliquoted in respective well labeled micro centrifuge tubes and 300µl of phage suspension was mixed in each tube. These tubes were incubated for 1 hour at room temperature. After incubation, 100µl of log phage (OD 0.25) host *Pseudomonas* bacteria was mixed with pH treated phage solution and DLAA was done. Now the plates were incubated overnight. After overnight incubation, phage plaques were counted and PFU/ml was calculated. Then graph of PFU/ml was plotted against different pH values.

3.8 Biofilm Production

The method described by Christensen et al. was used for biofilm production. Bacteria is inoculated in broth and incubated at 37^{o}C for 24 hours. The culture was diluted with fresh medium to the make the OD of 1. Now sterile polystyrene tissue culture plates were filled with 200µl of diluted culture. Only sterile broth was filled in culture plates for negative control. Strong biofilm producing strain (PAO1) was used as positive control. Now these plates were incubated at 37^{o}C for 24 hours. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 ml of Phosphate buffered saline (pH 7.2) for four times. Now the well of culture plate is fixed with 2% Sodium Acetate. Then the wells were stained by 0.1% Crystal violet. Excess stain was removed by using deionized water. Plates were then kept for drying. This is followed by addition of 95% ethanol in each well. OD of stained well was measured by using ELISA plate reader at wavelength 620 nm. The experiment was performed in triplicate. Optical density Cut Off (ODc) was determined.

ODc = Average OD of negative control + 3 X SD of Negative control

On the basis of ODc, the biofilm forming abilities of bacteria was classified as strong, moderate, and weak.

OD > 4ODc = Strong Biofilm Producer

2 ODc < OD ≤ 4 ODc =Moderate Biofilm Producer

ODc < OD ≤ 2 ODc = Weak Biofilm Producer

3.9 Biofilm Disruption

Biofilm disruption was done according to procedure mentioned by Forti et al. Bacteria were grown in broth overnight. The broth was then diluted to make OD of 1.200µl of bacteria was inoculated into polystyrene plate. Plate was then incubated at 37^{o}C for 24 hours for biofilm formation. Broth was removed and the wells were washed with LB broth (200µl) for two times. Then 200 µl of phage lysate was added. It was then

incubated for 4 hours. After incubation, the wells were emptied and washed with PBS for 2 times. The bacteria adhering to well of plate was fixed with sodium acetate (200µl) for 30 minutes at 60^{o} C. The content was then discarded. Finally, the adhered cells were stained with 200µl of crystal violet (0.1%) for 10 minutes. Excess stain was removed by using deionized water. Plates were kept for drying. 95% ethanol was added to each well. Then OD was measured at 620 nm. LB broth was used as negative control.

3.10 Synergistic effect of antibiotic and phage in the reduction of biofilm

The method explained by Chaudhry et al (2017) was used with some modifications to observe the synergistic effect of phage and antibiotic in reduction of biofilm.

A) Determination of Minimum Inhibitory Concentration (MIC) of the bacteria:

i) Preparation of culture:

Overnight culture of *Pseudomonas aeruginosa* (209295) was prepared. Its turbidity was matched with 0.5 Mac Farland solutions by adding distilled water. One ml of this will be used for MIC.

ii) Preparation of antibiotic solutions and its ranges:

Antibiotic solutions of the range 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 mg/L are needed. Later, 1 ml of this antibiotic solution was mixed with 1 ml of bacterial culture. So, to prepare solution of above concentration, solutions with concentration 0, 1, 2, 4, 8, 16, 32, 64, 128, 256 mg/L was prepared.

First stock solution of antibiotic was prepared. 0.2 gram of antibiotic was dissolved in 20 ml of distilled water. This gives stock solution of concentration 10, 000 mg/L. From this 1 ml was taken and mixed with 9 ml of distilled water. It gives stock solution of 1000 mg/L. Again, from stock of 10, 000 ml/L, 100µl was taken and it was mixed with 9.9 ml of distilled sterile water which gives stock of 100 mg/L. Now using these stock solutions, antibiotic solutions of the concentration 0, 1, 2, 4, 8, 16, 32, 64, 128 and 256 ml/L was prepared.

iii) Incubation:

Now 1ml of each of these ten antibiotic solutions was taken in ten separate labeled test tubes. To each of them, 1 ml of above prepared bacterial culture was added. Then these tubes were incubated overnight and observed for the tubes showing visible growth.

B) Synergistic effect of antibiotic and phage in biofilm reduction:

Bacteria were grown in broth overnight. The broth was diluted to make the OD of 1. 200µl of bacteria was inoculated into microtiter plates. Plates were then incubated at 37^{o}C for 24 hours for biofilm formation. Broth was removed and the wells were washed

with LB broth (200µL) for two times. Now equal volume of antibiotic solution (corresponding to MIC value) and phage lysate was mixed. Now 200µl of this resulting phage- antibiotic mixture was added to each well of the plate. Now the plate was incubated for four hours. After incubation, the wells were washed with PBS for two times. The bacteria adhering to well of plate was fixed with sodium acetate for 30 minutes at 60^{o}C. The content was then again discarded. Finally adhered cells were stained with 0.1% crystal violet for 10 minutes. Excess stain was removed by using deionized water. Plate was then kept for drying. 95% ethanol was added to each well. Then OD was measured at 620nm. LB broth was used as negative control.

3.11 Live cell count

The procedure for live cell count was followed from Sharma et al (2021) with slight modification. Bacteria were grown overnight. It was then diluted to OD of 1. 100µl of bacteria was spread over coverslip. It was dried for 30 minutes at room temperature inside biosafety cabinet. Then 100µl of phage lysate was added over the coverslip and allowed for 45 minutes. After incubation, the coverslip was transferred to 500µl of fresh Tryptic Soy Broth (TSB) and vigorously vortexed for 10 seconds to dislodge the attached bacteria from the surfaces. Now 100µl from this was taken and serial dilution was done. Then 10^{-9} dilution was taken and Double Layer Agar Assay was done in Tryptic Soya Agar (TSA) plates. The plates were incubated for overnight and the number of colonies was counted. For control, 100µl of SM buffer was used instead of phage lysate. Percent reduction in bacterial count was calculated in comparison to that of control.

3.12 Effect of Calcium ions on phage adsorption:

The procedure for the effect of calcium ions on phage adsorption was followed from Adnan et al with slight modification. The effect of Calcium ions in phage adsorption was observed. For this Calcium Chloride solution of 10 mM was prepared. Now 1 ml of this Calcium Chloride solution was mixed with 1 ml of phage (10^{-9} dilution) and 100µl of fresh bacterial culture (OD 0.25). This was allowed to incubate for 5 minutes. It was then centrifuged at 12,000 rpm for 5 minutes. The pellet contains bacteria and phages are adsorbed to it. The supernatant contains unadsorbed phage. Now this supernatant was taken and Double Layer Agar Assay was performed. Now these plates were incubated overnight at 37^{o}C. The number of colonies was counted and PFU/ml was also calculated. For control, only the phage solution was mixed with bacteria and DLAA was done.

Now the same process was done for 10 minutes and 15 minutes of incubation period.

3.13 Stability of phage against external factors

To see the effect of external effects on phage stability, procedure mentioned by Jurczak-Kurek et al was followed.

a) Organic solvents

i) Ethanol

Equal volume of phage lysate was mixed with 63 % ethanol. Then it was incubated for 1 hour at 22^{o}C. Serial dilution of this mixture was done in SM buffer. Now the dilution that gives the countable plaques (10^{-9}) was taken and plating was done by double layer agar method. The plate was then incubated overnight and after that number of plaques was counted. For control, only phage lysate was taken and same above process was done.

ii) Acetone

Equal volume of phage lysate was mixed with 90 % acetone. Then it was incubated for 1 hour at 22^{o}C. Serial dilution of this mixture was done in SM buffer. Now the dilution that gives the countable plaques (10^{-9}) was taken and plating was done by double layer agar method. The plate was then incubated overnight and after that number of plaques was counted. For control, only phage lysate was taken and same above process was done.

b) Detergents

i) SDS

Equal volume of phage lysate was mixed with 0.1 % SDS. Then it was incubated for 20 minutes at 45^{o}C. Serial dilution of this mixture was done in SM buffer. Now the dilution that gives the countable plaques (10^{-9}) was taken and plating was done by double layer agar method. The plate was then incubated overnight and after that number of plaques was counted. For control, only phage lysate was taken and same above process was done.

ii) CTAB

Equal volume of phage lysate was mixed with 0.1 % CTAB. Then it was incubated for 1 minute at 22^{o}C. Serial dilution of this mixture was done in SM buffer. Now the dilution that gives the countable plaques (10^{-9}) was taken and plating was done by double layer agar method. The plate was then incubated overnight and after that number of plaques was counted. For control, only phage lysate was taken and same above process was done.

c) Osmotic shock

First phage was incubated in SM buffer containing Sodium Chloride (NaCl) (final concentration 4.5 M) at room temperature for 15 minutes. Next the phage lysate was rapidly diluted in SM buffer without Sodium Chloride. The appropriate dilution (10^{-9}) was done and Double Layer Agar Assay was done. The plate was then incubated overnight and number of plaques was counted. For control, phage incubated in SM buffer without NaCl was used as control.

3.14 Protein profiling of phage by SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

15 microliter of phage solution was mixed with 20 microliter of loading dye. The sample-dye mixture was heated at 95^{o}C for 10 minutes. The sample is now ready to run.

Now gel was prepared in between two glass plates provided in the apparatus. 10ml resolving gel (12%) was poured first and it was allowed to set which makes the lower layer. One top of it, 2 ml stacking gel (5%) was poured and it was also allowed to set. Now the glass slab was mounted in the electrophoresis tank and running buffer was added to the tank.

Now the sample (10 microliter) was loaded carefully in the well along with protein ladder (5 microliter). The gel was then run at 120 voltages for 2 hours.

After completion of electrophoresis, the glass plate is removed from the tank. It was then stained with Comassie Blue (staining buffer) on a shaking incubator at room temperature for 1-2 hours until protein bands are observed. After completion of staining, the gel was placed in the destain solution to remove the excess stain. Now the gel was visualized for the presence of phage protein bands. Genei protein molecular weight marker – Broad range (50 lanes, 3.5 KDa to 205 KDa) was used as standard marker.

3.15 Phage DNA extraction

Phage DNA was extracted by using Norgen Biotek Corp Phage DNA isolation kit. One ml of phage lysate was transferred into a 15 ml tube. 500 microliter of lysis buffer B was added to the tube and it was vortexed vigorously for 10 seconds. The tube was then incubated at 65^{o}C for 15 minutes. Occasionally the lysate was mixed 2-3 times during incubation by inverting the tube. Now 320 microliter of isopropanol was added and briefly vortexed. A spin column was assembled to one of the collection tubes provided in the kit. 650 microliter of the above lysate was applied to a column and it was centrifuged for 1 minute at 8000 RPM. The flow through was then discarded. The column and the collection tube were then reassembled. The above process (from spin column assembly) was repeated till the entire lysate has passed through the column. Now 400 microliter of wash solution A was added to the column and it was centrifuged for 1 minute at 8000 RPM. The flow through was discarded and the spin column was reassembled with its collection tube. The column was then again washed for a second and third time by wash solution A by above mentioned procedure. Now the column was spinned for 2 minutes in order to dry at 14000 RPM. The collection tube was then discarded.

Now the column was placed into an elution tube provided with the kit. 75 microliter of elution buffer B was added to the column. Now it was centrifuged for 1 minute at 8000

RPM. Now the phage DNA will be collected in elution tube. The DNA sample was then stored at -20^{o}C.

3.16 Electrophoresis of Phage DNA

1 percent of agarose gel was prepared. 0.75 gram of agarose gel was mixed with 50 ml of 1X TAE buffer. The mixture was then heated till the agarose is completely dissolved. Then 0.25 microliter of EtBr was added to the solution. Then the gel was poured into the casting tray and was allowed to solidify. Now 5 microliter of phage DNA was mixed with 1 microliter of loading dye and it was placed into the well. DNA ladder was also run along the sample. Fermentas O' Gene Rule 1 KB DNA ladder was used. Now electrophoresis was done at 70 volts for one hour. After completion of electrophoresis, the gel was observed under UV illuminator to visualize the DNA band.

3.17 Whole Genome Sequencing of Phage DNA

After the confirmation of phage DNA by gel electrophoresis, whole genome sequencing of the phage DNA was done. Phage DNA was sent to CMDN (Center for Molecular Diagnosis Nepal) Thapathali, Kathmandu for sequencing.

Library preparation: Library preparation need to be done before sequencing. Whole DNA was broken into smaller fragments. Then adaptors were added to the both ends of the DNA fragments. Indexing was also done to identify the samples. Now sequencing of these DNA fragments will be done in Illumina platform.

After library preparation, the DNA fragments are placed in NGS MiSeq Illumina Platform for sequencing. The DNA fragments are attached in flow cell. Inside flow cell, there occur PCR of DNA segments through bridge amplification. After PCR, cluster of DNA will be generated. This is followed by sequencing of DNA. Illumina does sequencing by synthesis. It is based on reversible dye terminator chemistry. Single base is added at a time. The bases are fluorescently labeled. After base addition, it will be excited to release signal and then which base was added will be recorded by the computer. After this, excess bases are washed followed by deblocking step. Then the same cycle continues until whole DNA fragment is sequenced. The signal from sequencing is stored in the form of BCL files. Computer software converts these BCL files to FastQ files which is the standard format required for downstream analysis. But these FastQ files contain errors such as base calling error, low quality reads, adaptor contamination, sequencing errors. These errors need to be removed before analysis. This step is called quality control. FastQC checks and corrects the quality of reads whereas FastP does filtering of the reads. FastP is faster than other tools and it is written in C++. After filtering, filtered short reads will be obtained. Now de novo genome assembler (SPAdes) will assemble these short reads into contigs. Contigs are DNA segments with overlapping ends. Now from these contigs we can identify viral sequences. This can be achieved by using Virfinder tool. Virfinder is based on machine learning method. It uses k-mer frequency to identify viral sequence. It does not depend on homology based searches and gene

finding. Now we can also annotate viral genes by using tools such as Prokka. Prokka refers to 'prokaryotic annotation'. It is software tool to annotate bacterial and viral genes. First protein coding regions is identified and then its product is identified. It is done by comparing with database. The comparison is done in hierarchical manner. First of all, protein is searched in user provided set of annotated proteins, then searched in bacterial proteins present in uniport followed by all proteins from finished bacterial genome (RefSeq) for a specified genome and then in Hidden Markov Model Database. If protein cannot be found, it is then labeled as 'Hypothetical protein'. It generates ten files in the output directory. So, file is produced in ten different suffixes such as gbk, fna, txt, etc. Prokka relies on external tools such as Prodigal. Now these output files (genebank and Fasta) are further analyzed by other tools to derive information from viral genome. UGENE, Phaster, Prokka were the tools that were used to analyze phage genome.

4. RESULT AND DISCUSSION

4.1 Identification of host bacteria

4.1.1 Biochemical test of host bacteria

Pseudomonas aeruginosa was revived in Nutrient Agar. *Pseudomonas aeruginosa* formed greenish, flat, large sized colonies in Nutrient agar.

After this, biochemical tests of *Pseudomonas aeruginosa* were performed. Methyl red (MR), Voges Proskaur (VP), Citrate, Urease, Sulphur Indole Motility (SIM), TSI (Triple Sugar Iron) tests were performed.

Table 2: Biochemical tests of *Pseudomonas aeruginosa*

Test	Result	Inference
MR	Negative	*Pseudomonas aeruginosa*
VP	Negative	
TSI	Red/Red, Gas negative, H_2S negative	
SIM	Indole negative, H_2S negative, motility positive	
Citrate	Utilized	
Urease	Negative	

The result from the table confirmed that the bacterium was *Pseudomonas aeruginosa*. The result showed MR negative, VP negative, TSI Red/Red, Indole negative, Citrate positive and Urease Negative.

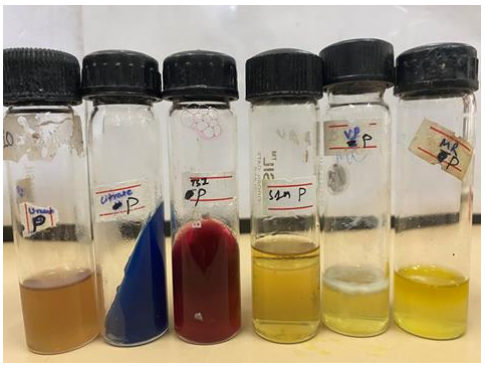


Figure 1 : Biochemical tests of *Pseudomonas aeruginosa*

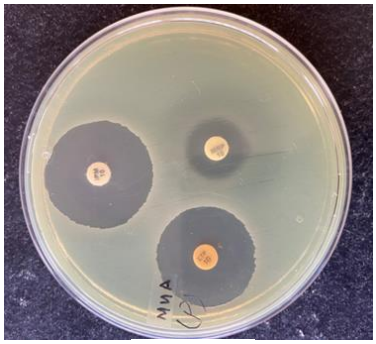
4.1.2 Antibiotic Susceptibility Test

The antibiotic susceptibility *Pseudomonas aeruginosa* was tested by using the various antibiotic discs. The clear zone around the antibiotic disc represents the zone of inhibition of the given antibiotic. The diameter of the zone of inhibition was measured in millimeter (mm). This value was then compared with the guidelines provided by CLSI which helps to interpret the bacteria as resistance, sensitive and intermediate against that antibiotic.

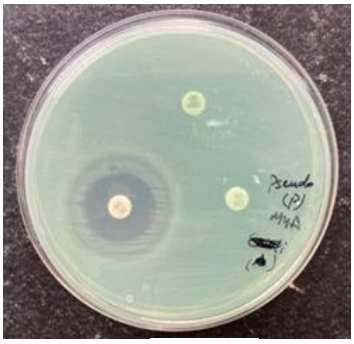
Table 3: AST pattern of *Pseudomonas aeruginosa*

Bacteria	Antibiotic	Std zone of resistivity (mm)	Zone (mm)	Inference
Pseudomonas aeruginosa	MRP(10)	≤15	15	Resistant
	NA(10)	≤13	0	Resistant
	CTX(30)	≤22	0	Resistant
	PI(100)	≤14	19	Sensitive
	CX(30)	≤14	0	Resistant
	AK(30)	≤14	18	Sensitive
	GEN(10)	≤12	20	Sensitive
	IMP(10)	≤15	28	Sensitive

Pseudomonas aeruginosa was resistant to Meropenem (carbapenem group of antibiotic), Nalidixic Acid (NA), Cefotaxime (CTX), and Cefoxitin (CX) whereas it is sensitive to Piperacillin (PI), Amikacin (AK), Gentamicin (GEN) and Imipenem (IMP).



(A)



(B)

(C)

Figure 2: A, B, C: Antibiotic susceptibility test of *Pseudomonas aeruginosa* showing zone of inhibition to different antibiotics.

4.1.3 Genomic DNA extraction

Genomic DNA of *Pseudomonas aeruginosa* was extracted by CTAB method. Gel electrophoresis of extracted DNA was performed and then DNA was viewed under UV illuminator. Distinct band of DNA was observed under UV light.

Figure 3: DNA band of *Pseudomonas aeruginosa*

4.2. Bacteriophage isolation and manipulation

4.2.1 Bacteriophage isolation

Waste water sample was collected from three different rivers (Balkhu river, Kalimati river and Bishnumati river).

Bacteriophage against *Pseudomonas aeruginosa* was isolated from all three rivers. Phage isolated from Balkhu was further characterized.

Table 4: Initial screening of bacteriophage

Sample source	Host bacteria	Name of the phage	Initial no of plaques	Plaque opacity	Plaque morphology
Balkhu river	*Pseudomonas aeruginosa*	Phage TU_pse1B	19	Clear	Small, pin head
Kalimati river	*Pseudomonas aeruginosa*	Phage TU_pse1K	3	Clear	Large
	Pseudomonas	Phage	6	Clear	Large, bull eye

Bishnumati river	*aeruginosa*	TU_pse1N			

Various factors affect plaque size such as adsorption rate, lysis time, virion morphology. High adsorption rate would result larger plaque size and low adsorption rate would result smaller plaque size. Greater lysis time and smaller lysis time would result in smaller plaque size while medium lysis time would result large plaque size. Smaller lysis time is correlated with small burst size. If burst size is small, fewer number of phage particles are available for infecting surrounding host cells thus leading to smaller plaque. If a virion is of larger size, it would take more time to diffuse thus resulting in smaller plaque size (Gallet et al., 2011).

Clear as well as turbid plaques were observed. Lytic bacteriophage produces clear plaques whereas lysogenic phage produces turbid plaques. Lytic phages produce clear plaques on a bacterial lawn as they kill all of the bacterial cells they infect. For bacteriophage showing lysogenic cycle, its genome integrates into the host so no new phage progeny are produced to infect surrounding host cells. Due to this, temperate phages like λ leave turbid plaques on a bacterial lawn. It is because they do not lyse all bacterial cells they infect (Gudlavalleti et al., 2020).

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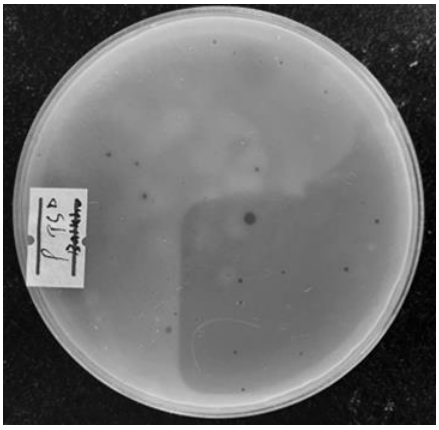


Figure 4: Distinct plaques produced by bacteriophage against *Pseudomonas aeruginosa*

4.2.2 Bacteriophage purification

Purification of bacteriophage was done by three rounds of streaking of a single plaque. Bacteriophage that has clear morphology was selected for purification. *Pseudomonas aeruginosa* phages isolated from three rivers were purified. Single clear plaque was

selected and it was streaked in agar plate. Clear lysis was seen over the streaking line. Same procedure was done for next two times.

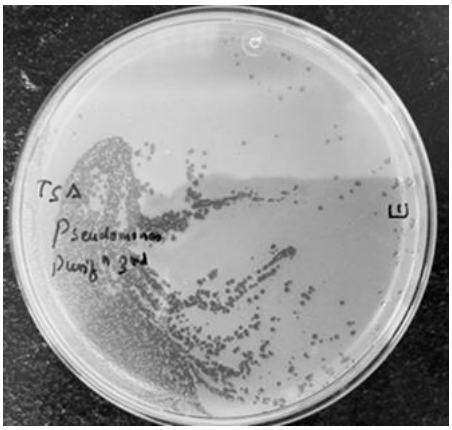


Figure 5: Purification of *Pseudomonas* phage

Finally, after three rounds of streaking, the phage stock was prepared by filtering the phage suspension through 0.22 µm pore sized syringe filter.

Pure phage lysate is also important for characterization of phage. Techniques other than filtration can inactivate phage so filtration technique was used to obtain pure phage lysate. Purification is very important as it remove contaminants derived from the bacterial host, such as lipopolysaccharides, flagella, proteins and peptidoglycan fragments. Various techniques can be used to purify bacteriophage such as clarification with organic solvent, polyethylene glycol (PEG) precipitation, ultracentrifugation and chromatography. These help to obtain phage solution with high level of purity. However purification procedures possess high risk of inactivating the phages. Filtration method through 0.22 µm pore filters can be done for lipid enveloped phages which are sensitive to chloroform (Jończyk-Matysiak et al., 2019).

Figure 6: Stock solution of three *Pseudomonas aeruginosa* phages

4.2.3. Bacteriophage concentration determination

Before determination of phage concentration, spot assay was done. During spot assay, countable plaques were observed at 10^{-9} dilutions. Double Layer Agar Assay (DLAA) was then done by using this dilution. Seventy clear, pin head, small sized plaques were observed.

Phage concentration was then determined by using the following formula:

$$\text{PFU/ml} = \frac{\text{No of plaques}}{\text{Volume of Phage} \times \text{Dilution}}$$

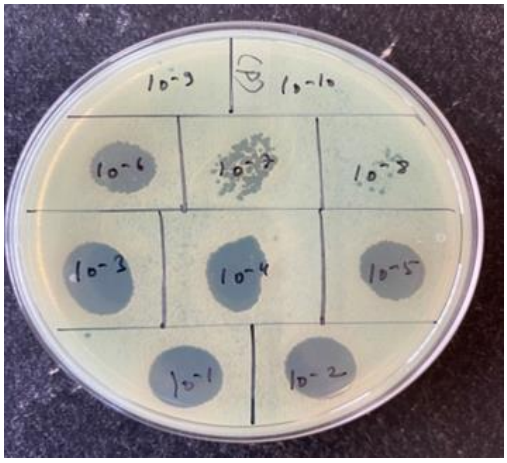
$$= \frac{70}{1 \text{ ml} \times 10^{-9}}$$

$$\text{PFU/ml} = 70 \times 10^{9}$$

(Where PFU/ml = plaque forming unit per milliliter of sample)

Phage titer is the quantitative measurement of the biological activity of the phage. It is expressed as PFU/ml. Determination of the phage titer is very important during the study of phage. It helps to determine the number of phages present at different dilutions. This is also required for the further characterization of the phage. Dilution that gives countable plaques is generally taken during characterization.

The phage formulation should contain appropriate phage concentration. If we use preparation with high concentration of phage, it may become viscous so it will possess difficulty in applying. Phage concentration is one of the important factors that influence phage therapy. For therapeutic use, phage should have a titer of 10^{9} pfu/ml (Jończyk-Matysiak et al., 2019).



(A)

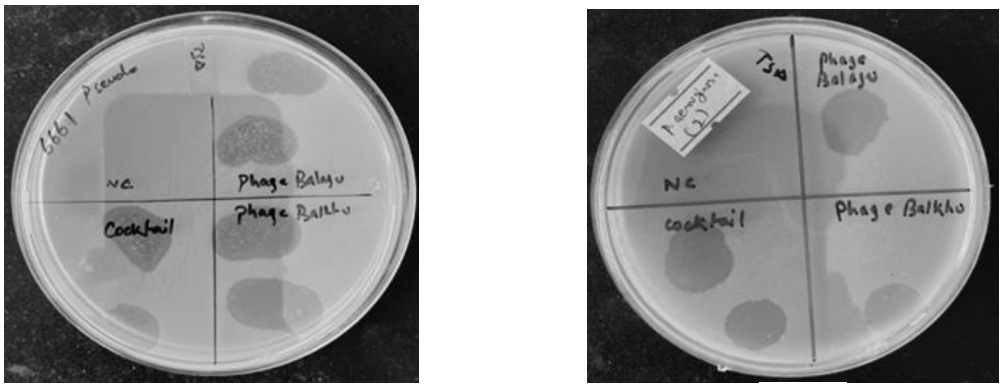
(B)

Figure 7: (A) Spot assay showing 10^{-9} dilution with countable number of plaques (B) Countable number of plaques produced by 10^{-9} dilution

4.3 Multiple Host range analysis

Lysis was seen against *P. aeruginosa* (209205) and *P. aeruginosa* (6661). Both of the phages and their cocktail showed the lysis against these two *Pseudomonas aeruginosa* strains. So the phage showed intraspecific host range.

Lysis was not observed for bacteria of other genus. Phage did not show inter specific host range.



(A) (B)

Figure 8: A, B: Lysis seen against two *Pseudomonas aeruginosa* strains (intraspecific host)

A phage killing only one species is desirable because it prevents the killing bacteria of other species. It helps to keep rest of the host's micro biome intact. This may also kill nonpathogenic members of the normal flora. Furthermore it can cause dilution of effective concentration of the phage toward the target bacteria. So a phage should have a narrower host range regarding the number of species it can infect. However if a phage can infect many strains within a bacterial species, it is considered useful. It means within a target species, phage that can kill most of the strains is desirable (Hyman, 2019).

The greater the range of a phage within the target pathogen species (referring to number of different strains within a species that can be infected), the more likely that particular phage can be used to treat infection caused by target bacteria (Hyman, 2019).

Table 5: Host range for phage TU_ pse1B, TU_ pse1N and its cocktail

Bacteria	Phage TU_pse1B	Phage TU_pse1N	Cocktail
P. aeruginosa (209205)	Yes	Yes	Yes
P. aeruginosa (6661)	Yes	Yes	Yes
Staphylococcus aureus (8299)	No	No	No
Klebsiella pneumoniae (6697)	No	No	No
Klebsiella pneumoniae (5615)	No	No	No
Klebsiella pneumoniae (A)	No	No	No
Escherichia coli (2778 2nd)	No	No	No
Escherichia coli (2278 3rd)	No	No	No
Escherichia coli (1380)	No	No	No
Escherichia coli (13164)	No	No	No
Escherichia coli (1137)	No	No	No
Escherichia coli (10146)	No	No	No
Citrobacter	No	No	No
Acinetobacter	No	No	No
Pseudomonas aeruginosa 1 (lab strain)	No	No	No
Pseudomonas aeruginosa 2 (lab strain)	No	No	No
Staphylococcus aureus (lab)	No	No	No
Klebsiella pneumonia (lab)	No	No	No

4.4 Characterization of phage

Phage TU_pse1B was selected for further characterization.

4.4.1 Bacterial growth curve analysis

One step growth curve of the phage was performed starting with infection of the host cell up to the release of phage progeny from the host cell. The change in phage number along different time was noted. The PFU/ml at each time was calculated. PFU/ml was plotted against time from which latent period and burst size of the phage was determined.

Table 6: PFU/ml produced by phage at different time interval

Time	PFU/ml
5	5
10	9
20	15
30	18
40	29
50	45
60	47

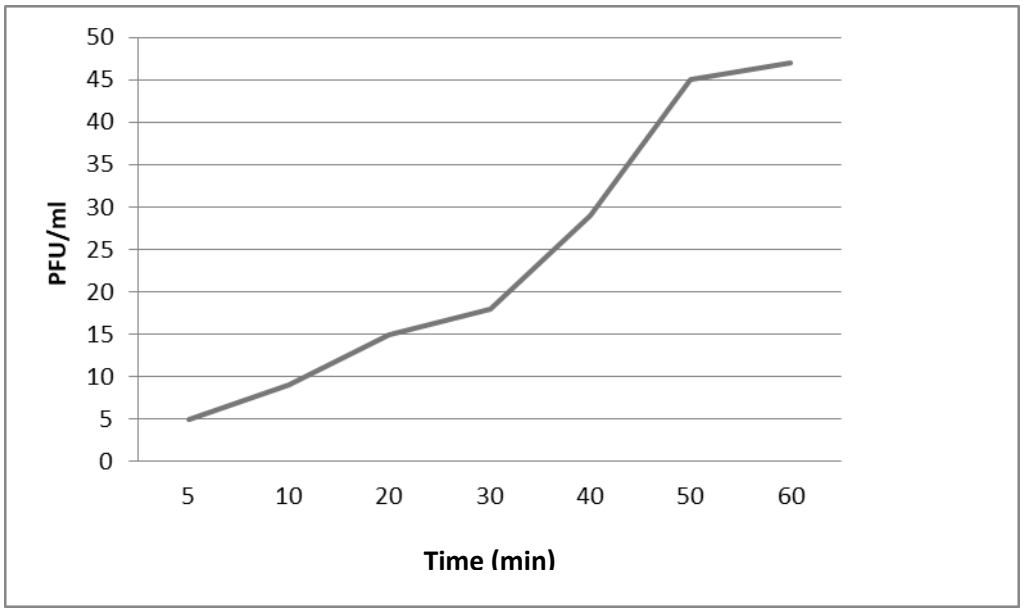


Figure 9: Growth curve of phage TU_pse1B

Latent period is the time taken by bacteriophage from infection of the host cell to the release of the phage from the host cell. After release of the phage progeny there will be sharp increase in phage titer. So the time period just before the occurrence of sharp increase in phage titer is the latent period for the phage. There was steady increase in the number of the phage from 5 minutes up to 30 minutes. After 30 minutes, there was sharp increase in PFU/ml of the phage. Thus for our phage, the latent period is 30 minute.

Burst size of the phage is also determined. Burst size is calculated by subtracting PFU/ml of post-rise period to that of pre-rise period of the phage growth. For our phage, the phage number starts to increase sharply after 30 minutes and it stabilized after 50 minutes. The PFU/ml for 30 minute was 18 and for 50 min it was 45. So the burst size of our phage is 27(45-18).

Burst size produced by phage varies significantly. Infection of *E.coli* with its bacteriophage could produce burst size ranging from 20 to over 1000 pfu per cell (Choi et al., 2010). Great fluctuation in the magnitude of bursts size was observed such that they vary from a few particles to two hundred or more (Ellis & Delbrck, 1939).

Various factors plays role in determining the burst size of the phage such as size of the cell, physiological state, growth rate. Cells of smaller size have smaller burst sizes compared to larger cells. Larger cells likely have greater burst size (Choi et al., 2010).Phage growth parameters such as latent period and burst size depend on cell maturation. If infection of bacteria occurs at young stage (immediately after division), then the latent period will be longer and burst size will be lower. The opposite happens when bacterial cells are infected near cell division. The physiological state of the bacterial cell determines the burst size and latent period of the phage (Šivec & Podgornik, 2020). The latent period and burst size of T4 bacteriophage depend on bacterial growth rate. Different phages have demonstrated that if we increase bacterial growth rate, it shortens the latent period, while increasing the burst size. (Nabergoj et al.,2018). A phage with a large burst size is preferred because such phage can substantially multiply within target bacterial cells in short period of time (Liao et al., 2019).

4.4.2 Stability of phage at different pH range

Phage was exposed to different pH values and PFU/ml was determined.

Table 7: PFU/ml of phage at different pH value

pH	PFU/ml
2	11
3	20
4	26
5	30
6	34
7	42

8	47
9	60
10	49
11	40
12	36

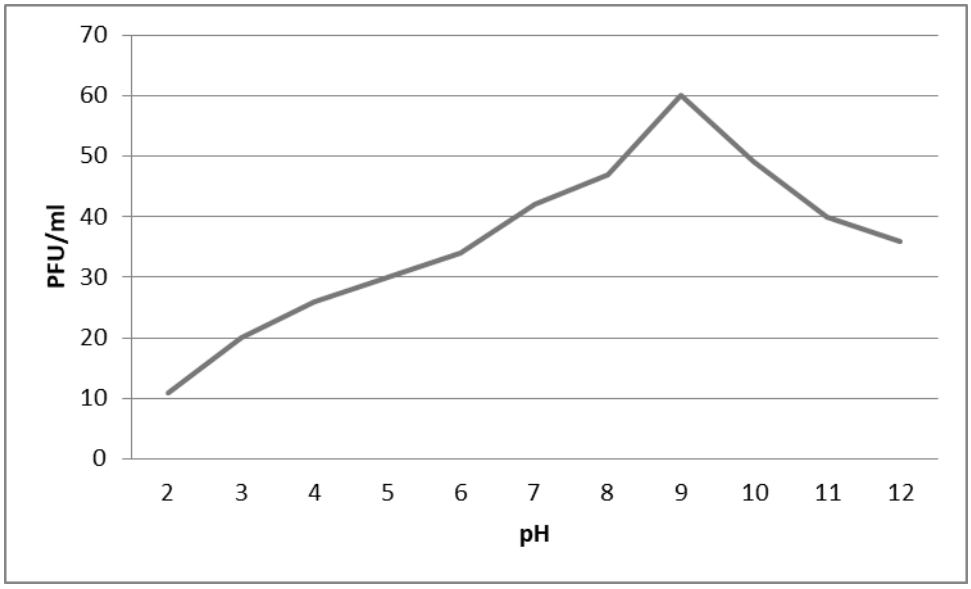


Figure 10: PFU/ml against different pH values

Phage growth was seen from pH 2 to pH 12. There was significant decrease in PFU/ml at lower pH values. This suggests the phage is susceptible to lower pH. From pH 5, there was continuous rise in PFU/ml up to pH 9. Highest PFU/ml (60) was observed at pH 9. From pH 9 to pH 12, the PFU/ml goes on decreasing. The phage showed good survivability from pH 5 to pH 11.

This result is similar to findings of Yuan et al., 2021 where the phage showed good stability within pH range of 5 -11. Phage showed good stability at pH levels from 5 to 11 (Yuan et al., 2021). Similarly, our finding is also relatable to result from Adnan et al., 2020. The phage showed stability at pH 5, 7 and 9 and 11 whereas highest stability was seen at pH 7. No plaque formation was observed at pH 1 (Adnan et al., 2020).

Factors such as acidity and temperature affect phage viability and survivability. This parameter causes modifications on phage's structural components and nucleic acids. Low pH values have shown to reduce phage titer and proliferation significantly. The instability of phage at acidic condition contributes to a poor efficacy of phage therapy. Exposure to low pH values can cause irreversible damage to phage. Acidic pH causes

aggregation of phage due to protonation effect which in turn adversely impact phage activity (Nobrega et al., 2016).

4.4.3 Thermal stability of phage

Phage was exposed to different temperatures and PFU/ml was determined.

Table 8: PFU/ml produced by phage after exposure to different temperatures

Time (min)	PFU/ml 37^{o} C	PFU/ml (50^{o}C)	PFU/ml (60^{o}C)
10	111	68	60
20	109	65	54
30	108	54	46
40	106	50	40
50	92	45	38
60	83	40	25

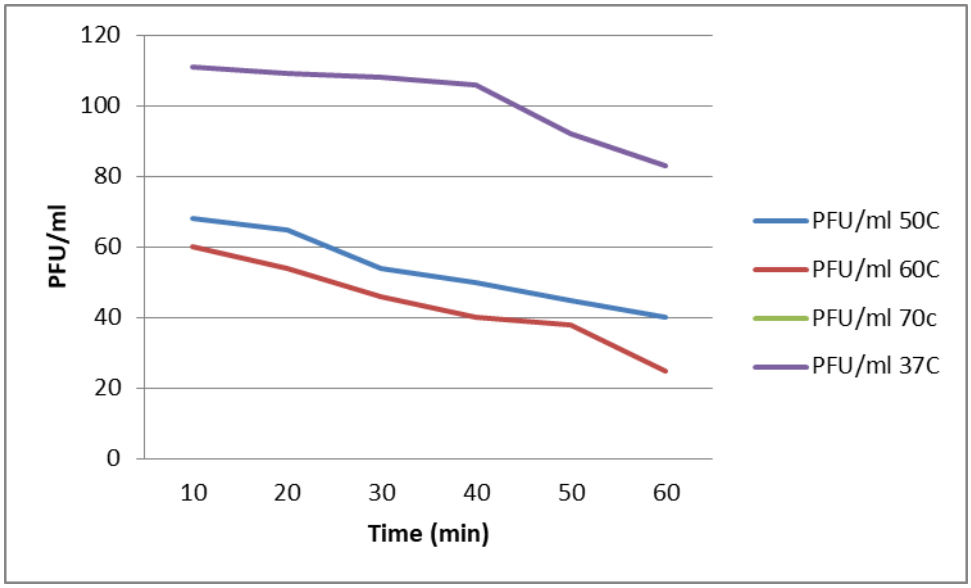


Figure 11: Graph showing PFU/ml against different temperatures

Phage showed greatest survivability at 37^{o}C. The PFU/ml was significantly highest at 37^{o}C than other temperatures (50^{o}C and 60^{o} C). The phage also showed survivability at 50^{o}C and 60 ^{o}C, the survivability being least at 60^{o}C. Phage growth was not observed at 70^{o}C.

There was gradual decrease in PFU/ml with increase in exposure time. Lower exposure time is better for phage survival.

The result showed that phage growth was best at 37^{o}C. With increase in temperature, the phage survivability decreases. The phage survivability also decreases with increase in exposure time. This concludes, higher temperature and higher exposure period is detrimental for phage survival.

This result is similar to study conducted by Sharma et al (2021). *Pseudomonas aeruginosa* phage was substantially stable at 37 °C temperature, while it showed moderate stability at 50 °C temperature. At 60 °C and above, the phage stability decreased (Sharma et al., 2021).

Phages with extreme thermal resistance have been isolated from thermal habitats and also from aquatic environments. Phage isolated was active on high temperatures. The phage remained viable up to 60 °C and maximum infectivity was observed at 37 °C (Piracha et al., 2014).

Thermal treatments lead to morphological changes to those phages that are heat-sensitive. These changes include phage DNA release from viral capsids, breakdown of phage into its head and tail structures, and aggregation of phage tails. However, *Lactococcus lactis* phages were found to be highly thermo-resistant. Recently few phages of *Lactococcus lactis* have emerged with high thermal stability (Geagea et al., 2018).

4.5 SDS Page of phage proteins

Three distinct bands of phage proteins were observed after SDS-PAGE. Two proteins were of smaller size (35 and 40 KDa) whereas one of the phage proteins was of larger size (100 KDa).

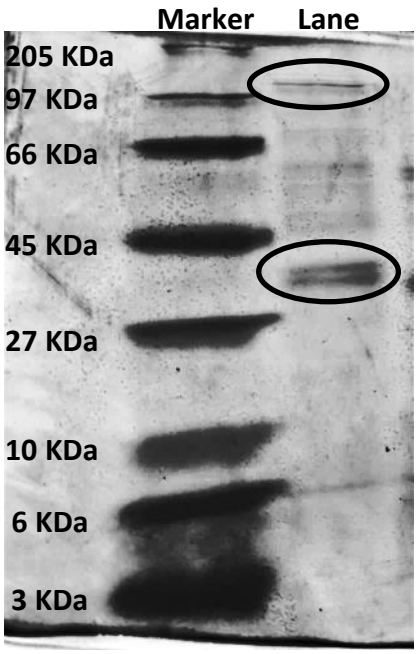


Figure 12: SDS of phage proteins showing distinct bands

The larger size protein can be attributed to tail fiber protein whereas the smaller size proteins can be attributed to capsid proteins and internal virion proteins.

Similar finding was reported by Sillankorva et al (2008). Five distinct protein bands were identified according to their size representing the T7 tail fiber protein, the minor capsid protein, the major capsid protein 10A, the capsid assembly protein, and the internal virion protein (Sillankorva et al., 2008).

4.6 Electrophoresis of Phage DNA

Distinct band of phage DNA was observed after gel electrophoresis. Fermentas o Gene Rule 1KB DNA ladder was run along the phage DNA. The size of phage DNA was more than 10 KB.

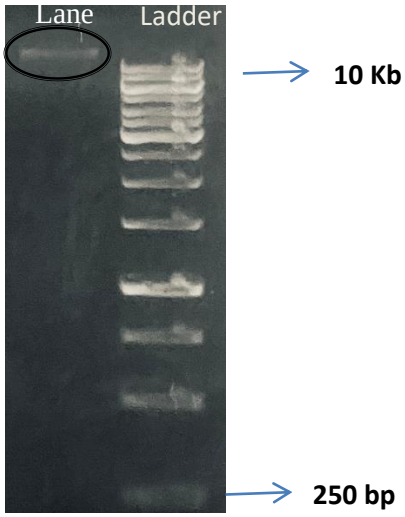


Figure 13: Gel electrophoresis of phage DNA showing distinct band

Phage genome shows enormous variation in size. It ranges from 3300 nucleotide to the almost 500 Kbp genome of Bacillus megaterium phage G (Hatfull & Hendrix, 2011).

4.7 Concentration determination of phage DNA

The concentration of the phage DNA was determined by using nanodrop (Thermo Scientific Nano Drop One). The concentration of phage DNA was found out to be 17.7 ng/µl.

A260/A280 ratio was 1.44. It was less than the normal value of 1.8. A low A260/A280 ratio may be caused by residual phenol or other reagent associated with the extraction protocol. A260/A230 ratio was 0.63. It was also less than expected normal value (2.0-2.2). A low A260/A230 ratio may be the result of residual phenol from nucleic acid extraction, residual guanidine (often used in column based kits), etc.

4.8 Whole Genome Sequencing of Phage DNA

Library Preparation:

The extracted Phage genomic DNA was first purified using 0.8X AMPure beads. The purified DNA was then tagmented using 0.5µl Amplicon Tagment Mix (ATM) and 2.5µl Tagment DNA Buffer (TD). The tagmentation was perfomed at 55°C for 10 minutes followed by neutralization adding 1.25µl of Neutralization Tagment Buffer (NT) at room

temperature for 5 minutes. The entire tagmented DNA was subjected to indexing using 1.25µl each of i5 and i7 indexes and 3.75µl of Nextera PCR Master Mix (NPM). The thermocycling condition for indexing was as: 72°C for 3 minutes, 95°C for 30s followed by 18 cycles of 95°C for 10s, 55°C for 30s and 72°C for 30s. The final extension was performed at 72°C for 5 minutes. The entire indexed PCR products were purified using 0.7X AMPure beads and quantified using Qubit HS DNA kit. The products' fragment size was determined using Bioanalyzer and all the products were normalized to 4nM and pooled together to obtain final library pool of 4nM. The library was spiked with 5% PhiX and denatured using equal volume of 0.2N NaOH. The denatured library was neutralized using Hyb buffer and 10pM of the final library was taken for Sequencing in MiSeq Reagent V2 kit.

Data Analysis:

After library preparation, the DNA fragments were placed in NGS MiSeq Illumina Platform for sequencing. The DNA fragments were attached in flow cell. Inside flow cell, PCR of DNA segments was performed through bridge amplification. After PCR, cluster of DNA will be generated. Sequencing of these clusters of DNA was then performed inside flow cell. The signal from sequencing was stored in the form of BCL files. BCL files wre converted to FastQ files by computer software which is the standard format required for downstream analysis. Low quality reads were removed by FastQC whereas filtering was done by FastP tool. After filtering, filtered short reads were obtained. Now de novo genome assembler (SPAdes) assembled these short reads to generate contigs. Contigs are DNA segments with overlapping ends. Viral sequences were then identified from these contigs by using Virfinder tool. This is followed by annotation of viral genes. Viral genes were annotated by using another tool called Prokka. Prokka identifies viral sequence by comparing to database.

The result of whole genome sequencing of phage DNA was obtained in two file formats i.e., fasta format and gene bank format. Interpretation of the sequence obtained was then done by using UGENE, Phaster and Proksee tool.

4.8.1 Genome analysis by UGENE

4.8.1.1 General Statistics

Table 9: General Information of phage genome from whole genome sequencing

Length	43,428 nt (43 Kbp)
GC content	62.16%
Molecular weight	26839484.57 Da
Extinction coefficient	692404973 L/mol cm
µg/OD260	38.76
Melting temp 90.37C	90.37^{o}C
ORF	234

Annotated regions	42

The length of the whole genome of *Pseudomonas aeruginosa* was 43,428 nucleotides (43 Kb) long. The GC content was 62.16 %.The melting temperature of the DNA was 90.37 °C. Molecular weight of the double stranded DNA was 26839484.57 Da.

Extinction coefficient (molar attenuation coefficient or molar absorption coefficient) of the DNA was 692404973 Liter/mole^{-1}cm^{-1}. The molar attenuation coefficient or molar absorption coefficient is a measure of how strongly a chemical species absorbs light at a given wavelength and it is an intrinsic property of the species (Banihashemian et al., 2013).

The µg/OD260 for the DNA was 38.76. It means the concentration of DNA was 38.76 µg per ml.

4.8.1.2 Character occurrence

Table 10: Number of nucleotides and their percentage

Nucleotide	Number	Percentage
A	7339	16.9%
C	13543	31.2%
G	13454	31.0%
T	9092	20.9%

The total number of Adenine was 7339. Its percentage was 16.9%. The number of Cytosine was 13543 and its percentage was 31.2%. Guanine account for 31.0% and its number was 13454. The number of Thymine was 9092 and its occurrence was 20.9%.

4.8.1.3 Amino acids

Table 11: Different amino acids and their total number in genome

Amino acid	Number	Amino acid	Number
Alanine(A)	8816	Lysine(K)	1470
Arginine(R)	10810	Methionine(M)	1261
Asparagine(N)	1335	Phenylalanine(F)	1485
Aspartic acid(D)	2870	Proline(P)	7645
Cysteine (C)	2762	Serine (S)	8114
Glutamic acid (E)	2652	Threonine(T)	5013

Glutamine(Q)	3202	Tryptophan(W)	1980
Glycine(G)	7646	Tyrosine(Y)	1426
Histidine(H)	2746	Valine(V)	5013
Isoleucine(I)	2103	Stop codon (UAA, UAG and UGA)	1935
Leucine(L)	6568		

Arginine was the amino acid with the highest occurrence (10810) followed by Alanine and Serine. The amino acid with lowest number is Methionine (its number was 1261).

The total number of stop codon was 1935.

4.8.1.4 Complements (genes)

The gene bank format of the whole genome contains information about complement (genes) present in the phage. Information of all together 59 complements was present. Phage genome lacked toxic genes as well as integrase genes. Genes such as phage DNA directed RNA polymerase, phage protein p25, phage endonuclease, phage DNA helicase, phage non contractile tail fiber protein Gp17, phage major capsid protein Gp10A, etc were present in phage genome. However, few of the genes in the phage genome were not identified and they were named as hypothetical proteins. Eight such hypothetical proteins were present in our phage genome.

The gene bank format of phage genome contained information of the location of the gene in the genome, the amino acid sequence and the protein product. For example, the information of one gene is shown below:

Complement (24582...24887)

Translation:
MSKKQTASAERLGLLHELVCTAIERNFKWYMDNDIPIPASDIAAATKFLKDNEITCDPSDTINIDRLRE
EMRQAQKENRRIALEGFIAGETDDEMERLYTH

Product = Phage terminase small subunit Gp18, DNA packaging

4.8.1.5 Circular form of genome

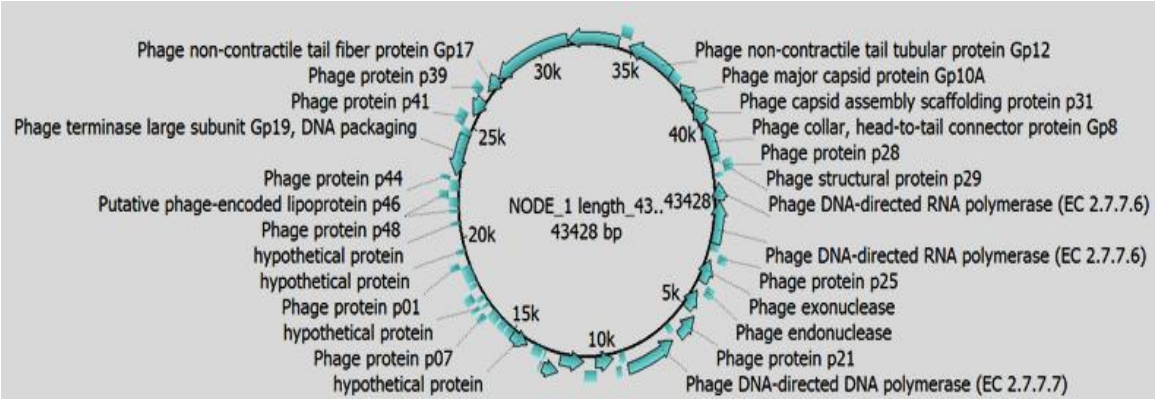


Figure 14: Circular representation of phage genome derived from UGENE

Circular form of phage genome was derived from UGENE software. The circular representation shows the name of different genes present in phage genome and their respective position on phage genome. For example phage major capsid protein Gp10A is located between 35000 and 40000 base pair.

4.8.2 Circular map of phage genome derived from Proksee:

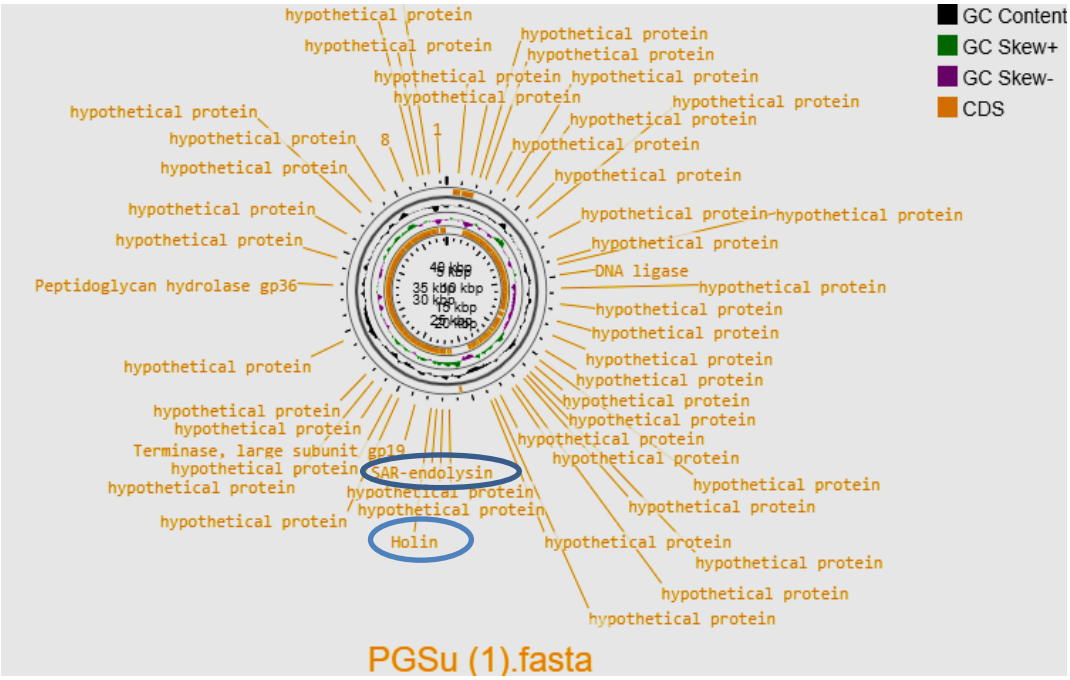


Figure 15: Circular map of phage genome showing holin and endolysin gene

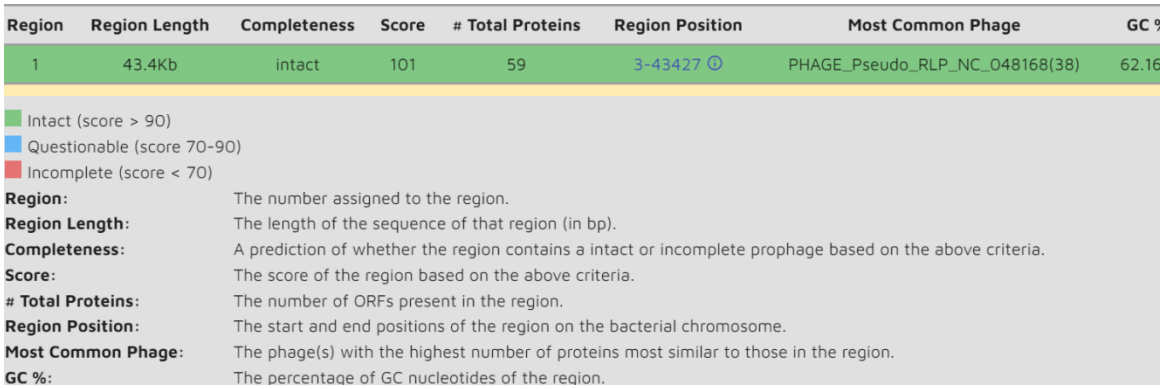
Proksee tool was also used to develop circular genomic map of phage genome. The circular map showed GC content of phage in the diagram. It showed holin and endolysin

gene of phage along with other genes. Toxic and integrase gene were not present in the phage genome map.

4.8.3 Genome analysis by PHASTER

PHASTER (Phage Search Tool Enhanced Release) was used for phage genome analysis. PHASTER helps to generate circular genome of sequenced phage with its genome size, CDS region, GC content and predicted location. The genome was intact and no significant break was reported throughout the region. The total length of the phage was 43.4 Kb. Total number of proteins was 59. The GC content was 62.16%. The start and the end position were 3-43427.

PHAGE_Pseudo_RLP_NC_048168 showed highest similarity with our phage on the basis of protein similarity. This also further conformed that our sequenced phage belongs to that of *Pseudomonas aeruginosa*.



Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
1	43.4Kb	intact	101	59	3-43427	PHAGE_Pseudo_RLP_NC_048168(38)	62.16

Intact (score > 90)
Questionable (score 70-90)
Incomplete (score < 70)

Region: The number assigned to the region.
Region Length: The length of the sequence of that region (in bp).
Completeness: A prediction of whether the region contains a intact or incomplete prophage based on the above criteria.
Score: The score of the region based on the above criteria.
 # Total Proteins: The number of ORFs present in the region.
Region Position: The start and end positions of the region on the bacterial chromosome.
Most Common Phage: The phage(s) with the highest number of proteins most similar to those in the region.
GC %: The percentage of GC nucleotides of the region.

Figure 16: General information of phage genome derived from PHASTER

Total 59 genes were successfully hit and identified by PHASTER annotation. Genes encoding endolysin, u-spanin, methyltransferase type 11, tail tip protein, tail fiber protein, exonuclease, HNH endonuclease, putative transcriptional regulator, DNA helicase, minor tail protein, DNA adenine methyltransferase, head-tail connector protein, putative phosphoesterase were successfully identified. Apart from these genes several hypothetical proteins were also present.

There was no integrase gene or any virulence or bacterial gene within the phage genome. Absence of integrase gene rules out the possibility of transfer of phage genome to bacterial genome. Similarly the absence of lytic cycle repressor protein (cI group of protein) makes it impossible to produce lysogenicity in the phage. Also, all of the 59 genes were hits against Virus and Prophage Database confirming they lack bacterial genes. Thus, the phage is lytic and lacks toxic genes.

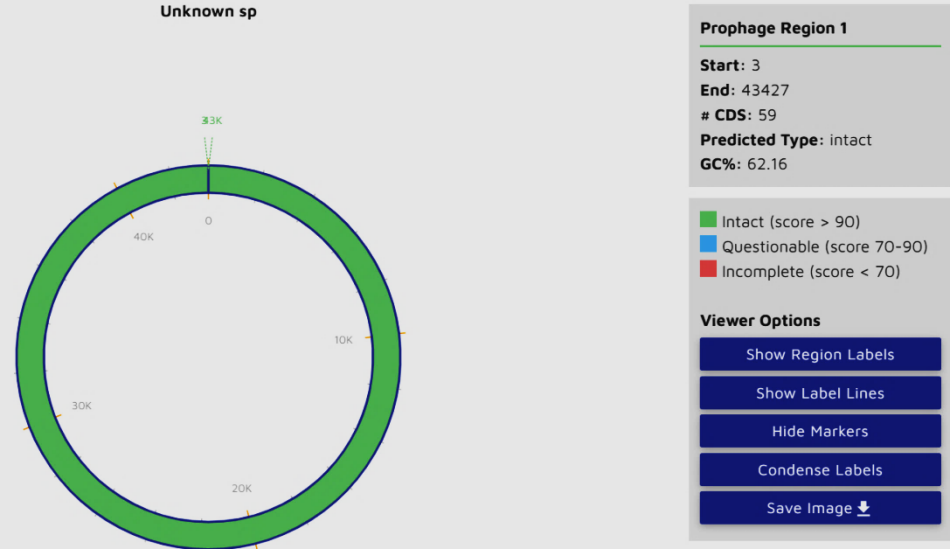


Figure 17: Circular representation of phage TU_pse1B genome generated by PHASTER

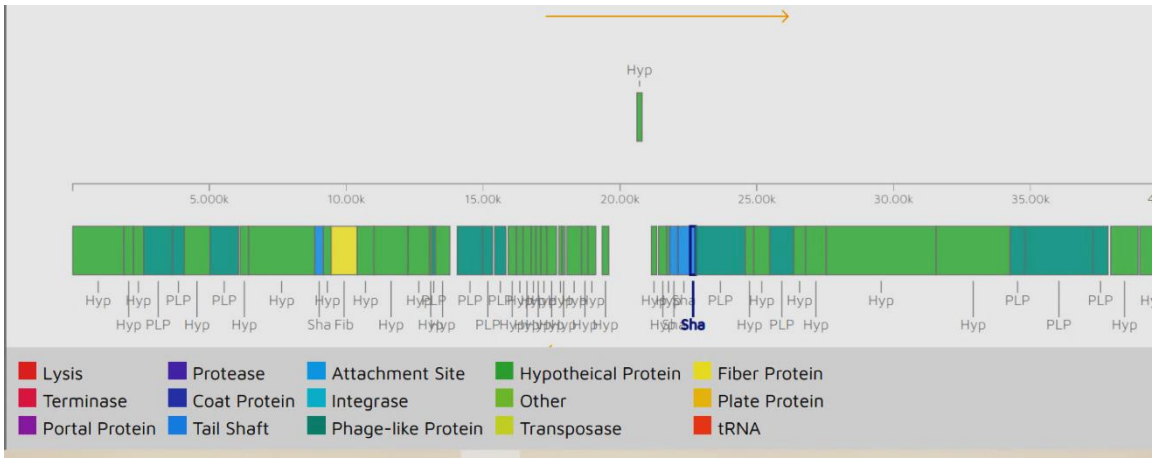


Figure 18: Linearized genome of phage TU_pse1B generated by PHASTER

4.8.4 Multiple sequence alignment and phylogenetic tree construction

BLAST was performed in NCBI to look for the phage genomes that possess similarity with our phage genome. The BLAST generated list of *Pseudomonas aeruginosa* phage that has similarity with our phage genome. Top ten phage genome showing highest percentage similarity was selected for multiple sequence alignment. Highest percent identity (97.82%) was shown against *Pseudomonas* phage MPK7 and lowest percent identity was found against *Pseudomonas* phage PAXYB1 (92%). The FASTA format of these ten phage genome was downloaded. Multiple sequence alignment of our phage genome was done with these ten phage genomes before the construction of phylogenetic tree.

After completion of multiple sequence alignment, phylogenetic tree construction was done in UGENE software. The figure below shows phylogenetic relationship of our phage genome with other ten *Pseudomonas* phage genomes.

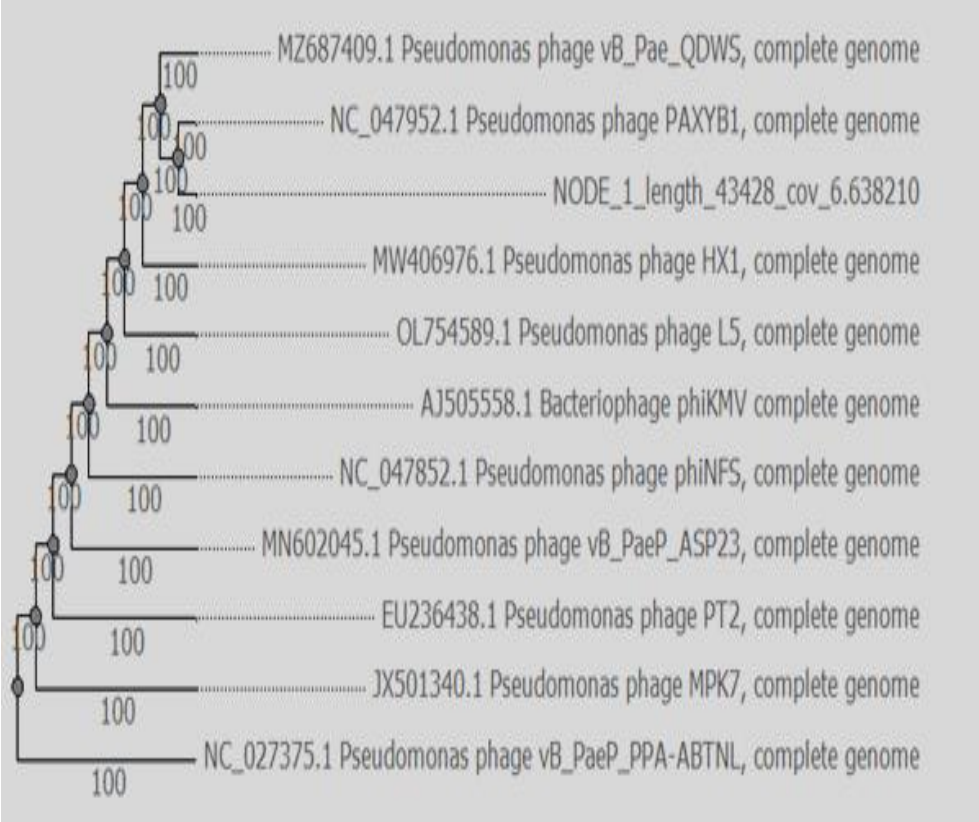


Figure 19: Phylogenetic relationship of phage genome with other ten *Pseudomonas aeruginosa* phages

Our phage genome showed closest phylogenetic relation with *Pseudomonas* phage PAXYB1. It was then followed by *Pseudomonas* phage vB_Pae_QDWS. The phage with farthest phylogenetic relation was *Pseudomonas* phage vB_PaeP_PPA-ABTNL.

4.8.5 Open Reading Frame

Total number of ORF in our phage genome was 234. Presence of ORF can indicate a possible gene. ORF can code protein but all of the ORF are not protein coding regions. Similar information can be implied from our result because the number of ORF (234) is far higher than the number of genes (59) obtained from the result of our whole genome sequencing. The probability for an ORF to code a protein can be estimated by determining its length and by comparing with standard database.

Proteins are encoded in Open Reading Frames (ORFs) delimited by a start and stop codon, although the number and the typical length of ORFs may vary. An ORF is defined as the longest string of triplets beginning with a start codon (Mir et al., 2012).The length of an open reading frame (ORF) is one important piece of evidence often used in locating new genes. A sufficiently long open reading frame (ORF) is one of the most commonly noted indications for the existence of a gene (Fickett, 1995). A substantial fraction of open reading frames (ORFs) are short, suggesting that many are not genes but random stretches of DNA. For the majority of species, perhaps 10–30% of recognized ORFs do not actually encode proteins. The reading frame of protein coding

regions can usually be deduced from alignments of homologous sequences in closely related organisms. Many short, annotated ORFs are not genuine protein-coding regions (Ochman, 2002).

4.8.6 Annotated regions

The number of annotated regions was 42 which were determined from UGENE software. DNA annotation is the process of finding the locations of genes and coding regions in the genome. It also determines the function of gene. Genome annotation mainly consists of predicting genes and attaching biological information (such as gene name, intron, exon, protein products regulatory sequences, repeats, etc) to the DNA. DNA annotation can be done by searching the homologous genes in databases by using search tools such as BLAST. DNA annotation takes help of both biological experiments and computer (in silico) analysis. Genome annotation is based on detecting homology between newly identified genes and sequences that have been previously annotated. Genes predicted in newly sequenced genomes are compared against reference databases to find the homologues and then annotated. When predicted protein sequences cannot be functionally annotate, these protein sequence are classified as 'hypothetical' proteins (Lobb et al., 2020).

4.9 Live cell count

The number of colonies produced by bacteria without phage treatment and after phage treatment is shown in table below.

Table 12: Number of bacterial colonies in phage treated and untreated sample

	Phage treated	Phage untreated
No of colonies	79	181

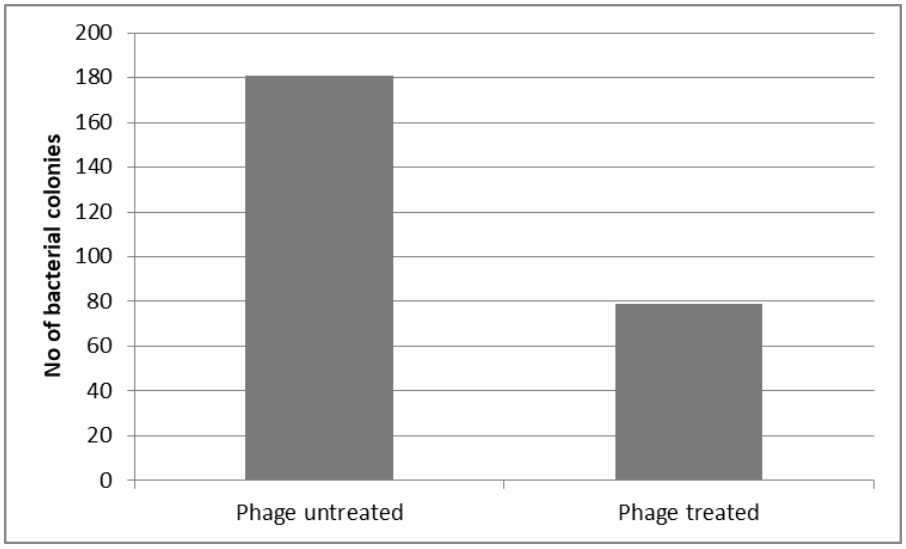
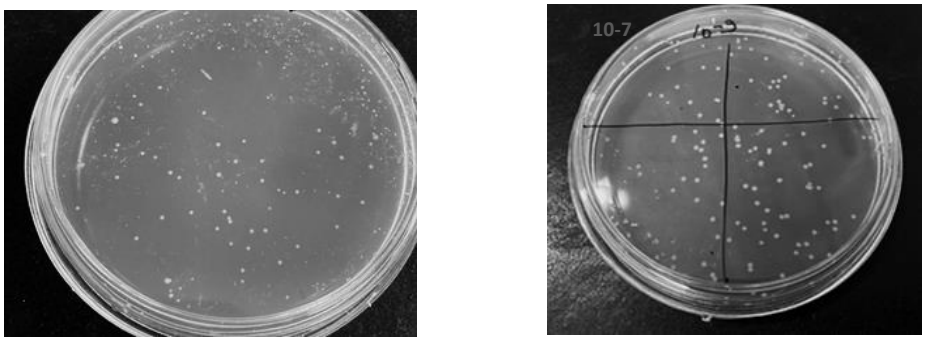


Figure 20: Number of colonies produced by phage treated and phage untreated *Pseudomonas aeruginosa*

Phage treated bacteria produced 79 colonies; whereas phage untreated bacteria produced 181 colonies. The reduction in number of bacterial colonies was by 102. The percentage reduction in bacterial count was 56.35%.

Phage concentration was delivered to the lung tissue and then bacterial concentration was determined. Bacterial load in the lungs was significantly reduced after the treatment. Phage was administered intra nasally to test its efficacy. It was followed by bacterial count in the lung tissues. There was complete clearance of bacteria in seventy percent of mice and there was significant reduction of bacterial load in the thirty percent of mice (Chang et al., 2018).



(A) (B)

Figure 21: (A) Number of bacterial colonies in phage treated petri plate. (B) Number of colonies in phage untreated plate.

4.10 Effect of Calcium ions on phage adsorption

The number of plaques in Calcium treated plates was lower than that of non-Calcium treated plates. This suggests calcium ions increase the rate of phage adsorption to the host cell.

Table 13: Number of plaques in Calcium treated and untreated plates at 5, 10 and 15 minutes of time interval

	No of plaques		
	No $CaCl_2$	$CaCl_2$	Difference
5 min	6	2	4
10 min	8	4	4
15 min	9	6	3

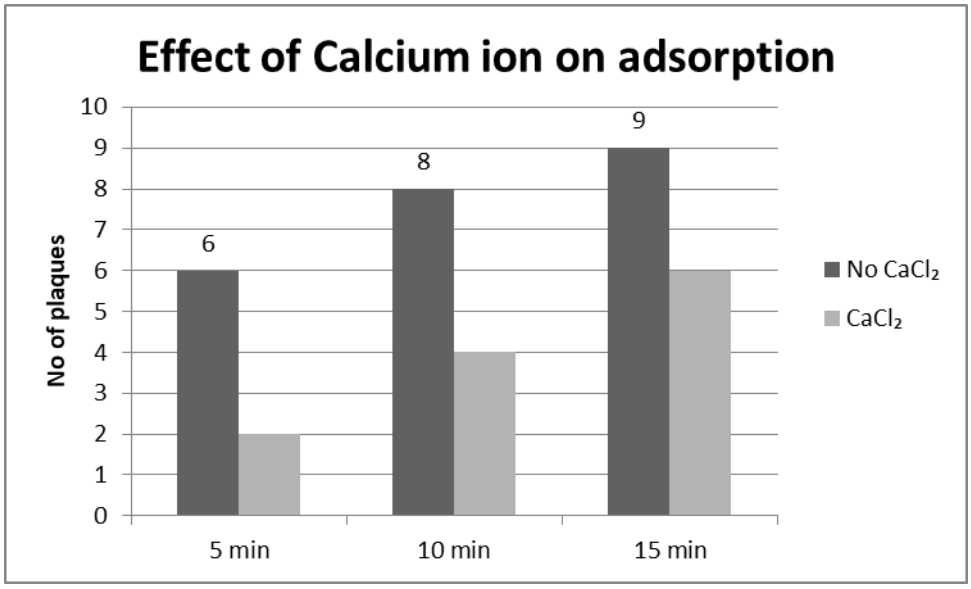
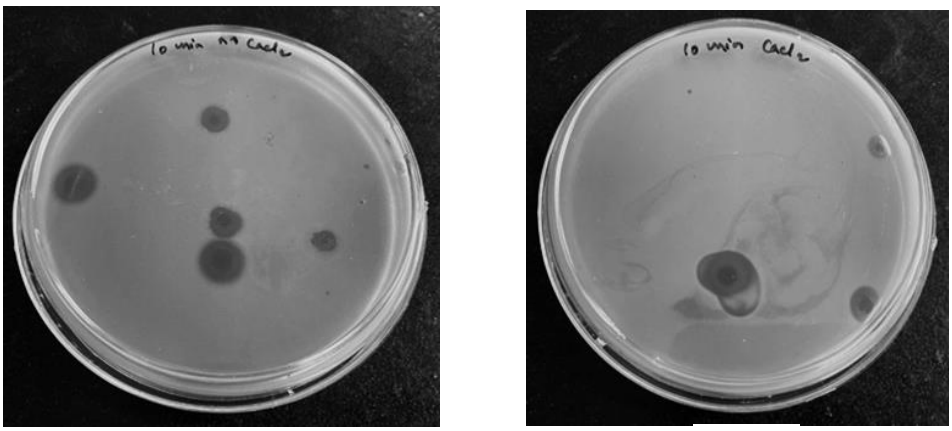


Figure 22: Number of plaques produced by Calcium treated and Calcium untreated phage at three different time intervals.

The number of plaques in Calcium treated plates was lower than that of Calcium untreated plates. This shows calcium ions increase the rate of phage adsorption to the host cell. It is because when more number of phage is adsorbed to the host cell; there will be less number of phage in the supernatant and double layer agar assay was done by taking this supernatant. This will produce less number of plaques compared to that of control (Calcium untreated plates).

The difference in number of plaques in Calcium ion treated and untreated plates were almost similar for 5 minute, 10 minute and 15 minute interval. This proves that by increasing the time period there will not be more adsorption of phage to the host cell. So increasing the time interval will not increase the phage adsorption. An optimum exposure time will be sufficient for the phage to adsorb to the host cell. In our case this time was 5 minutes.

It has been observed in several phage systems that Calcium ion is required for adsorption of bacteriophage in host cell. Penetration of phage into the host cell requires calcium ions. Divalent ions are needed for either adsorption of phage or its penetration-synthesis process. Divalent ions such as calcium and magnesium also control phage synthesis mechanisms apart from aiding in penetration process (Shafia & Thompson.,1964)



(A) (B)

Figure 23: (A) $CaCl_2$ untreated and (B) $CaCl_2$ treated plates after exposure period of 10 minutes.

4.11 Effect of external factors on phage survivability

i) Organic solvents (ethanol and acetone)

Phage did not showed survivability against ethanol. For ethanol treated phage, the number of colonies was zero. For control, the number of plaques was 126.

The number of plaques in acetone treated phage sample was 23 whereas for acetone untreated phage the number of plaques was 126. The percent survivability of phage in acetone was 18.25%.

ii) Detergents (CTAB and SDS)

The number of plaques in control plate was 126 whereas for CTAB treated phage, the number of plaques was just 5. The percent survivability of phage after CTAB treatment was only 3.96%.

The number of plaques in SDS treated plate was 97 whereas for control, the number of plaques was 126. The percent survivability of SDS treated phage was 76.98 %.

iii) Effect of osmotic shock

The number of plaques in NaCl treated plate was 108 whereas for control, the number of plaques was 126. The percent survivability of phage when exposed to osmotic shock was 85.71%.

Table 14: Percent survivability of phage against various external factors

External Factors	Percent survivability
63% Ethanol	0
90% Acetone	18.25
0.1% SDS	76.98
0.1% CTAB	3.96
Osmotic shock	85.71

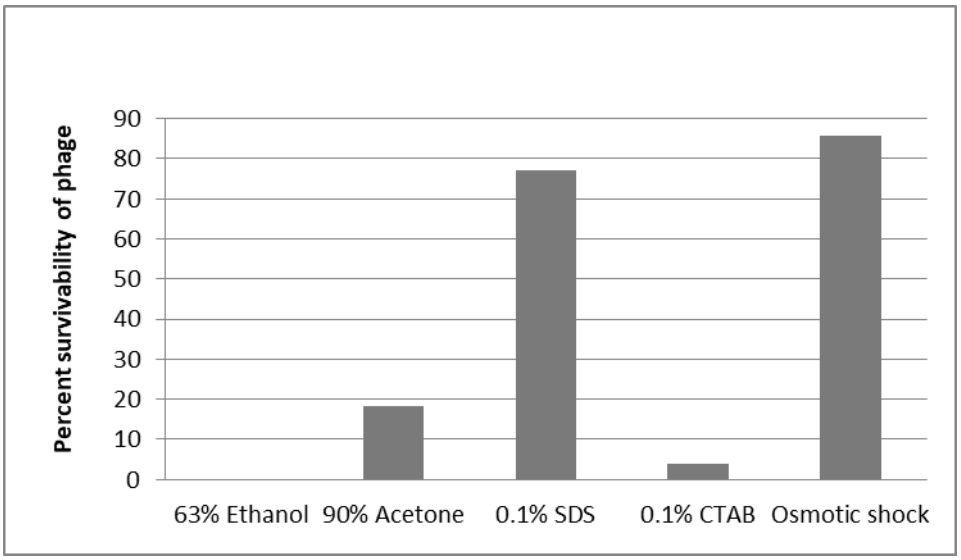


Figure 24: Percent survivability of bacteriophage against five different external factors

Phage showed good survivability when exposed to SDS and osmotic shock whereas it was susceptible to ethanol, CTAB and acetone.

The toxic effects of SDS depend on the lipid/surfactant molar ratios and absolute concentrations. SDS was found to have a dose-dependent anti-HSV-1 impact on VERO cells, which are kidney cells from an African green monkey. Low doses (≤ 50 µM) had no effect on viral glycoprotein production (de Sousa et al., 2019).

Linderoth et al claim that phage can exhibit SDS resistance. One of the mechanisms is brought on by a mutation in a phage protein, in this case protein IV (pIV) for filamentous phage. Extreme SDS and heat stability are conferred by a mutation on pIV (Linderoth et al., 1996). Some of the native phage proteins (such as tail spike proteins) are resistant to SDS denaturation. Studies have established that a number of proteins can efficiently refold in vitro after denaturation (Goldenberg et al., 1982). Tail Spike Protein is a kinetically stable protein that exhibits significant resistance to SDS unfolding. Proteins that are kinetically stable are stabilized in their final state and require significant disturbances to alter their structural characteristics. SDS resistance has been demonstrated to be the result of stiff protein structures with oligomeric beta-sheets.

Energy barriers are known to trap these proteins in specific conformations in their final natural states (Ayariga et al., 2021).

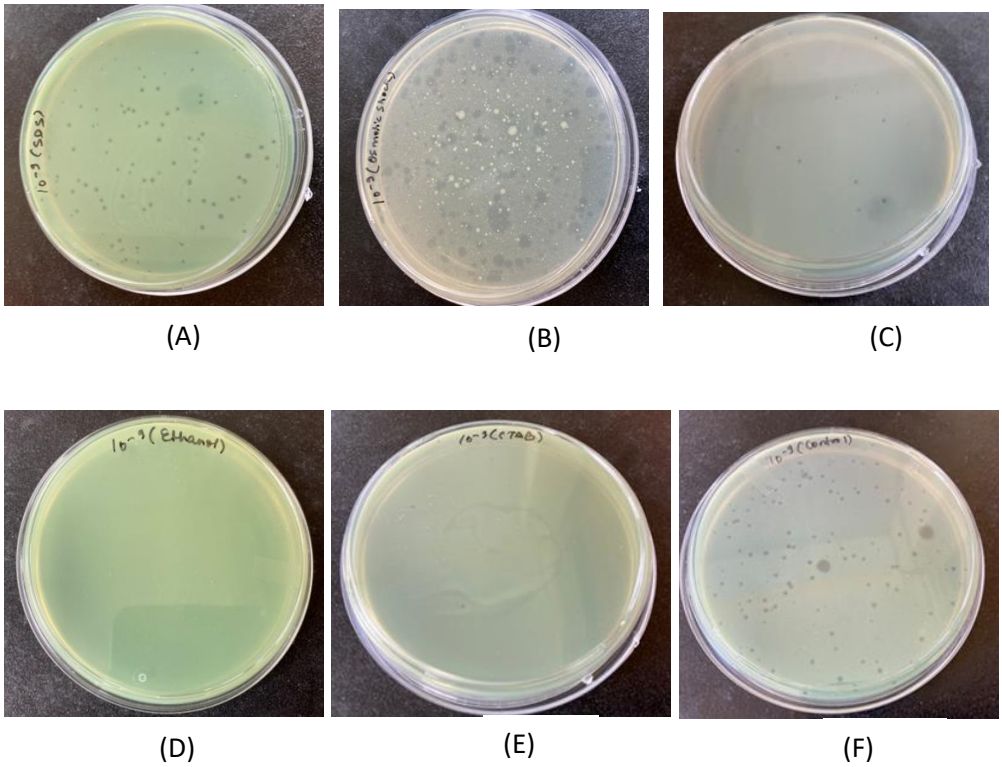
The genome of many bacterial viruses is under intense pressure, meaning that the closed capsid must be able to endure a sizable force per unit of area exerted by the enclosed RNA or DNA. Whether they are single or double stranded RNA or DNA, nucleic acid segments are crammed onto one another due to their confinement at crystalline-like densities. Strong short-range repulsions between molecules as a result of this molecular crowding put strain on the capsid walls. According to studies, interior pressures are thought to be around 50 atmospheres, which means that the capsid strengths must be at least this high. The viral genome's twisted state is another factor contributing to pressure buildup. These factors make it necessary for capsids to be strong, and their strength enables them to tolerate osmotic pressure (Cordova et al., 2003).When cultured in sufficiently high concentrations of salt and then rapidly diluted, phages (Odd-numbered T phages such as T1, T3, T5, and T7) can survive intact and remain completely infectious particles. It can be explained in terms of the various viral capsids' different permeability to water and salt ions. Permeable capsids, such as odd-T and lambda, let both salt and water to pass through on relatively fast time scales, allowing them to withstand osmotic pressure (Cordova et al., 2003).

The overall result of the effect of the external factors on phage survivability is similar to that of Jurczak-Kurek et al. Against the 10 phages of *Pseudomonas aeruginosa,* most of them showed good survivability against osmotic shock. Six of them showed 100 % survivability against osmotic shock. The least survivability was 70 %. Most of the phages were sensitive to SDS. However four out of ten showed survivability against SDS. They showed 100 %, 68 %, 42 % and 1.2 % survivability against SDS. All of the phages were sensitive to acetone except one which showed 27.8 % survivability. Out of ten phages, seven showed survivability against CTAB. The survivability of these seven phages were 100%, 100%, 86.4%, 69.6%, 46.7%, 19% and 1%. Out of ten phages, three phages showed survivability against ethanol. The survivability of these three phages was 36.6%, 11.7% and 3.7%.

This phage can be used in formulations containing SDS to kill the bacteria synergistically. SDS is used in cleaning applications as it has detergent property. So the phage can be used in combination with SDS as disinfectant particularly in hospital setting which is a common place for acquiring nosocomial infection as well as place to harbor multidrug resistant bacteria.

Phage treatment followed by chemical disinfection inactivated *P. aeruginosa* cells better than either treatment alone. So phage and chemical disinfectant can be combined to inactivate surface-associated *P. aeruginosa*. This makes phages as promising agents such as disinfection agents for controlling opportunistic pathogen in the built environment. Drug-resistant *Staphylococcus aureus*, *E. coli,* and *P. aeruginosa* isolates collected from

hospital surfaces on ceramic, plastic, and glass test surfaces were removed effectively by phage treatment. Sequential treatment with phages and then chemical disinfectants could result in greater removal of bacterial biofilms than either treatment alone (Stachler et al., 2021).



(A) (B) (C)

(D) (E) (F)

Figure 25: (A) Plate after treatment with SDS. (B) Plate after exposure to osmotic shock. (C) Plate after treatment with acetone. (D) Plate after treatment with ethanol. (E) Plate after treatment with CTAB. (F) Control plate

4.12 Biofilm formation

The interpretation of biofilm formation was done according to Stepanovic et al.

Biofilm formation capacity of *Pseudomonas aeruginosa* (209205) and *Pseudomonas aeruginosa* (6661) was analyzed. Strong biofilm producer strain (PA01) was used as positive control.

This experiment was done in 96 well polystyrene tissue culture microtiter plate. Bacteria were inoculated in the well for overnight and OD was measured at 620 nm in ELISA reader. Only the culture media was used as negative control. Test was performed in triplicate.

The data obtained from ELISA reader was interpreted to determine whether the two bacteria were biofilm producers or not according to following parameter:

OD > 4ODc = Strong Biofilm Producer

2 ODc < OD ≤ 4 ODc =Moderate Biofilm Producer

ODc < OD ≤ 2 ODc = Weak Biofilm Producer

Optical Density Cut Off (ODc) value needs to be determined first. So it was calculated first. The formula for calculation of ODc is given below

ODc = Average OD of negative control + 3 X SD of Negative control

Average OD of negative control: 0.25

Standard deviation of Negative control = 0.0918

ODc = 0.25 + 3 X 0.00918 = 0.5254

2 ODc = 1.0508

4 ODc = 2.1016

Optical density of *Pseudomonas aeruginosa* (209205) = 0.542

Optical density of *Pseudomonas aeruginosa* (6661) = 0.479

Optical density of Positive control = 2.69

The OD for *Pseudomonas aeruginosa* (209205) was greater than ODc but it was less than 2ODc (0.52). So this bacterium is weak biofilm producer. The ODc for *Pseudomonas aeruginosa* (6661) is 0.479 which is less than ODc (0.52) so this bacterium is not biofilm producer. *Pseudomonas aeruginosa* (209205) was selected for biofilm disruption experiment.

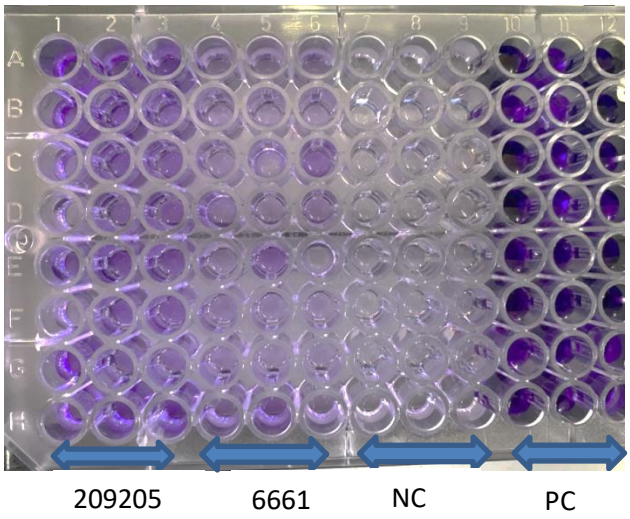


Figure 26: Microtiter plate showing the biofilm staining of two *Pseudomonas aeruginosa* strains i.e., 209205, 6661, Negative control (NC) and Positive control (PC)

4.13 Biofilm disruption

The interpretation of biofilm disruption was done according to Forti et al.

Bacteria was grown overnight in microtiter plate and it was treated with phage and their OD was measured at 620 nm in ELISA reader. Phage untreated bacterial well in microtiter plate was used as control. Two phages i.e., Phage TU_pse1B and Phage TU_pse1N were used to disrupt biofilm.

OD of Phage TU_pse1B treated bacteria = 0.416

OD of Phage TU_pse1N treated bacteria = 0.422

OD of Phage untreated bacteria = 1.065

Percent reduction of biofilm by Phage TU_pse1B = 60.99 %

Percent reduction of biofilm by Phage TU_pse1N = 60.37%

OD of negative control = 0.176

Phage TU_pse1B reduced the biofilm by 60.99% whereas Phage TU_pse1N reduced the biofilm by 60.37%. Both phages reduced the bacterial biofilm significantly and to the same extent.

4.14 Synergistic effect of antibiotic and phage in biofilm reduction

A) Minimum Inhibitory Concentration (MIC) determination

Antibiotic solutions of concentration 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 mg/L were prepared. Visible growth was seen up to concentration of 4 mg/L. So the minimum inhibitory concentration for our bacteria was 8 mg/L. MIC is the lowest concentration of an antibacterial agent which completely prevents visible growth of the test organism and it is expressed in mg/L or µg/mL (Kowalska-Krochmal & Dudek-Wicher, 2021).

B) Synergistic effect of antibiotic and phage in biofilm reduction

The average OD of only phage treated well was 1.20. Whereas the OD of phage-antibiotic treated well was 0.88. There was significant reduction in OD of phage antibiotic treated well compared to that of only phage treated well i.e., by 0.32 (26.67%). The biofilm further decreased by 26.67% when phage-antibiotic solution was used.

The OD for bacterial control was 2.45 whereas the OD for negative control was 0.37. LB was used in negative control wells.

Similar result was also mentioned by Li et al., 2021. When Gentamicin was used along with phage to treat *Pseudomonas aeruginosa* (PAO1) biofilm, there was 10-50 % enhancement in phage-antibiotic synergistic effect compared to phage alone. Higher synergistic effect was seen when different antibiotics was used with different strains (Li et al., 2021).

Phage can help break up the biofilms that frequently impede antibiotic therapy and boost the effectiveness of antibiotics used to treat these illnesses. Phages and antibiotics can sometimes have profoundly positive synergistic interactions. More bacteria in biofilms can be eliminated by combining antibiotics and phages than by either agent working alone. Some antibiotics may work better at lower doses than larger ones when coupled with phage (Chaudhry et al., 2017).

The main objective of using phage and antibiotic in combination to reduce biofilm is due to the reason that bacteria can develop resistance to phages too. There is continuous evolutionary race between bacteria and phage which leads to phage resistant bacteria (Ong et al., 2020).The resistance of bacteria to phages is not addressed properly with few exceptions. If we are to use phage in clinical setting then we need to understand the resistance of bacteria to phages in detail. (Oechslin et al., 2016).

Phage therapy alone was active in treating animals with endocarditis but it was highly synergistic with antibiotics. Combination of phage with antibiotic (ciprofloxacin or meropenem) inhibited the growth of bacteria that are resistant to phage. So, the combination of phages with ciprofloxacin showed good synergistic effect. (Oechslin et al., 2016). Phage cocktail together with antibiotic (either CIP or MEM) is more effective than a phage cocktail or antibiotic alone to prevent the growth of resistant *P. aeruginosa*. This showed the possibility to use phage cocktail together with antibiotic to treat Pseudomonal infection (Ong et al., 2020).

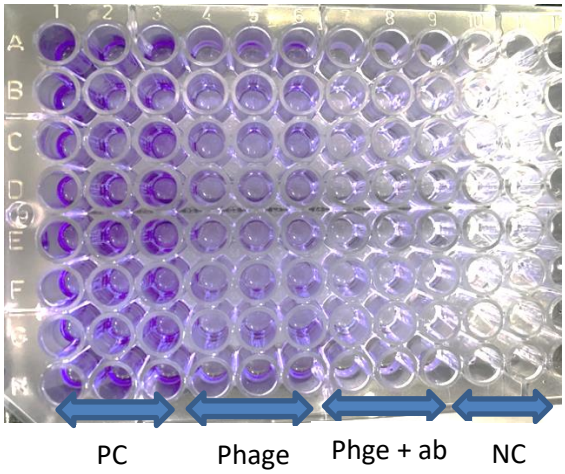


Figure 27: Microtiter plate showing Positive control (PC), phage treated, phage plus antibiotic treated and negative control wells.

5. SUMMARY

Identification of *Pseudomonas aeruginosa* was done through biochemical test. AST was done to determine whether *Pseudomonas aeruginosa* was antibiotic resistant or not. *Pseudomonas aeruginosa* was found to be resistant to Meropenem, Nalidixic acid, Cefotaxime, and Cefoxitin. Lytic bacteriophages against *Pseudomonas aeruginosa* were isolated. Phage TU_pse1B was characterized. It showed intraspecific host range.

The stability of the phage was highest at 37^{o}C and stability decreases with increase in temperature. Phage showed survivability up to 60^{o}C. The phage showed good stability from pH 7 to pH 11 with highest survivability at pH 9. So this phage showed good thermal and pH stability. The latent period of the phage was 30 minutes whereas the burst size was determined to be 27. SDS PAGE of phage proteins was performed and it showed three distinct bands. Two proteins were of smaller size (35 and 40KDa) whereas one protein was of larger size (100KDa). Phage DNA was extracted by using kit and its agarose gel electrophoresis was done. The size of the phage DNA was found to be larger than 10 Kb from electrophoresis. The concentration of phage DNA was determined by using nanodrop and it was found to be 17.7 ng/µl.

The effectiveness of phage in killing bacteria was determined by performing live cell count. Application of phage reduced the bacterial population by 56.35 % percentage showing the bactericidal potential of phage. Calcium ion increased the phage adsorption shown by the decrease in PFU/ml of calcium ions treated phage. Stability of phage was checked against different solvents. Phage showed good stability to SDS and osmotic shock whereas it was susceptible to ethanol, acetone and CTAB. The biofilm forming capacity of *Pseudomonas aeruginosa* (209205) was assessed and it was found to be weak biofilm producer. The biofilm was produced by using this strain and phage was used to disrupt the biofilm. Phage TU_pse1B reduced the biofilm by 60.99% whereas Phage TU_pse1N reduced the biofilm by 60.37%. So both phages reduced the bacterial biofilm significantly. The effectiveness of phage in reducing biofilm in synergism with antibiotics was also analyzed. When phage plus antibiotic are both used, the biofilm further decreased by 26.67% compared to phage treatment only. Whole genome sequencing of phage DNA determined the length of phage DNA to be 43 Kb. The GC content was 62.16%.It contained 234 ORF and the number of genes was 59. Phage genome lacked toxic and integrase genes. There were also several hypothetical proteins present in the phage genome. Our phage showed closest phylogenetic relation to NC_047952.1 Pseudomonas phage PAXYB1 which was inferred from phylogenetic tree.

6. CONCLUSION

Lytic bacteriophage against MDR *Pseudomonas aeruginosa* was isolated from sewage sample. The phage showed good survivability at higher temperatures (50^{o}C and 60^{o}C) and it also showed stability at wider range of pH (pH 5-11). Phage reduced the bacterial population significantly as shown by live cell count. So this phage can be an effective alternative to kill drug resistant bacteria. The phage was also equally effective in disrupting bacterial biofilm. Phage application greatly reduced bacterial biofilm. The biofilm reduction was further enhanced when phage plus antibiotic was used in synergism. Calcium ions increased phage adsorption. Thus phage efficiency can be increased by applying it along with calcium ions. Phage showed stability to some external factors such as SDS and osmotic shock. Thus, this phage can be used to control the biofilm produced by multi drug resistant *Pseudomonas aeruginosa*.

7. RECOMMENDATIONS AND FUTURE PERSPECTIVES

i) Phage and antibiotic can be used in combination to treat antibiotic resistant bacteria. It is more efficient than using antibiotic or phage alone. It will decrease the chance of development of resistance by bacteria to phage as well as antibiotic.

ii) Our phage showed good stability to some of the external factors (SDS and osmotic shock) as well as it was good in reducing biofilm. So it can be also used to kill surface associated bacteria.

iv) Further analysis of whole genome can be done to yield more useful information and conclusions.

v) Endolysin and Holin gene of phage code proteins that makes pore in bacterial cell wall and causes its lysis. So cloning of holin and endolysin gene to create recombinant bacteria that can kill other antibiotic resistant bacteria would be a great achievement.

8. LIMITATION OF THE STUDY

i) Electron microscopy of phage was not possible in our laboratory. Electron microscopy would have generated information regarding phage morphology.

ii) Less number of bacteria was available for intraspecific host range analysis. Host range analysis done with more *Pseudomonas aeruginosa* strains would have given the better picture regarding the host range of our phage.

iii) Further bioinformatics analysis of phage whole genome could not be performed.

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10.APPENDICES

i) Composition of urea broth

Ingredients	Gram/liter
i) Dextrose	1.0
ii) Peptic digest of animal tissue	1.5
iii) Sodium chloride	5.0
iv)Monopotassium phosphate	2.0
v) Phenol red	0.012
Vi) Agar	15

24.52 gram of Urea Agar base (Christensen Agar) is dissolved in 950 ml of distilled. It is heated to dissolve the medium and then autoclaved at 15 psi (pound per square inch) (121^{o}C for 15 min). Then it is allowed to cool. To this solution, 50 ml 0f sterile 40% urea solution is added. The medium is then dispensed into tubes and set in position to obtain agar slants.

ii) SM buffer

i) Water	800ml
ii) Sodium Chloride	5.8 gram
iii) Magnesium Sulphate	2 gram
iv) 1 Molar Tris Cl (pH 7.5)	50 ml
V) 2% gelatin	5 ml

Then final volume is made 1000 ml

1 Molar Tris base composition (for 100 ml)

Tris base	12.11 gram
Water	80 ml
HCL	7 ml

iii) Phosphate Buffered Saline (PBS)

Salt	Concentration (g/L)
i) NaCl	8
ii)KCL	0.2
iii) Disodium hydrogen phosphate	1.42
iv) Potassium dihydrogen phosphate	0.24

All of these are dissolved in 800 ml of water and the final volume is made 1 liter.

iv) TAE buffer

i) Tris Base	242 gram
ii) Acetic acid	57.1 ml
iii) EDTA	18.612 gram

50× stock solution is prepared by dissolving 242 g Tris base in water, adding 57.1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0) solution, and bringing the final volume up to one liter. This stock solution is then diluted 49:1 with water to make a 1× working solution.

v) SDS reagents

A) 30 % Acrylamide solution

Constituents	Weight
i) Acrylamide	29 gram
ii) Bis Acrylamide	1 gram
iii) Distilled Water	Maintain 100 ml

B) Lower Tris Buffer (pH 8.8)

i) Tris Base	18.17 g
ii) Distilled Water	Maintain 100 ml

C) Upper Tris Buffer (pH 6.8)

i)Tris base	3.03 gram
ii) Distilled water	Maintain 50ml

D) Loading Dye

i) Upper Tris (pH 6.8)	1.25 ml
ii) 10% SDS	3.0 ml
iii) Glycerol	4.75 ml
iv) Beta-mercaptoethanol	0.5 ml
v) 0.1% Bromophenol blue	0.5 ml

E) Comassie Brilliant Blue (500 ml)

i) Comassie Brilliant Blue Color	500 mg
ii) Glacial acetic acid	25 ml
iii) Methanol	250 ml
iv) Distilled Water	225 ml

F) Destain Solution (500 ml)

i) 7.5 % Glacial acetic acid	37.5 ml
ii) 5% Methanol	25 ml
iii) Distilled water	437.5 ml

G) Running Buffer (1000ml) (pH 8.4)

i) 39 mM Tris	4.72 g
ii) 48 mM glycine	3.603 g
iii) 0.1% SDS	0.37 g

H) 12% Resolving Gel (for 10 ml)

i) Water	3.3 ml
ii) 30 % Acrylamide	4 ml
iii) 1M Tris HCL (pH 8.8)	2.5 ml
iv) 10 % SDS	0.1 ml
v) 10%(NH4)2S208	0.1 ml
vi) TEMED	0.004 ml

I) 5% Resolving Gel (for 5 ml)

i) Water	3.4 ml
ii) 30% Acrylamide	0.83 ml
iii) 1M Tris-HCL (pH 6.8)	0.63 ml
iv) 10 % SDS	0.05 ml
v) 10% (NH4)2S2O8	0.05 ml
vi) TEMED	0.005 ml

vi) TE buffer

i) Tris Base (1M) (pH 10-11)	1 ml
ii) EDTA (0.5 M)	0.2 ml

These two are mixed in the required volume as above and the final volume is made 100 ml with distilled water.

vii) Table showing data for synergism of phage and antibiotic

PC			Phage treated			Phage plus ab treated			NC		
2.340	2.381	2.521	1.525	0.917	0.542	1.066	0.631	0.542	0.357	0.253	0.249
2.471	2.866	2.693	0.457	0.582	1.031	0.727	1.067	0.752	0.299	0.256	0.286
2.711	2.658	2.176	1.065	0.678	0.879	0.752	0.682	0.761	0.304	0.340	0.352
2.553	1.972	2.889	1.184	2.097	1.003	0.436	0.467	0.692	0.284	0.302	0.355
2.274	2.435	2.873	1.395	2.080	1.839	1.043	0.633	0.608	0.314	0.318	0.341
1.851	2.373	2.643	1.203	1.614	1.167	1.967	0.958	0.919	0.291	0.615	0.843
1.917	2.316	2.664	1.322	1.118	0.824	1.368	0.860	0.784	0.694	0.359	0.350
2.144	2.634	2.581	1.130	2.499	0.852	1.282	0.932	1.253	0.313	0.449	0.589

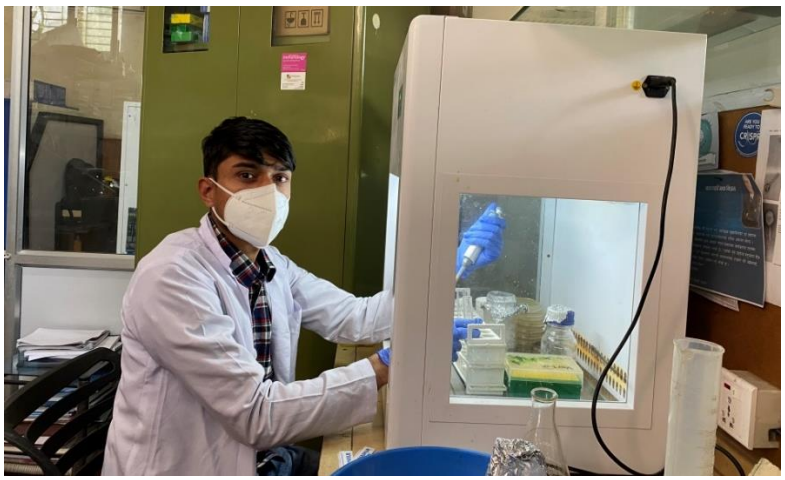
vii) Table showing data for biofilm formation

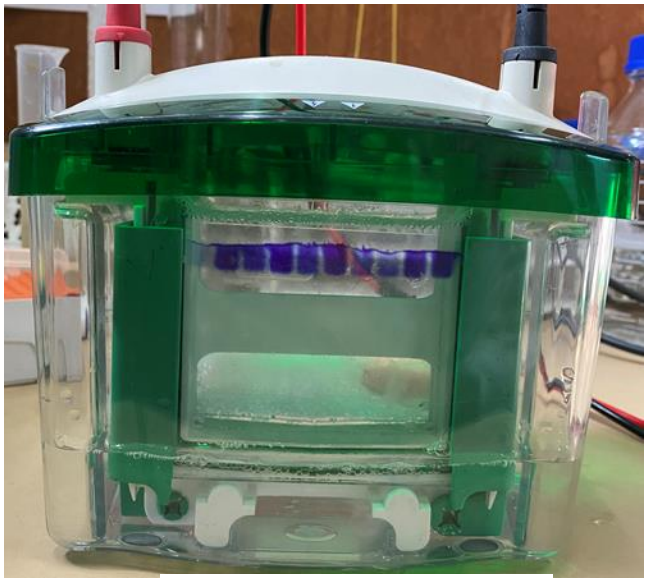
PC			209205			6661			NC		
2.842	2.939	2.937	1.481	0.659	0.754	0.541	0.446	0.466	0.283	0.366	0.270
2.984	2.035	2.660	0.502	0.530	0.608	0.427	0.912	0.442	0.306	0.257	0.279
2.922	3.009	2.922	0.544	0.511	0.511	0.418	0.416	0.434	0.307	0.227	0.187
2.748	2.697	2.228	0.472	0.519	0.490	0.607	0.395	0.422	0.214	0.297	0.207
2.803	2.338	2.937	0.455	0.446	0.415	0.372	0.487	0.576	0.281	0.229	0.175
2.102	3.013	2.758	0.516	0.491	0.533	0.746	0.543	0.347	0.248	0.315	0.174
2.755	2.609	2.836	0.427	0.388	0.486	0.424	0.385	0.372	0.256	0.215	0.210
2.847	2.744	2.113	0.482	0.403	0.388	0.455	0.600	0.387	0.268	0.236	0.202

viii) Table showing data for biofilm disruption

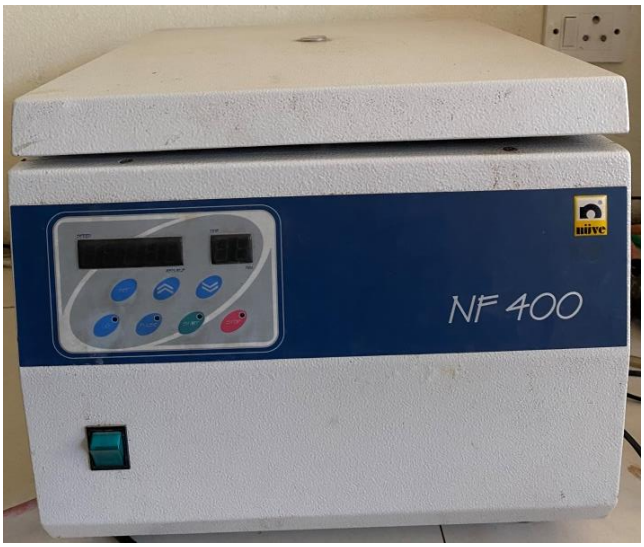
TU_pse1B treated			TU_pse1N treated			PC			NC		
0.367	0.302	0.475	0.302	0.296	0.205	1.073	2.731	0.175	0.142	0.163	0.152
1.046	0.298	0.351	0.275	0.312	0.248	1.244	2.332	1.042	0.189	0.167	0.169
0.885	0.670	0.382	1.098	0.466	0.273	1.393	2.049	0.935	0.210	0.185	0.190
0.303	0.260	0.376	1.042	0.283	0.360	0.952	2.036	0.500	0.165	0.160	0.138
0.882	0.306	0.325	0.255	1.155	0.591	0.292	1.697	0.703	0.178	0.175	0.207
0.285	0.215	0.407	0.266	0.268	0.635	0.692	2.094	0.273	0.169	0.184	0.175
0.218	0.220	0.501	0.200	0.214	0.517	0.269	0.212	1.154	0.190	0.217	0.163
0.225	0.232	0.457	0.222	0.234	0.418	0.181	0.231	1.911	0.186	0.181	0.169

PHOTOGRAPHS





SDS Electrophoretic tank



Centrifuge

ELISA Plate Reader