Isolation and Characterization of *E. coli* Bacteriophage from Raw Meat and Environment Water

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Bachelor of Science in Biotechnology and Bachelor of Science in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Ethics Statement

No human and environmental hazardous model was used in this study.

Abstract

Several diseases such as stomach cramps, urinary tract infections, and diarrhea are caused by the bacterium *Escherichia coli*. One of the five subtypes of E. coli which are ETEC, EPEC, EAEC, EHEC/STEC, and EIEC are responsible for intestinal infections. *E. coli* outbreak is a major public health concern. One of the key elements that can put an end to outbreaks could be bacteriophages. Since their discovery in 1915, they have been beneficial to humans in a variety of ways, including phage therapy, genetic screening tools, diagnostic weaponry, pathogenic bacteria detectors, therapeutic agents, and more. Phage research has gained attention because of the recent rise in antibiotic resistance because of their capacity to infect and destroy bacteria without endangering humans. This experiment is designed to isolate and characterize bacteriophage specific to *E. coli*. It includes procedures that have been used in the lab to check for the presence of phages and the results of the experiments. In this research, *E. coli* bacteria and bacteriophage was isolated from the Raw meat. Unfortunately, only one bacteriophage was possible to isolate from Raw meat and which is why water samples are collected from various regions of Mohakhali, Gulshan and Hatirjheel and from the samples, four more bacteriophages were successfully isolated. Phage ECPW4.4, ECPW5.1 and ECPW12.3 were chosen for further analysis and characterization. In characterization, three tests were done, which were; Temperature test, salinity test and pH test. On these three tests, the three bacteriophages showed better results overall. Furthermore, more tests and analysis are needed to be done but because of the closure of BRAC University Lab, further research was not possible.

Keywords: Bacteriophage; phage; phage isolation; phage characterization; phage therapy.

Dedication

Dedicated to our beloved Parents

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First of all, we would like to give our heartiest gratitude to our Almighty Allah for everything He has allowed us to achieve and for giving us patience, knowledge and strength to make this project a success.

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List of Abbreviations

g	Gram
h	Hour
C	Celsius
S	Sample
ml	Milliliter
E. coli	*Escherichia coli*
µL	Microliter
LA	Luria-Bertani Agar
LB	Luria-Bertani Broth
NS	Normal Saline
CFU	Colony forming unit
PFU	Plaque forming unit
EMB	Eosin Methylene Blue
DLA	Double layer assay
rpm	Rotation per minute
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
psi	Pound-force per square inch
min	Minute
MCT	Microcentrifuge tube

Chapter 1

Introduction

1.1 Objective

The primary goal of this thesis project was to isolate bacteriophage against *Escherichia Coli* and characterize its numerous physiological and molecular traits to use it as the optimal phage for additional research with a broader perspective.

1.2 Bacteriophage

A virus known as a bacteriophage, or simply phage, replicates and infects bacteria. The word "phage" comes from the Greek word "phagein," which means "to devour." Phages are thought to be the most prevalent and genetically varied biological species on Earth; their estimated global population is between 10^{30} to 10^{32}. Since they are essential to preserving the microbial balance of our environment, they have a significant impact on it. Phages are omnipresent, existing in all-natural environments where their bacterial hosts are present, including both aquatic and terrestrial systems. More than 6,000 distinct phages have been recognized and given morphological descriptions. The phages that have been isolated thus far have been effectively employed to treat plant bacterial diseases in a variety of agricultural settings. It also established a strong basis for continuing aquaculture and livestock. In addition, the dentistry department also started using phages to remove bacterial contamination. (El-Shibiny & El-Sahhar, 2017).

As of late, scientists have been attempting to use phages as molecular tools for gene therapy, vaccine delivery, and the identification of particular bacterial species in environmental and clinical samples. But despite all of these uses, phages are an intriguing substitute antimicrobial agent in situations where chemically produced antibiotics might not work because of their

capacity to lyse particular bacterial cells, particularly those that are resistant to antibiotics, and to prevent or treat bacterial infections. Furthermore, it is estimated that bacteriophages eliminate 20–40% of marine bacteria daily, are essential to an ecosystem's nutrient and energy cycles, and comprise the most genetically diverse group of "life forms" on the planet. (Kasman & Porter, 2020)

1.2.1 Early History and Discovery of Bacteriophage

The Ganges and Jumna rivers in India have antibacterial activity against Vibrio cholera, according to a report by Ernst Hankin in 1896. Two years later, a Russian bacteriologist working with Bacillus subtilis reported a similar incident. Frederick Twort did not postulate that a virus could be the cause of this antibacterial activity until 1915. The British bacteriologist observed the development of "glassy" colonies of micrococci during his attempts to cultivate the Vaccinia virus on cell-free agar media. He came up with his theory after looking at those colonies under a microscope and seeing granules of bacterial degeneration**.** (El-Shibiny & El-Sahhar, 2017)

Unfortunately, Twort's work was put on hold due to funding shortages and the World War. The unfinished work of Twort recognized by a French-Canadian microbiologist Félix d'Hérelle, led to the discovery of an antagonistic microbe of the dysentery bacillus that was invisible. D'Hérelle declared, "In a flash, I had understood: what caused my clear spots was an invisible microbe, a virus parasitic on bacteria," so there was no doubt about the significance of his discovery. (Kutter & Sulakvelidze, 2004). The virus was given the name a bacteriophage, or bacteria-eater, by d'Hérelle. Researchers from all around the world have applied D's theory or research to new levels of phage research and conducted new studies in its stead. Phage therapy, which has the potential to be a groundbreaking scientific discovery of this century, is being developed and put into practice based on D'Hérelle's study.

Furthermore, Max Delbrück, Alfred Hershey, and Salvador Luria were granted the 1969 Nobel Prize in Physiology or Medicine for discovering the genetic structure and replication of viruses.

1.2.2 Structure of Bacteriophage

All phages share certain common characteristics or properties, even though their types vary depending on the type and group of bacteria they infect. Among these traits or attributes of bacteriophages are:

- Bacteriophages are extremely species-specific about their host cell, just like all other viruses. Only one species of bacteria, or even particular strains within a species, are infected by bacteriophages.
- All bacteriophages share the same fundamental structure. They are composed of a protein capsid enclosing a core of nuclear material.
- Three fundamental structural forms of bacteriophages are known to exist: the icosahedral head with a tail, the icosahedral head without a tail, and the filamentous form.
- Both DNA and RNA, which can both be single- or double-stranded, can be the genetic material, or nuclear material, of bacteriophages.
- As obligatory intracellular parasites, bacteriophages lie dormant outside of their host cells and depend on the host's cellular apparatus for their metabolic processes.
- Similar to bacteria, bacteriophages are categorized into various orders and families based on their genetic makeup and morphology. Among the families that are frequently researched are the Rudiviridae, Microviridae, Tectiviridae, and Inoviridae.

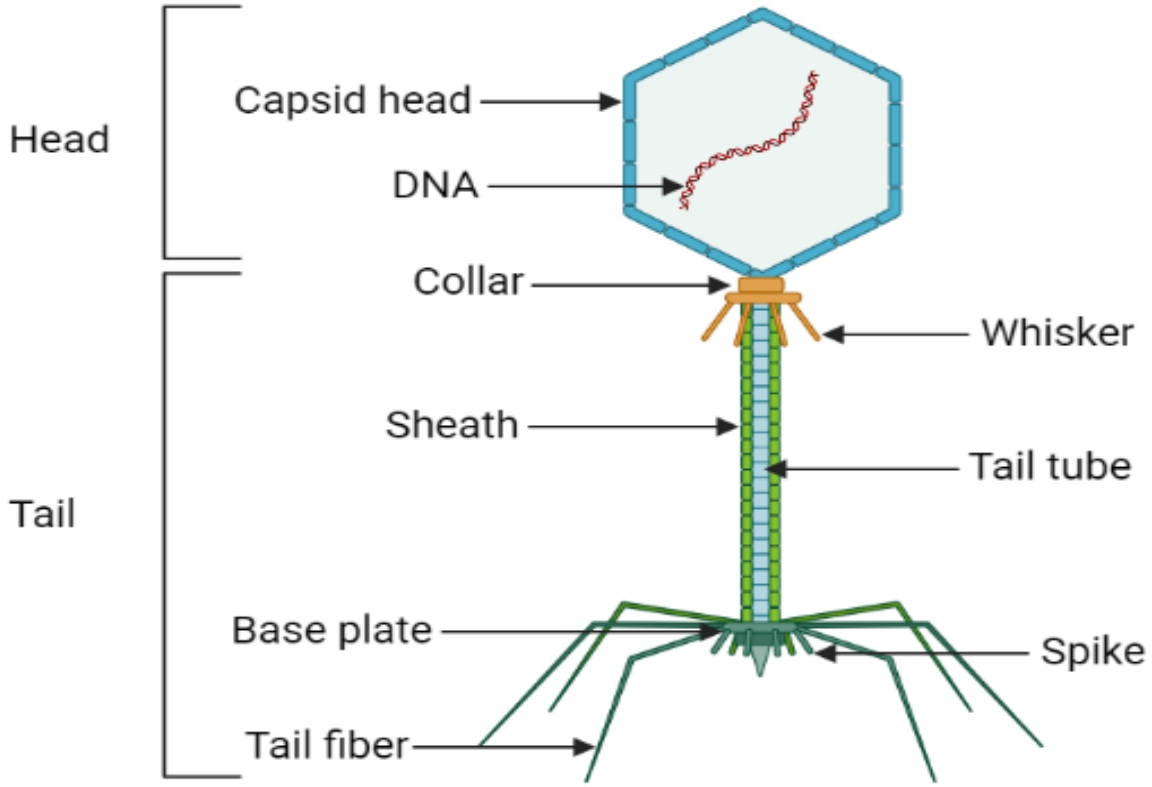


Figure 1: Structure of Bacteriophage (T4) (Life Cycle of Bacteriophage | Lytic Cycle | Lysogenic cycle | Induction, n.d)

1.2.3 Types and classifications of bacteriophage

Phage types can differ based on the mode of reproduction. For example, temperate and virulent phages

Virulence: Bacteriophages that reproduce via the lytic cycle, such as T-phages, are considered virulent.

Temperature: Temperate viruses, such as lambda virus, reproduce by using both cycles.

Bacteriophages are categorized according to their morphological features and amount of nucleic acid. Two of the 19 families of bacteriophages that have been discovered are made up of RNA bacteriophages. examples of the main families and traits of bacteriophages are shown below

T4 (*Escherichia* virus *T4*)	Myoviridae	Linear dsDNA, 169kbp long	Infects *E. coli.* Nonenveloped, Icosahedral head, tail is hollow with complex contractile structure and has tail fibers. It undergoes only a lytic life cycle, no lysogenic cycle.
T2 (*Enterobacteria phage T2*)	Myoviridae	Linear dsDNA, 170kbp	Infects *E. coli.* The famous Hershey and Chase experiment to prove that DNA is a genetic material was done with T2 phage infecting *E. coli.* Nonenveloped, icosahedral head and have a contractile tail. It undergoes a lytic life cycle.
λ (Coliphage, *Escherichia* virus lambda)	Siphoviridae	Linear dsDNA, 48502bp	Infects *E. coli.* The virus particle is made up of head, tail, and tail fibers. Nonenveloped and have a noncontractile long tail. It undergoes both lytic and lysogenic cycles.

			Commonly used as a vector in recombinant DNA technology.
M13 (*Escherichia virus M13*)	Inoviridae	Circular ssDNA, 6407 nucleotides long	Filamentous, nonenveloped. Infects *E. coli.* M13 plasmids are used in genetic engineering.
ΦX174 or phi x 174 (*Escherichia virus ΦX174*)	Microviridae	Circular ssDNA, 5386 nucleotides	Isometric and non-enveloped. Infects *E. coli.* It was the first DNA genome to be sequenced and also the first genome to be assembled *in vitro*. It encodes for 11 proteins. The transcriptome of ΦX174 was generated in 2020.

Qβ, MS2, etc.	Leviviridae	ssRNA, 3500-4200 nucleotides	They are nonenveloped with icosahedral or spherical geometry. It contains the '+' sense strand of RNA, which codes for four proteins, namely for coat, replicase, lysis, and maturation. Viral RNA works as mRNA and codes for phage proteins inside the host cell. They infect *Enterobacter, Pseudomonas, Acinetobacter,* etc.
Φ6, Φ7, Φ8, etc.	Cystoviridae	dsRNA, 14 kbp	They are enveloped with icosahedral or spherical geometry. They contain protein and lipid outer layers. They mostly infect *Pseudomonas* bacteria.

Table 1: Classification of Bacteriophage (Bacteriophage: Definition, Structure, Examples and Life Cycle, n.d)

1.2.4 Life cycles of bacteriophage

Viruses enter the host cell to reproduce, during which time they infect the host cell in various ways. The lifecycle of a virus is the complete sequence of events that includes its entry, replication, and exit from the host cell. Like other viruses, bacteriophages enter their bacterial host cell and then proceed similarly to replicate. There are two different kinds of lifecycles; in one, the viral DNA replicates independently of the host DNA, while in the other, the viral DNA is integrated into the host DNA. These lifecycles may transpire in various types of bacteriophages in an independent or alternative manner.

Lytic cycle: One of the two bacteriophage lifecycles, the lytic cycle, is characterized by the viral DNA replicating independently of the bacterial DNA while remaining a free-floating molecule. When virulent phages release their viral particles and cause the infected cell membrane to be destroyed, the lytic cycle typically takes place. Because the lytic cycle causes a cell to be destroyed, it is a virulent infection**.**

Attachment and penetration

- The first step in the lifecycle of a bacteriophage is attachment, where the ligands on specific molecules on the surface of the viral particles bind to the receptor molecules on the plasma membrane of the host cell.
- The receptors depend on the type of viruses as most orthomyxoviruses use receptors like terminal sialic acid on an oligosaccharide side chain of a cellular glycoprotein.
- The ligand, however, is an aperture at the distal end of each monomer of the trimeric viral hemagglutinin glycoprotein.
- Even though there is a high degree of specificity between the receptors and the ligands, several viruses might use the same receptors.
- Besides, some bacteriophages might use other membrane glycoproteins as their receptors.
- Once attached, the virus injects its nuclear material into the cytoplasm of the bacterial cell.
- The viral genome (either DNA or RNA) remains in the cytoplasm, and in some cases becomes circular and resembles the bacterial plasmid.
- The virus inserts its nuclear material into the bacterial cell's cytoplasm after attaching.
- The viral genome, which can be either DNA or RNA, stays in the cytoplasm and occasionally takes on the shape of a circular bacterial plasmid

Biosynthesis and transcription

- After entering the cytoplasm, the viral genome uses the host's cellular machinery as a means of replication.
- When a DNA virus is present, its DNA goes through transcription to create messenger RNA, which instructs the host cell's ribosome.
- When it comes to the lytic cycle, the mRNA codes for several polypeptides, the first of which annihilates the DNA of the host.
- An enzyme known as reverse transcriptase is involved in the transcription of viral RNA into DNA in the case of RNA viruses**.**
- After that, the DNA is transcribed back to mRNA, which instructs the host DNA to be destroyed.
- After seizing control of the host cell, the viral DNA produces various proteins needed for the assembly of new viruses.
- To create more genetic material for new viral particles, the viral DNA also replicates.
- Different genes and enzymes mediate the replication of DNA and the process of biosynthesis.

Assembly and lysis

- Numerous viral proteins and genomes are created as biosynthesis and replication proceed.
- The genetic material of the virus is integrated into the capsid, the viral protein, during the assembly process, which occurs once a sufficient number of virus particles have developed and matured.

- The lysin enzyme is released into the cytoplasm by the freshly assembled bacteriophages. Newly formed phage particles are released when the bacterial cell wall is lysed by the enzyme.
- Thus, the infected bacterial cell and cell membrane are destroyed at the end of the lytic lifecycle.

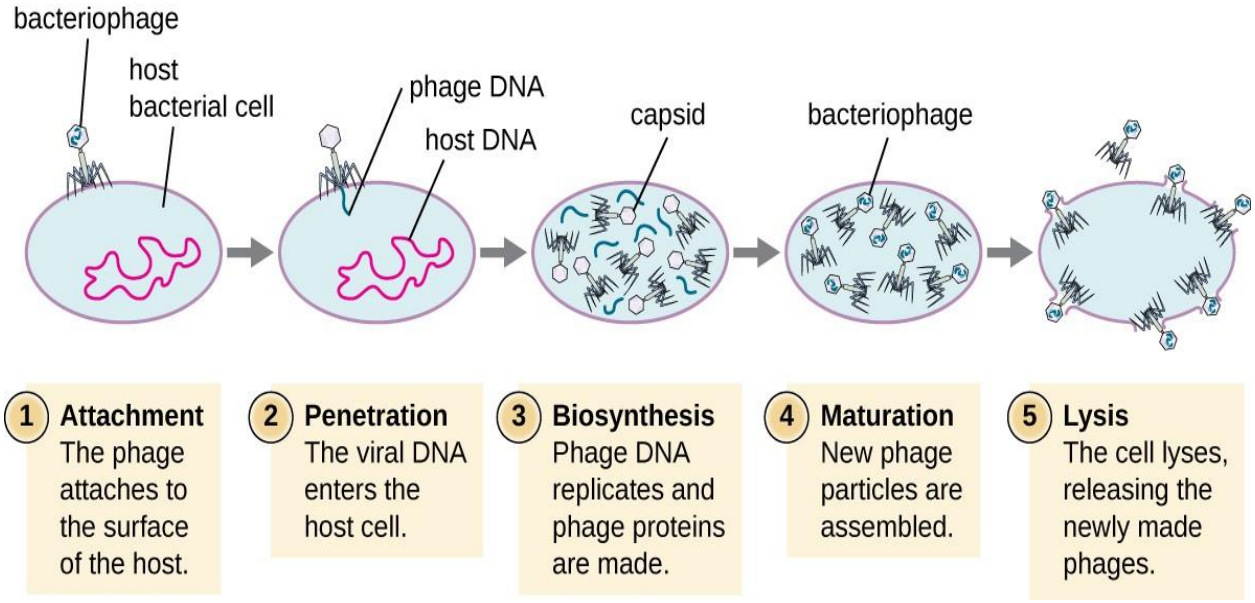


Figure 2: Lytic cycle of bacteriophage (The Viral Life Cycle | Microbiology, n.d.)

Lysogenic Cycle: One of the two bacteriophage lifecycles, known as lysogenic, is distinguished by the bacteriophage genome's integration with the host genome. Following the bacteriophage replication, the host bacteria continue to exist and proliferate normally during the lysogenic lifecycle. Prophages are bacteriophage genetic material that is integrated into bacterial DNA during the lysogenic lifecycle and can be passed on to daughter cells during bacterial cell division. Since the bacteriophage does not destroy the host cell, the lysogenic cycle is a mild and non-virulent infection.

Attachment and penetration

- There is no difference between the first stage of the lytic and lysogenic life cycles.
- The ligands of the bacteriophage bind to the surface receptors of the bacterial cell wall.
- The interaction between the ligands and the receptors on the surface of the bacterial cell wall determines the highly specific attachment.
- The viral genome is injected into the host cell's cytoplasm after attachment.
- After being integrated into the host chromosome, the infectious viral DNA or prophage changes from an infectious to a non-infective form

Replication

- During cell division, the viral DNA continues to replicate with the help of the host chromosomes by using the host machinery.
- The viral DNA may occasionally enter the lytic cycle and the prophage may be expelled from the host chromosome.
- In contrast to the lytic cycle, no viral protein is biosynthesized and the bacterial cellular mechanism is not taken over by the viral particles.
- However, during bacterial cell division, the prophage can be passed on to the daughter cells**.**
- Replication proceeds unabated until external factors, such as UV radiation, low nutritional status, or chemical stressors, occur. These factors have the potential to cause the lysogenic cycle to change into the lytic cycle.
- Viral proteins are produced by transcription of the viral DNA after it has been transformed into the lytic cycle. After the proteins and viral genome are put together, whole viral particles are created, which are lysis-released from the host cell.

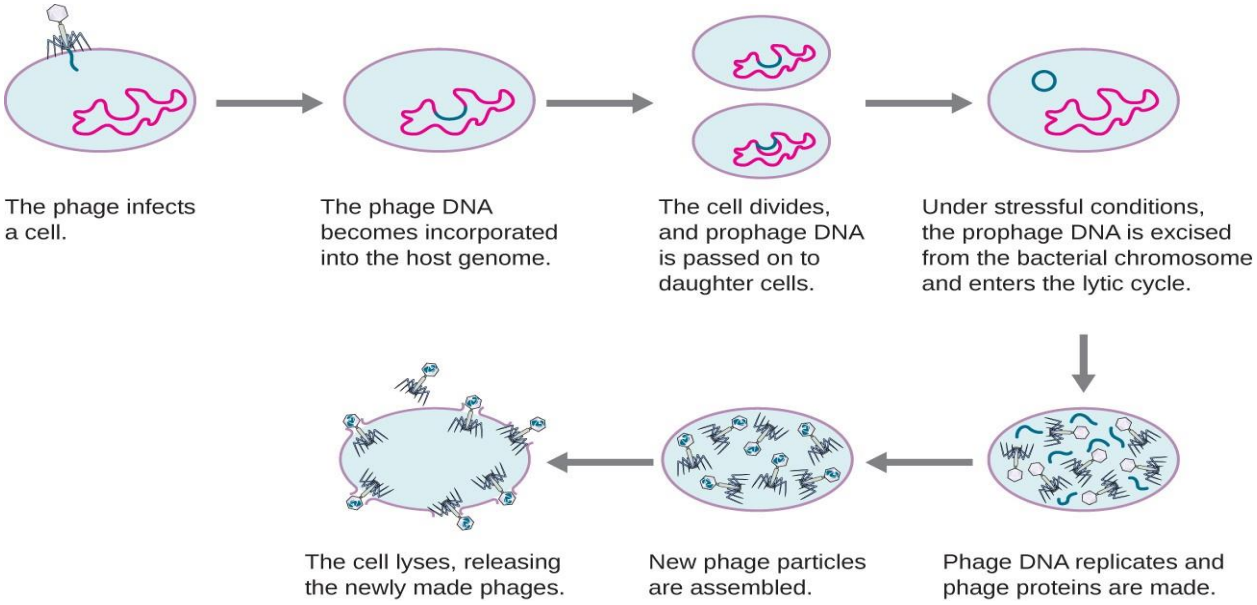


Figure 3: Lysogenic cycle of bacteriophage (The Viral Life Cycle | Microbiology, n.d.)

1.3 *Escherichia Coli (E. coli)*

One of the most well-known bacteria in the world, *Escherichia coli* is a species that contains a variety of bacterial strains. Numerous harmless strains of these bacteria can be found in nature and the gastrointestinal tracts of humans and other vertebrates. They aid in the absorption and digestion of food as well as the synthesis of vitamins K and B complex. On the other hand, infections caused by other strains can have detrimental effects. *E. coli* is crucial to the advancement of contemporary molecular biology and is frequently employed as a model organism in academic studies. Due to its rapid growth rates and genetic simplicity (*E. coli* contains only 4,400 genes, whereas human cells have approximately 25,000 genes), it is widely used in lab settings. The roundworm Caenorhabditis elegans, another model organism, is maintained in colonies because they are simple to establish and manage. *E. coli* has made significant contributions to our knowledge of DNA replication, genetic engineering, and bacterial physiology and genetics. On the other hand, Human intestinal and extraintestinal illnesses can also be caused by the gram-negative bacillus *Escherichia coli* or *E. coli*.

Numerous *E. coli* strains have been identified, and they can cause a wide range of illnesses, from mild, self-limiting gastroenteritis to renal failure and septic shock. Because of its virulence, *E. coli* can resist common antibiotics and evade host defenses. (*E. coli*, n.d.)

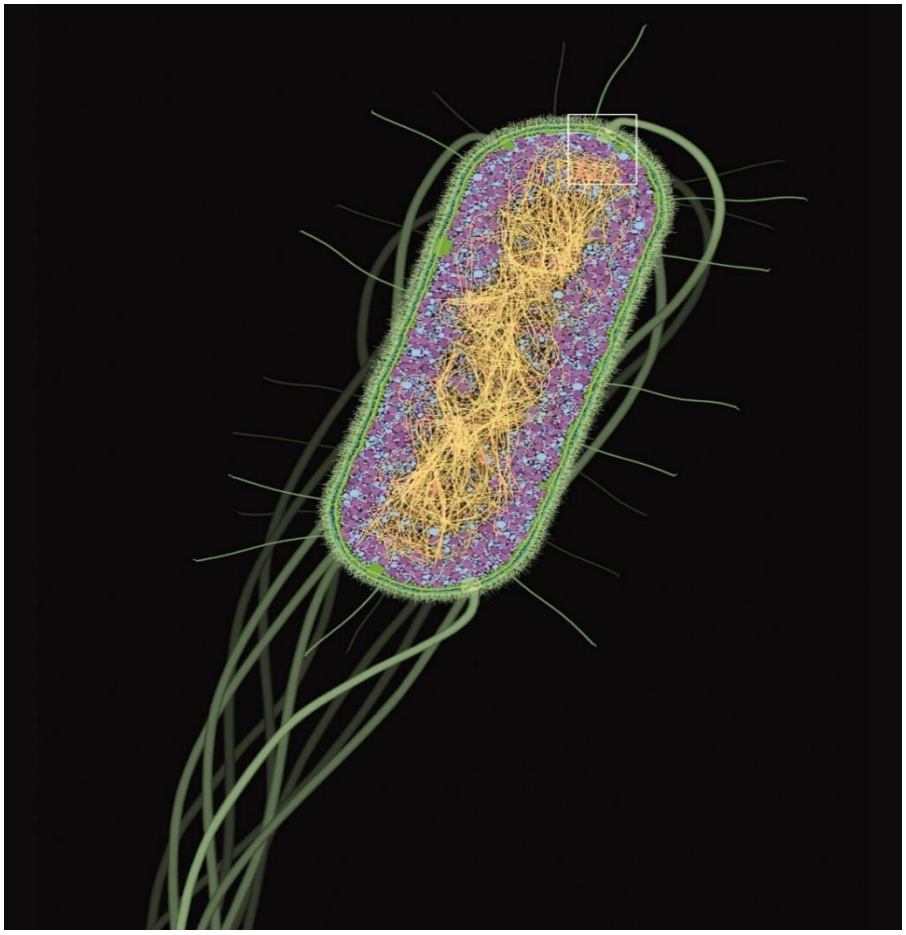


Figure 4: Section through a whole cell of Escherichia coli. The nucleoid is orange and yellow, the cytoplasm is blue and magenta, and the cell wall is green. (Goodsell, 2009)

1.3.1 Early History and Discovery of *E. coli*

German pediatrician Theodor Escherich (1857–1911) was a revolutionary physician who sought to enhance child care through an emphasis on nutrition and hygiene. While residing in Munich, Escherich started studying the bacteria in newborns' digestive tracts and how they alter after birth. He then used these findings to look into infant illnesses. He conducted research in labs, institutes, and even dairy industry facilities, learning how to characterize and culture

bacteria. He proved that within three to twenty-four hours of birth, an infant's environment could cause bacterial colonization. On July 14, 1885, Escherich made his formal presentation of his research on the *Bacterium coli* commune to the Society for Morphology and Physiology at this same time and gave it the name Bacterium coli commune. The term "bacterium" is a noun that is no longer in use, and "coli" refers to the fact that Escherich discovered the organism in the colon. Escherich received an offer to work as a professor and the director of a children's hospital in Graz, Austria, in 1890. He found that intestinal infections in infants are linked to coliform bacteria. Additionally, he pointed out that the presence of Bacterium coli commune in young girls' urine samples was common, which clarified the importance of urinary tract infections. Because of his achievements in Graz, Escherich was proposed in 1902 for the positions of Director of the St. Anna Children's Hospital in Vienna and Chair of Pediatrics at the University of Vienna. He established the Austrian Society for Children's Research and modernized the hospital while he was there. He is recognized today as the first pediatric infectious disease physician and an innovator in the field of pediatrics. To honor its discoverer, the bacterium was renamed *Escherichia coli* in 1919; however, this renaming was not formally approved until 1958. Numerous additional species belonging to the genus Escherichia are found within the gastrointestinal tracts of vertebrates. Most bacteria are known by their scientific names, but *E. coli* is so vital to human health and science that its name has also become widely used. (*E. coli*, n.d.)

1.3.2 Outbreaks of *E. coli around the world*

E. coli outbreak in Japan (1996)

The first outbreak of *E. coli* was found in Japan in 1996. About 10,000 cases of Escherichia coli O157:H7 infection linked to at least 14 distinct clusters were reported in Japan between May and August of 1996. The majority of cases involved school-age children. One cluster

included over 6,000 elementary school students in Sakai City, Osaka Prefecture, and involved a sizable outbreak. When the outbreak first occurred on July 13, 1996, radish sprouts were thought to be the most likely cause, according to an investigation. (Watanabe et al., n.d.)

E. coli outbreak in Germany (2011)

Then one of the biggest outbreaks of a food-borne illness caused by enterohaemorrhagic *Escherichia coli* (EHEC) serotype O104:H4 occurred in Germany during the summer of 2011. It is called the deadliest and second-largest outbreak. During a Shiga-toxin outbreak, more than 3000 cases of bloody diarrhea and gastroenteritis—including 810 cases of hemolytic uraemic syndrome—were reported. (Rogers, 2019) This high incidence of HUS cases in an outbreak caused by a food-borne pathogen has never been seen before. The EHEC O104:H4-related events that occurred in Germany during the summer of 2011 demonstrate strikingly how quickly an infectious agent can grow to pose a serious threat to national security. (Chaudhuri & Henderson, 2012)

E. coli outbreak in the United States

69% of *E. coli* outbreaks between 1998 and 2007 were linked to contaminated food, 18% to contaminated water, and 14% to contaminated animals or people. The Centers for Disease Control and Prevention (CDC) estimate that *E. coli* O157:H7 causes 95,400 of the estimated 265,000 E. coli infections that occur each year.

Year 2018: 235 cases of *E. coli* O157:H7 from Romaine lettuce and other leafy greens, 130 hospitalizations, and 6 deaths.

Year 2018: 18 cases of ground beef *E. coli* O26, 6 hospitalizations, and 1 death.

Other outbreaks include Cookie Dough (2009); Hazelnuts (2011); Sprouts (2016, 2014, 2012); SoyNut Butter (2017); Flour (2016); and Spinach (2012, 2006). (Nicholl, 2019)

***E. coli* outbreak in European Union**

5900 cases in 2014 (1663 in Germany, 1324 in the UK, and 919 in the Netherlands).

1616 Germany, 1328 UK, and 858 Netherlands cases totaling 5929 in 2015

635 Netherlands, 1367 UK, and 1843 Germany out of 6389 cases in 2016. (Nicholl, 2019)

***E. coli* outbreak in 2023**

In recent studies, numerous students at the University of Arkansas have reported symptoms of *E. coli* food poisoning and five of them require hospital treatment. Health officials are conducting investigations into this outbreak. Two 19-year-old sorority members who contracted the *E. coli* strain O157:H7 were among those impacted. They experienced a serious complication that could result in kidney failure. (Five People Hospitalized in *E. coli* Outbreak at the University of Arkansas, 2023)

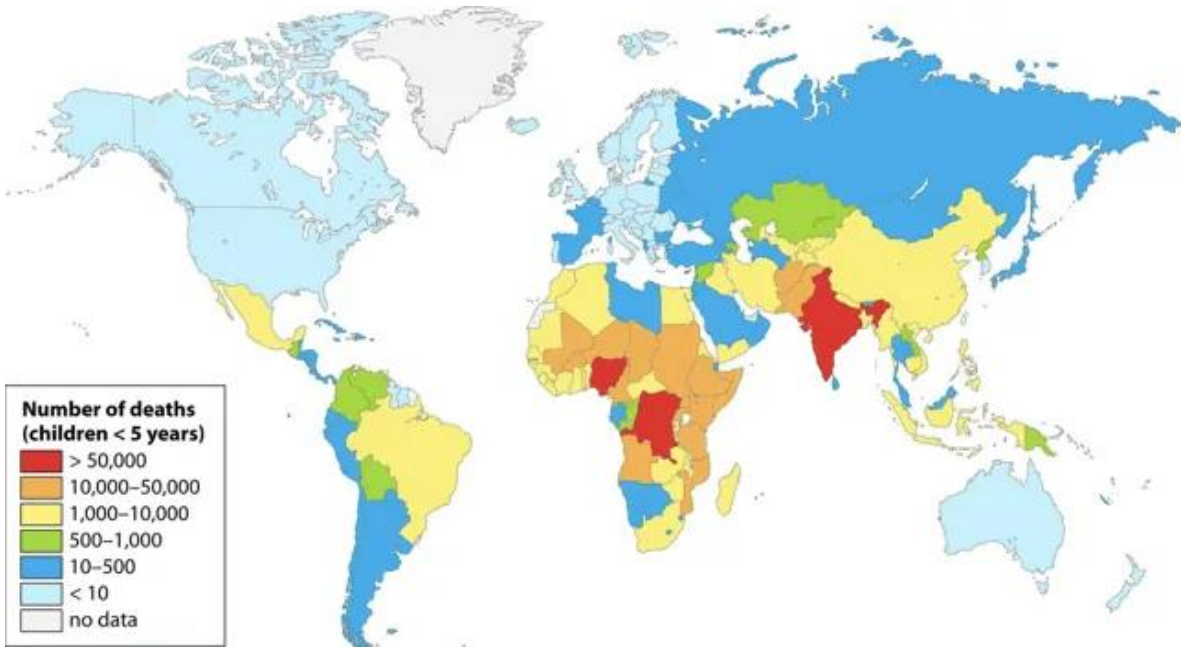


Figure 5: Major global outbreaks by the numbers around the world (Nicholl, 2019)

1.4 Bacteriophage Application

Bacteriophages have been given sufficient importance to be used as therapeutic agents due to their antibacterial capacity. However, interest in the therapeutic use of bacteriophages declined and for many years it was only thought of as a research tool in molecular biology due to the inadequate understanding of the biological mechanism of phage activity and the subsequent discovery and widespread application of broad-spectrum antibiotics in the late 1930s and 1940s**.**

Because of the rising trend in bacterial resistance and the development of modern biotechnology, which has made it possible to precisely evaluate the safety and effectiveness of phage use, bacteriophages have recently been given new consideration as antimicrobial tools. They are also being investigated for use in phage display technology, phage-derived vaccine development, biocontrol agents, and gene therapy delivery systems. (Gibb et al., 2021)

There are several applications for bacteriophages, which are thought to be safe for human use. Through genetic engineering or their life cycle, they have managed to remain one of the most broadly beneficial viruses to this day. The following are some applications for bacteriophages. (Author, 2022)

- production of vaccines
- Delivery of drugs, particularly with filamentous phages because of their size
- Using it as a biomarker
- Phage therapy (diseases caused by bacteria)
- keeping an eye on infections
- Identification of bacterial illnesses
- cleaning surfaces
- Bacteriophage as a biocontrol agent in food

- **Phage Therapy**: While there are some benefits to using bacteriophages as therapeutic agents instead of antibiotics, there are also some drawbacks. Phages have been used with variable degrees of success to treat humans, animals, and plants. Phages' host specificity is advantageous because it is less likely to disrupt the host's native flora. According to reports, phages spread quickly throughout the body after administration and get to the majority of organs. Nevertheless, because they contain lipid and/or protein structures, phages may provoke an immune reaction that causes them to be rapidly eliminated from the body. To get around the problem, more research can be conducted. Phage therapy has been used recently in the food industry and dentistry. (Lin et al., 2017)
- **Phage as vaccine vehicle:** There are two ways that bacteriophages have been used as vectors for vaccine delivery: 1) vaccination using phages that express vaccine antigens on their surface, and 2) insertion of a DNA vaccine expression cassette into the phage genome, which is then delivered by the phage particle. Target antigens in phage-display vaccinations can be produced by transcriptional fusion to coat proteins or by synthetically conjugating antigen protein to the phage surface, which allows for a broad range of antigen display. Because the phage coat protein protects the DNA vaccine more effectively and elicits a higher antibody response, it has been shown that unmodified phages deliver DNA vaccines more efficiently than standard DNA vaccine procedures**.** (González-Mora et al., 2020)
- **Nonclinical:** Bacteriophage treatments have been implemented across food production levels, in addition to their diverse clinical applications. Phage therapy, for instance, has been utilized in the veterinary care of animals that produce food to enhance food safety by lowering pathogens in fish and live animals. To control infections, phages have also been applied to meat in addition to fruits and vegetables. Large animals and poultry

with bacterial infections from various species, including *Salmonella*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium difficile*, *Escherichia coli*, and *Campylobacter*, have been effectively managed by them. They greatly lowered the colonization and horizontal transmission of Salmonella enterica. Bacteriophages have also been utilized as nanostructured material, to decontaminate food and surfaces, and as a method of identifying pathogenic bacteria in food and clinical samples. (El-Shibiny & El-Sahhar, 2017)

- **Antibiotic-resistant and Infection control:** The human microbiome's bacterial evolution is influenced by bacteriophages (Level II evidence). Antimicrobial medications and other forms of host cell stress can cause prophage to transition to the lytic replication cycle. As a result, it is reasonable to anticipate that antibiotic treatments, particularly those that target the gut microbial flora, will also affect the viral (phage) microbiome. Although they are more susceptible to UV inactivation, autoclaving, and routine hospital disinfection techniques, bacteriophages are not as easily inactivated as vegetative bacterial cells. (Kasman & Porter, 2020)
- **Bacterial Toxin Mediated Disease:** It is crucial for all members of the healthcare team tending to a patient suffering from a phage-borne toxin-mediated illness, like shigella or cholera, that the disinfection protocols employed are selected based on their capacity to deactivate viruses in addition to bacteria. The bacteriophages can facilitate the transfer of virulence genes from pathogenic to non-pathogenic bacterial strains, despite the fact that they do not directly infect human cells. (Kasman & Porter, 2020)
- **Bacteriophage as future savior:** If bacteriophages are shown to be effective, they can be a great substitute for antibiotics. Current research shows great potential for improving food safety, as evidenced by its promising results. Phages could be added to feed and drinking water to make it easier for poultry and animals to consume it. It has

been demonstrated that phages reduce the number of pathogenic bacteria in large animals and poultry before slaughter. They could also be added to food packaging materials as a sanitizer to clean the production line and as an immobilizer to increase the shelf life of food products. Phages can be encapsulated by drying under a vacuum after being adsorbed to the surface of skim milk powder, whey protein, and soy protein powder.

This improves its stability for various uses in human, veterinary, and agricultural medicine. The growth of the hosts on experimental meat was recently controlled using an immobilized cocktail of *E. coli* and L. monocytogenes phages on cellulose membranes; the outcomes were encouraging. (El-Shibiny & El-Sahhar, 2017)

Chapter 2

Methodology

2.1 Place of Research

The research was performed in the Biotechnology and Microbiology laboratory of the department of Mathematics and Natural Science, BRAC University, Dhaka, Bangladesh.

2.2 Location of the Sample collection

For performing research on raw meat and environment water, meat samples were collected from various butcher shops which are located in Mohammadpur, Dhanmondi, Mohakhali, Rampura and water samples were collected from Hatirjheel lake, Gudara Ghat, Gulshan-1 lake and Gulshan-2 lake.

2.3 Standard Laboratory exercises

During research, all plastic, steel and glass wares such as; falcons, Microcentrifuge tubes, vials, test-tube, conical flask, beakers, knife and chopping board which were used are washed nicely with tap water and soap once and rising with distilled water at the second to ensure no contamination. The medias (both agar & broth based) which were used for culture and tests, falcons, vials, test-tubes, microcentrifuge tubes and pipette tips were autoclaved at 121^{o}C at 15psi for 15min before use. Clean Lab coats and hand gloves were worn before conducting any experiment. For avoiding contamination, laminar were cleaned by using 70% ethanol before conducting experiment, spirit lamp was used during the experiment and fresh agar plates were incubated 24h before use.

2.4 Preparation of Reagent, Media and Solutions

For our research, we needed various medias and reagent for performing experiments. Those are as follows;

Luria-Bertani Broth (LB)

Luria-Bertani broth was mainly used in our project to enrich the bacteriophages and for making young culture of our bacteria which are used for Spot test & DLA test. LB contains a mixture of different components, which are; Tryptone, Yeast extract and Sodium Chloride. In 1 liter of distilled water, 10g of tryptone, 5g of yeast extract and 10g of NaCl was combined and heated at 100^0C temperature for proper mixture. Lastly, mixture was autoclave for 25 min at 120^0C. If there is a direct product where all the components are mixed together in powder form then follow the instruction of the product as how much gram of powder needed to make 1 liter of Luria-Bertani broth.

Luria-Bertani Agar (LA)

Luria-Bertani agar was used as our media plates for every tests. LA contains a mixture of same components as Luria-Bertani broth but with an additional mixture of agar. In 1 liter of distilled water, 10g of tryptone, 5g of yeast extract, 10g of NaCl and 15g of agar was combined and heated at 100^0C temperature for mixing properly. Mixture was then autoclaved for 25min at 120^0C. If there is a direct product where all the components are mixed together in powder form then follow the instruction of the product as how much gram of powder needed to make 1 liter of Luria-Bertani agar.

MacConkey Agar

MacConkey agar was used as media plates for only isolating *E. coli* from raw meat. MacConkey contains different varieties of components, which are; Peptone (Pancreatic digest of gelatin), Proteose peptone (meat and casein), Lactose monohydrate, Bile salts, Sodium

chloride, Neutral red, Crystal Violet and agar. In 1 liter of distilled water, 17g of peptone, 3g of proteose peptone, 10g lactose monohydrate, 1.5g of bile salts, 5g of NaCl, 0.03g of neutral red, 0.001g crystal violet and 13.5g of agar was combined and heated at 100^0C temperature for mixing properly. Mixture was autoclaved for 25min at 120^0C. If there is a direct product where all components are mixed together in powder form then follow the instruction of the product as how much gram of powder needed to make 1 liter of MacConkey agar.

Normal Saline

Normal saline was mainly used as a solution that helps to grind the raw meat. It helps to restructure proteins in meat and acts as binding & emulsifying agent. NS is composed of distilled water and sodium chloride. In 100ml of distilled water, 0.9g of NaCl is mixed and was autoclaved for 25min at 120^0C. Also, we have made 0.5%, 0.7% and 1.1% of saline which are used for salinity test.

SM buffer

SM buffer was used for routinely manipulation of phage suspension. It was also used for characterization process and storage of the phage. SM buffer contains sodium chloride (NaCl), magnesium sulfate (MgSO4), gelatin and Tris-HCl. In 100 ml of distilled water, 0.58g of NaCl, 0.20g of MgSO4, 0.1g of gelatin and 15.76g of Tris-HCl was combined and heated at 100°C for proper mixing. After the mixture, the media was autoclaved for 25min at 120°C so that if any bacteria do present in the media could be terminated.

Soft Agar

For performing Double Layer Assay (DLA) and spot test, 0.6% soft agar was used. In 1litre of distilled water, 10g of NaCl and tryptone, 5g of yeast extract and 6g of agar was combined and heated at 100°C for proper mixing. The mixed media was autoclaved for 25 minutes at 120°C, so that if any microorganism present in media, could be terminated properly.\

Eosin Methylene Blue (EMB)

The purpose of using EMB agar was to ensure about the collected bacteria whether it was *E. coli* or not. EMB contains peptone, lactose, di-potassium hydrogen phosphate, eosin (yellowish), methylene-blue and agar. For 1litre of distilled water, 10g of peptone and lactose, 2g of di-potassium hydrogen phosphate, 0.4g of yellowish eosin, 0.065g of methylene blue and 13.5g of agar was combined and heated at 100°C for proper mixing. Mixed media was autoclaved for 25 minutes at 120°C, so that if any microorganism present in media, could be terminated properly. If there is a direct product where all the components are mixed together in powder form then follow the instruction of the product as how much gram of powder needed to make 1 liter of EMB agar.

2.5 Isolation of *E. coli* from Raw meat

- A piece of raw meat weighting 25-50g which was collected from bazar and sliced into small pieces.
- The meat was grinded on a mortar-pastel by using normal saline (10-15ml).
- The liquid solution was collected to a falcon tube.
- 1ml from the liquid solution was taken for dilution (up to 10^{-5}) and the rest of the solution were needed for phage isolation.
- From each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) 100µL solution was taken and dropped onto MacConkey agar plates and used the spread plate technique to lawn the surface.
- The plates were incubated for 18-24h and result were observed.
- The dry pink (indication of *E. coli*) single colonies which grew on the plates were taken for subculture onto Luria Bertani agar (LA) plates. Later, these bacterial strains will be used for enrichment of bacteriophage.

By following the above process, 9 *E. coli* samples were collected and was named *ECM1.1*, *ECM1.3*, *ECM3.1*, *ECM3.3*, *ECM4.4*, *ECM5.1*, *ECM12.2.1*, *ECM12.2.2* and *ECM12.3*. Later, these *E. coli* samples were experimented for hosting bacteriophages.

2.6 Isolation of Bacteriophage from Raw meat and environment water

- The liquid solution which was collected from the grinded meat and the collected water sample both were centrifuged 3-4 times at 13500rpm for 15-20mins and then filtered using 0.22- or 0.2-micron filter.
- The sub cultured bacterial strains which are isolated from raw meat were inoculated into a falcon tube containing 8ml of Luria Bertani broth (LB) and incubated on a shaker incubator for 1.5-2h to make young culture.
- 1ml of that filtered liquid solution was added to the young culture and again incubated on a shaker incubator overnight for phage enrichment.
- Next day, the enriched solution was centrifuged at 13500rpm for 15-20min and filtered using 0.22- or 0.2-micron filter and kept into fridge at 4^0C.
- Spot test was conducted to ensure the presence of bacteriophage in the enriched solution.
- If there were plaque formed on to the plate then there would be presence of phage and so, phage were isolated from raw meat.

By following the above process, 5 phages were managed to be isolated from which 4 phages are from environment water and only 1 is from raw meat. The phages were named ECPW4.4, ECPW5.1, ECPW12.2.1, ECPW12.3 (First four were from environment water) and ECPM12.2.1 (Last one was from raw meat).

2.6.1 Spot Test

- Each sub cultured bacterial strain were young cultured in a vial containing 3ml of LB.
- 5ml of molten soft agar (55^{o}C temp) were taken into a test tube and 100µL of each young cultured bacterial strain were mixed with them.
- The bacteria mixed molten soft agar were poured and dried onto an LA plate.
- Onto each of those LA plates, 20µL of enriched solution was given as droplets and dried for 15-20mins.
- Incubate the plates for 10-12h and observe the result.
- If there was presence of plaque on the dropped zone of a plate, then there would be presence of phage which were specific for that bacterial strain. Therefore, cut the zone of plaque using backside of tips and put into a microcentrifuge tube (MCT) containing 500µL of SM buffer and 50µL of chloroform (Using 1:10 method).
- MCT was vortexed for 1-2mins straight and kept in the fridge at 4^{o}C.
- Next day, 8ml of bacterial young culture was prepared for that specific phage and 400µL of the phage lysate is added to it and left for 24h incubation.
- The enriched solution was centrifuge at 13500rpm for 15min and the supernatant was filtered with 0.22 filter and kept into fridge at 4^{o}C.

2.6.2 Double Layer Assay test (DLA)

- 100µL of enriched phage solution is added to 900µL of SM buffer/Normal saline in a MCT and dilute as desire.

- On 5ml of molten soft agar, 100µL of young cultured bacterial strain and 10µL of diluted phage was mixed with vortex machine and poured into an LA plate then dried for 20-25mins.
- The plates are incubated for 10-12h and after that, results are observed.
- Small plaques would be seen on to plate and as the number of dilutions increases, the number of plaques decreases.

2.6.3 Phage titer

- After conducting the DLA test, the results are checked and number of plaques which are formed on the plates were counted as per dilution.
- The number of plaques formed in each dilution is recorded in a hard or soft copy.
- By using the formula of phage titer that is shown below, the PFU/ml of a phage was determined.

  Titer (PFU/ml) = $\boldsymbol{Number\ of\ plaque\ (PFU)}$ **/ {dilution x volume of phage added to plate (ml)}**

2.7 Bacteriophage Characterization

On characterization of bacteriophage, three experiments were conducted, which are; Temperature test, Salinity test and pH test in order to observe whether out bacteriophages are tolerable or intolerable on high temperature, salinity, acidic and basic conditions.

2.7.1 Temperature test

In this test, the whole DLA test process was followed. The only difference was on the point, 200µL of SM buffer and 200µL of phage stock was added in MCT instead of 900µL of SM buffer and 100µL of phage stock. The MCT was then kept on the desired temperature which was; 25^{o}C and 50^{o}C. 25^{o}C is room temperature so, the MCT was kept on a room for 2h and for

50^{o}C, the MCT was kept on water bath for 2h. After the completion of 2-hour temperature test, the mixture of phage stock and SM buffer is diluted as desired. Lastly, the DLA test was conducted for the 3 temperatures and the number of plaques formed was recorded for each temperature.

2.7.2 Salinity test

In this test, 3 types of concentrated saline water are used, which were; 0.5% salinity, 0.7% salinity and 1.1% salinity. Firstly, by following the process of making normal saline, 0.5%, 0.7% and 1.1% of NaCl was added to each distilled water instead of 0.9% NaCl. After the completion of all the salinities, for each salinity, 200µL of saline was mixed with 200µL of phage stock in a MCT and kept in incubator for 2h. After the completion of 2-hour, the saline mixed phage was then diluted as desire and then process of DLA test conducted for each salinity and number of plaques formed was recorded for each concentrated saline.

2.7.3 pH test

In this test, 5 pH values were taken which were; 2,4,7,10 and 13. Firstly, SM buffer were made according to the process of making SM buffer. Furthermore, droplets of Glacial Acidic acid were given in order to make the buffer acidic and on the other hand, NaOH was given to make the buffer basic. After completion of the 5-buffer solutions, for each pH, 200µL of buffer was mixed with 200µL phage stock in an MCT. Moreover, the MCT was kept into incubator for 2h and after that, each buffer mixed phage is diluted as desire and DLA test was conducted. Lastly, after overnight incubation, result was checked and number of plaques was counted each pH buffer.

Chapter 3

Results

3.1 Isolation of *E. coli* from Raw meat

Out of 6 Raw-meat samples, 9 *E. coli* samples were collected and was named *ECM1.1*, *ECM1.3*, *ECM3.1*, *ECM3.3*, *ECM4.4*, *ECM5.1*, *ECM12.2.1*, *ECM12.2.2* and *ECM12.3*. Later, these *E. coli* samples were experimented for hosting bacteriophages. The details of 6 raw meat samples and their CFU counts are shown below;

Sample	**Date of Collection**	**Source**	**Growth of *E. coli***	**Colony forming unit per ml**
Sample-1: Meat(beef)	28th February 2023	Amtoli Kachabazar, Mohakhali	*E. coli* found in 10^{-1} (408 single colonies), 10^{-3} (2 single colonies)	CFU/ml for dilution 10^{-3} = $(2x10^{3})/0.1$ = $2x10^{4}$ CFU/ml
Sample-2: Meat(beef)	6th March 2023	Zakir Hossain road, Mohammadpur	No bacterial growth	-
Sample-3: Meat(beef)	13th March 2023	Wireless Kachabazar, Mohakhali	*E. coli* growth found in 10^{-1} (310 single colonies) and 10^{-3} (1 single colony)	CFU/ml for dilution 10^{-1} = $(310x10^{1})/0.1$ = $3.1x10^{4}$ CFU/ml

Sample-4: Meat(beef)	20th March 2023	Amtoli Kachabazar, Mohakhali	*E. coli* growth found in 10^{-3} (5 colonies)	CFU/ml for dilution 10^{-4} = $(5x10^4)/0.1$ = $5x10^5$ CFU/ml
Sample-5: Meat(beef)	3rd April 2023	Amtoli Kachabazar, Mohakhali	*E. coli* growth found in Raw(Numerous colonies) and in 10^{-1} (14 colonies).	CFU/ml for dilution 10^{-1} = $(14x10^1)/0.1$ = $1.4x10^3$ CFU/ml
Sample-12: Meat(beef)	12th June 2023	Butcher shop beside Arong, Lalmatia	*E. coli* growth found in 10^{-1} (Numerous colonies), 10^{-2} (14 colonies) and in 10^{-3} (1 colony)	CFU/ml for dilution 10^{-2} = $(14x10^2)/0.1$ = $1.4x10^4$ CFU/ml

Table 2: Isolation of E. coli from Raw meat and CFU per ml for each sample.

Also, the images of *E. coli* isolated from raw meat shown below;

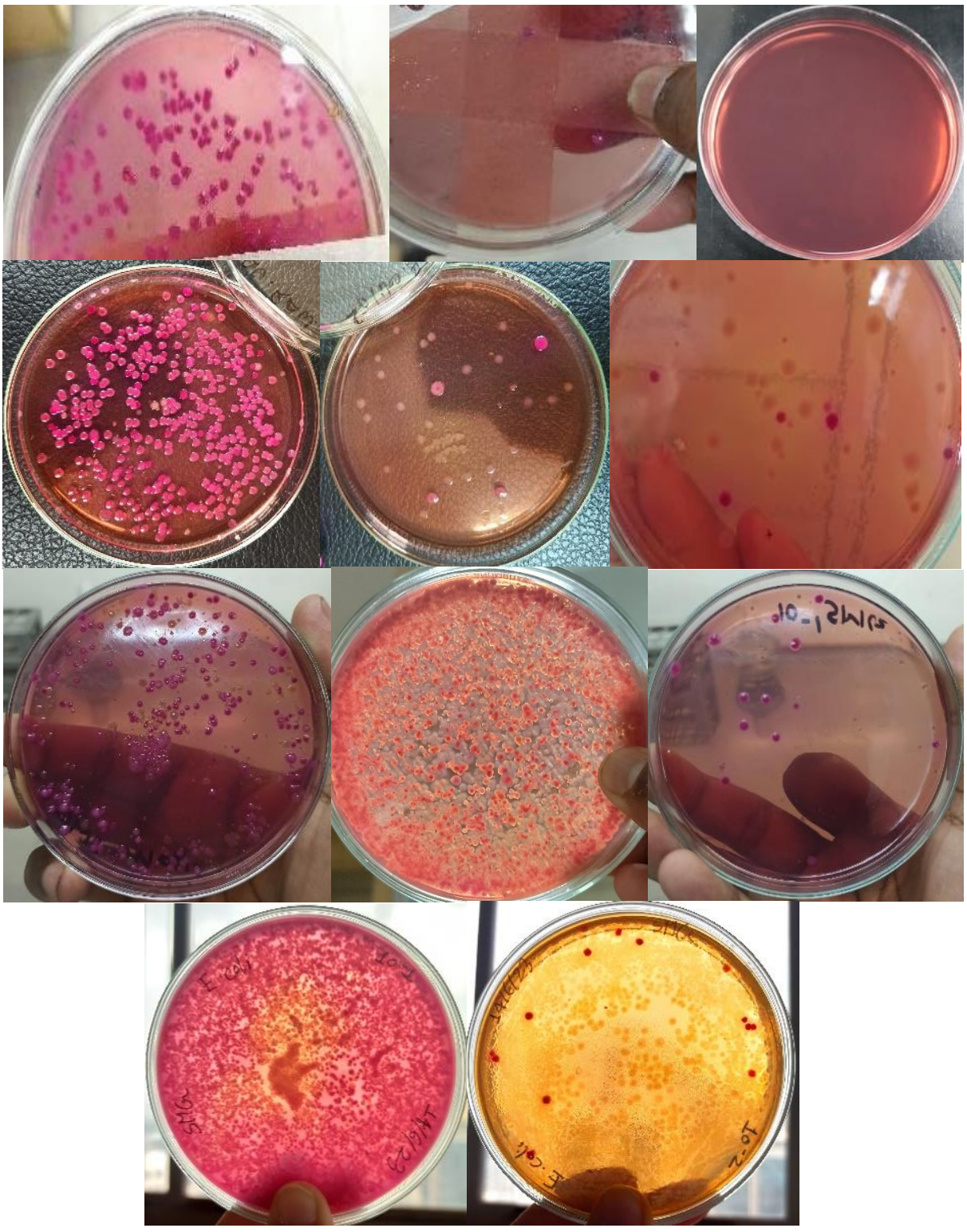


Figure 6: Isolated E. coli from Raw Meat.

3.1.1 Identification of *E. coli* on EMB agar

Identification of *E. coli* bacteria couldn't be fully determined by only using MacConkey agar, because unlike *E. coli*, there are more lactose-fermenting bacteria for MacConkey agar which are; *Klebsiella spp*, *Enterobacter*. Therefore, *E. coli* could be identified by using EMB agar because when *E. coli* bacteria grows into EMB, the color of the colony becomes a metallic green sheen. Here, a figure was shown below which indicates that the bacteria isolated from raw meat are none other than *E. coli*.

Figure 7: Four bacteria sample ECM4.4, ECM5.1, ECM12.2.1 and ECM12.3 are all identified as E. coli.

3.2 Isolation of Bacteriophage

3.2.1 Isolation of Bacteriophage from Raw meat

Out of ten samples of Raw Meat, only one phage was isolated from *E. coli* bacteria sample *ECM12.2.1*. The analysis was done by spot test. This phage was not taken for further analysis and characterization because of its abnormal growth in DLA test results.

Sample	**Date of Collection**	**Source**	**Bacteriophage isolated from *E. coli* sample**
Sample-4: Meat(beef)	20th March 2023	Amtoli Kachabazar, Mohakhali	-
Sample-5: Meat(beef)	3rd April 2023	Amtoli Kachabazar, Mohakhali	-
Sample-6: Meet(beef)	10th April 2023	Town-Hall Kachabazar, Mohammadpur	-
Sample-7: Meat(beef)	8th May 2023	Krishi-Market Kachabazar, Mohammadpur	-
Sample-8: Meat(beef)	15th May 2023	Mohanagar Project Kachabazar, Rampura	-
Sample-9: Meat(beef)	22nd May 2023	Lalmatia Kachabazar, Lalmatia	-
Sample-10: Meat(beef)	29th May 2023	Mohammadi Housing limited Kachabazar, Mohammadpur	-
Sample-11: Meat(beef)	5th June 2023	Amtoli Kachabazar, Mohakhali	-
Sample-12: Meat(beef)	12th June 2023	Butcher shop beside Arong, Lalmatia	*ECM12.2.1*

Sample-13: Meat(beef)	19th June 2023	Wireless Kachabazar, Mohakhali	-

Table 3: Bacteriophage isolated from Raw meat sample.

Bacteriophage which was isolated from *E. coli bacterial sample ECM12.2.1* was named as ECPM12.2.1. Also, a figure of the phage was shown to observe the structure of it.

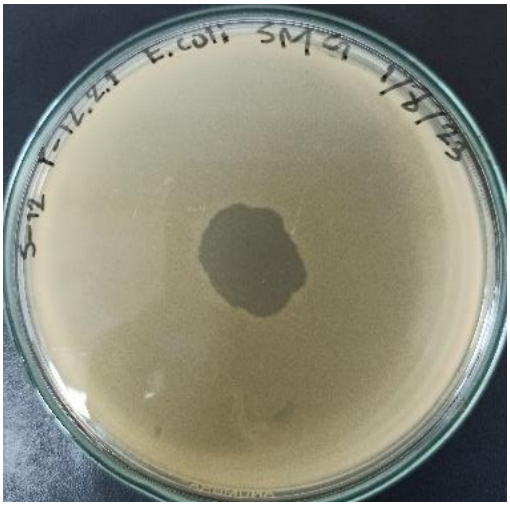


Figure 8: Plaque assay of phage ECPM12.2.1

3.2.2 Isolation of Bacteriophage from environment water

Out of three water samples, four bacteriophages were successfully isolated by using *E. coli* bacterial sample *ECM4.4*, *ECM5.1*, *ECM12.2.1* and *ECM12.3* from raw meat. These phages were isolated by following the spot test process. Later one was not used for further analysis and characterization because of abnormal growth in DLA tests but the other three phages were taken for further analysis and characterization.

Sample	**Date of collection**	**Source**	**Bacteriophage isolated from *E. coli* sample**
Sample-1: Water	5th August 2023	Hatirjheel lake	*ECM12.2.1*, *ECM12.3* & *ECM5.1*

Sample-2: Water	5th August 2023	Gudara Ghat	*ECM4.4*
Sample-3: Water	6th August 2023	Gulshan-1 lake	-

Table 4: Bacteriophage isolated from environment water by using bacteria from Raw meat.

The bacteriophages which were isolated from water by using *E. coli* sample *ECM4.4*, *ECM5.1*, *ECM12.2.1* & *ECM12.3* are named as ECPW4.4, ECPW5.1, ECPW12.2.1 & ECP12.3 respectively. Also, pictures of the phages are shown below to observe the structure of it.

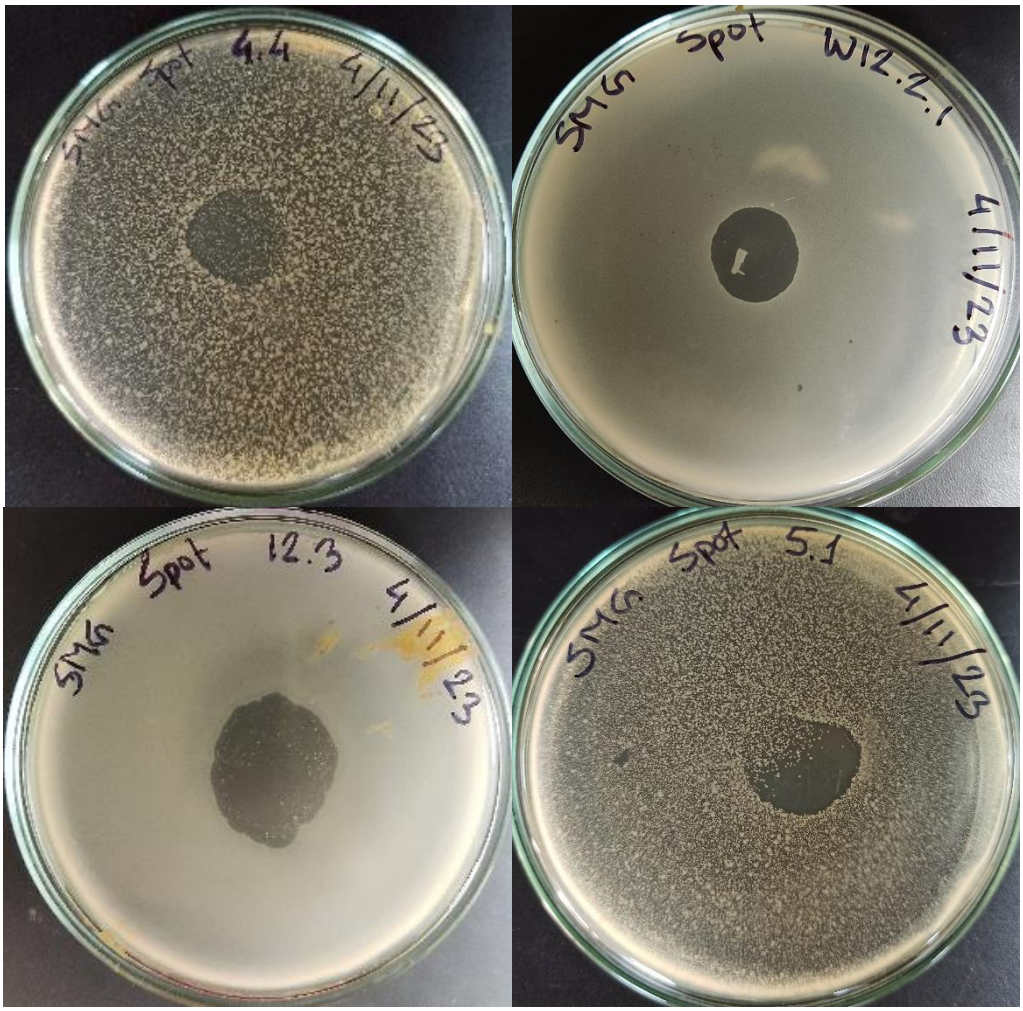


Figure 9: Plaque assay of phages ECPW4.4, ECPW5.1, ECPW12.2.1 & ECPW12.3.

3.3 Characterization

Characterization was conducted into three separate tests. First test was to check whether the bacteriophages are heat resistant for temperature test. Second test was to see if they could susceptible in more concentrated saline water and the last test was to see if the bacteriophages could withstand the high acidic solution and high basic solution. The whole three test was conducted with three phages, which were; ECPW4.4, ECPW5.1 and ECPW12.3 and their host bacteria ECM4.4, ECM5.1 and ECM12.3.

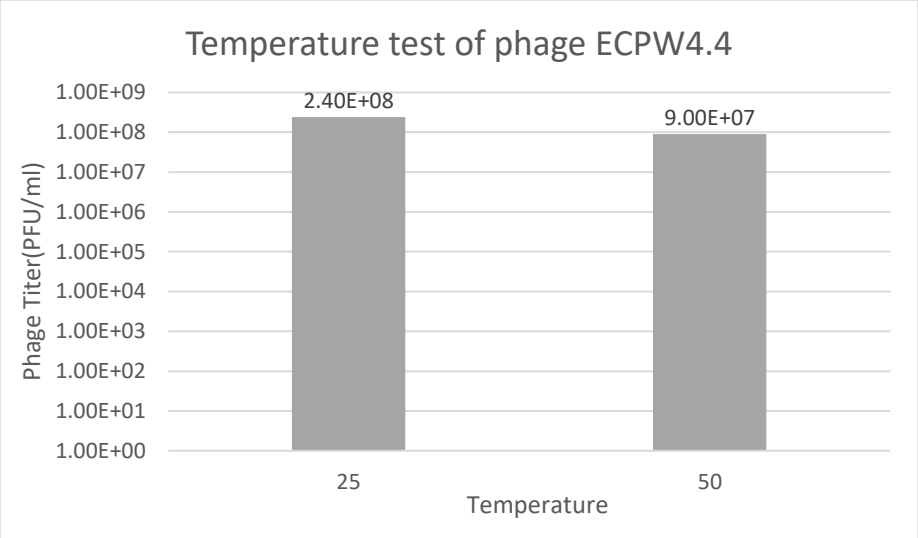
3.3.1 Temperature test

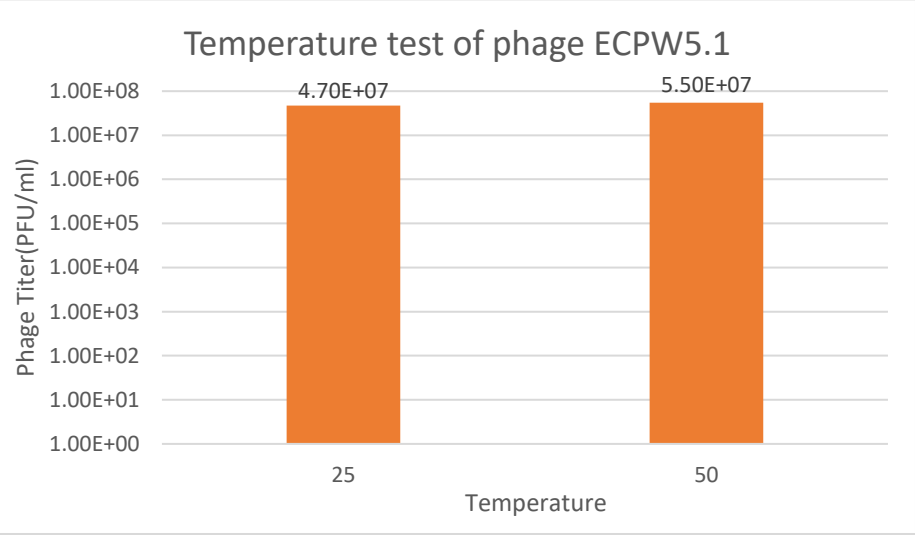
The temperature test was conducted in order to identify whether the three bacteriophages are resistance in room temperature (25^{o}C) and in 50^{o}C or not. The phage titer of the phages ECPW4.4, ECPW5.1 and ECPW12.3 in 25^{o}C and 50^{o}C are shown below;

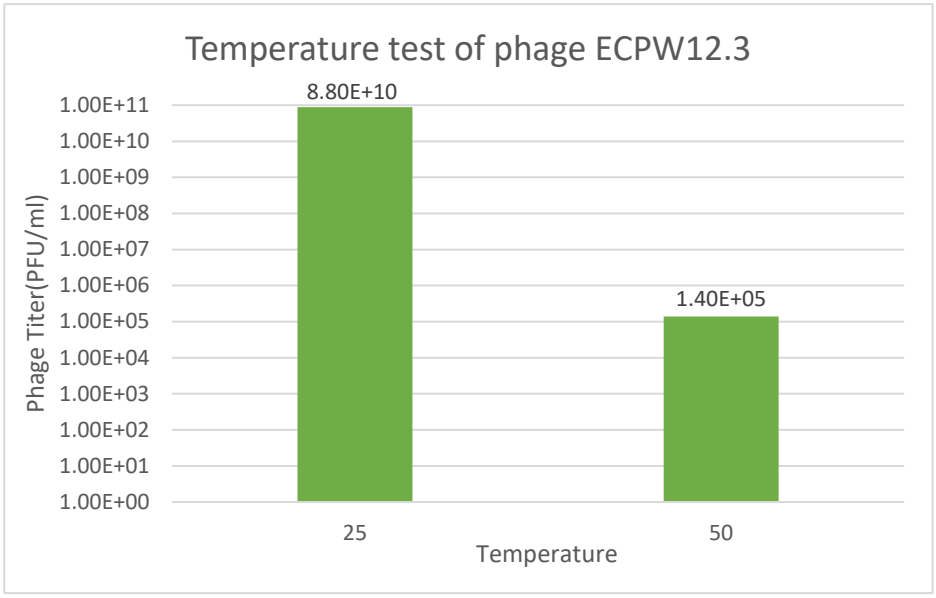
Temperature	**Phage ECPW4.4**	**Phage ECPW5.1**	**Phage ECPW12.3**
25^{o}C	$2.4x10^{8}$ PFU/ml	$4.7x10^{7}$ PFU/ml	$8.8x10^{10}$ PFU/ml
50^{o}C	$9x10^{7}$ PFU/ml	$5.5x10^{7}$ PFU/ml	$1.4x10^{5}$ PFU/ml

Table 5: Phage titer of Phages ECPW4.4, ECPW5.1 and ECPW12.3 in temperature test.

The temperature test graphs of the phages ECPW4.4, ECPW5.1 and ECPW12.3 and figure of the DLA results were shown below;







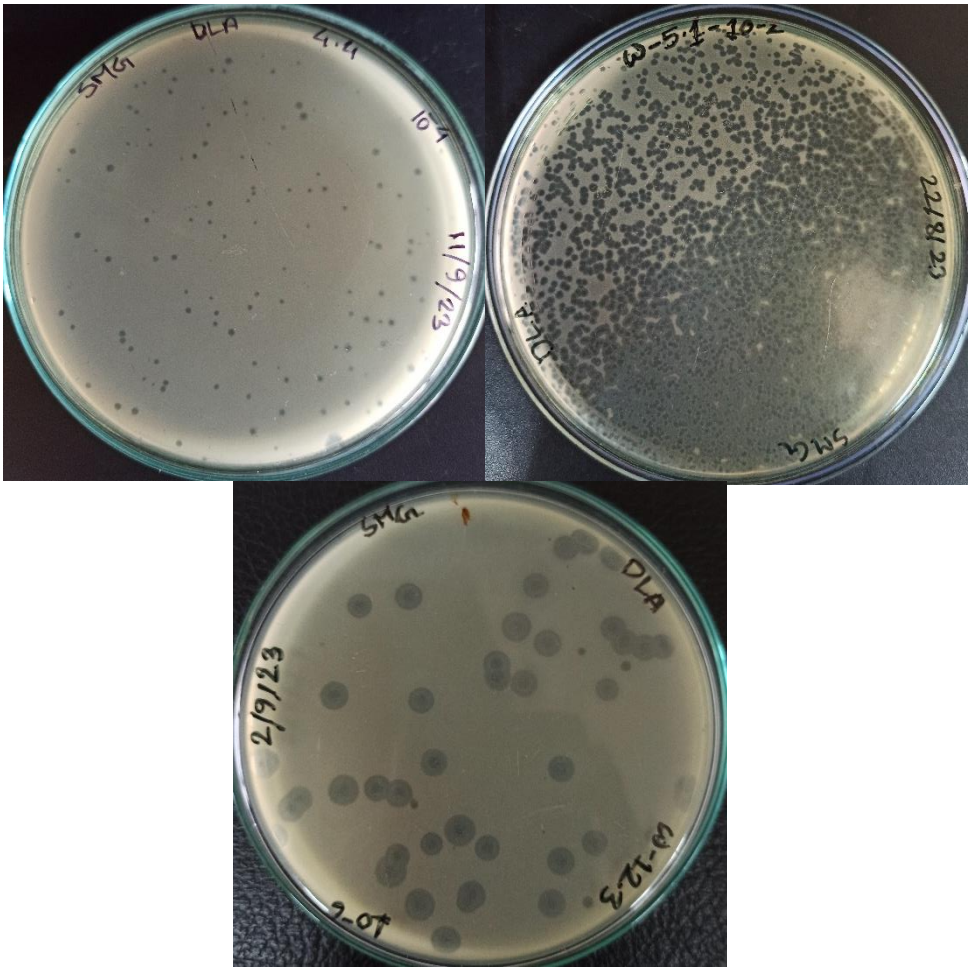


Figure 10: DLA of Temperature test of phages ECPW4.4, ECPW5.1 & ECPW12.3

Here, it was observed that, both ECPW4.4 and ECPW5.1 had a very slight change in phage titer from temperature 25^{o}C to 50^{o}C. On the other hand, phage titer of phage ECPW12.3 had gone down significantly from temperature 25^{o}C to 50^{o}C. Therefore, it could be said that, phage ECPW4.4 and ECPW5.1 were resistant to high temperature and ECPW12.3 was slight resistant to high temperature.

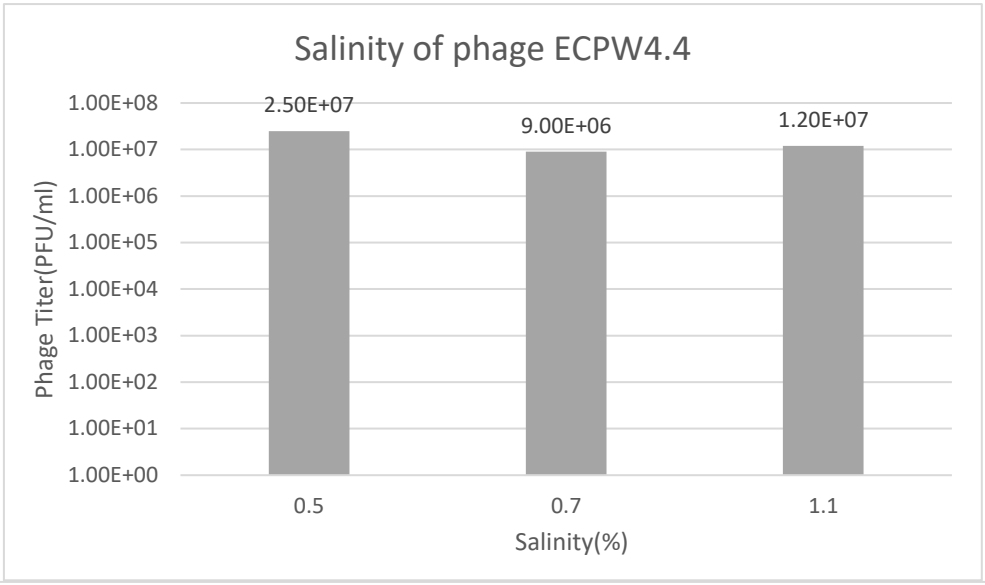
3.3.2 Salinity Test

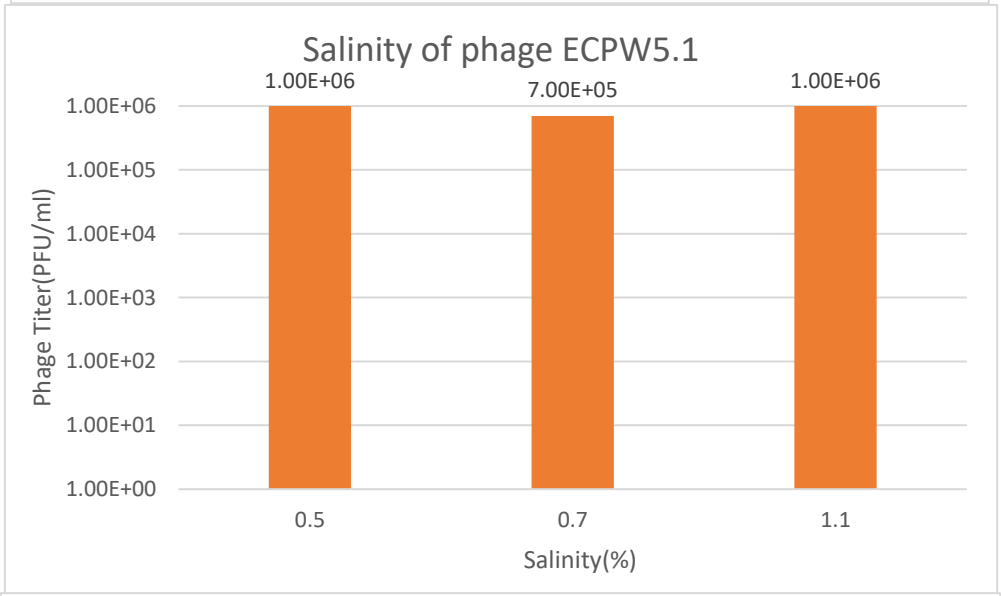
The salinity test was conducted to in order to identify whether the phages ECPW4.4, ECPW5.1 and ECPW12.3 can infect the host bacteria under a wide range of salinity condition. The range of the salinity were 0.5%, 0.7% and 1.1% respectively. The phage titer of the phages ECPW4.4, ECPW5.1 and ECPW12.3 in 0.5%, 0.7% and 1.1% salinity were shown below;

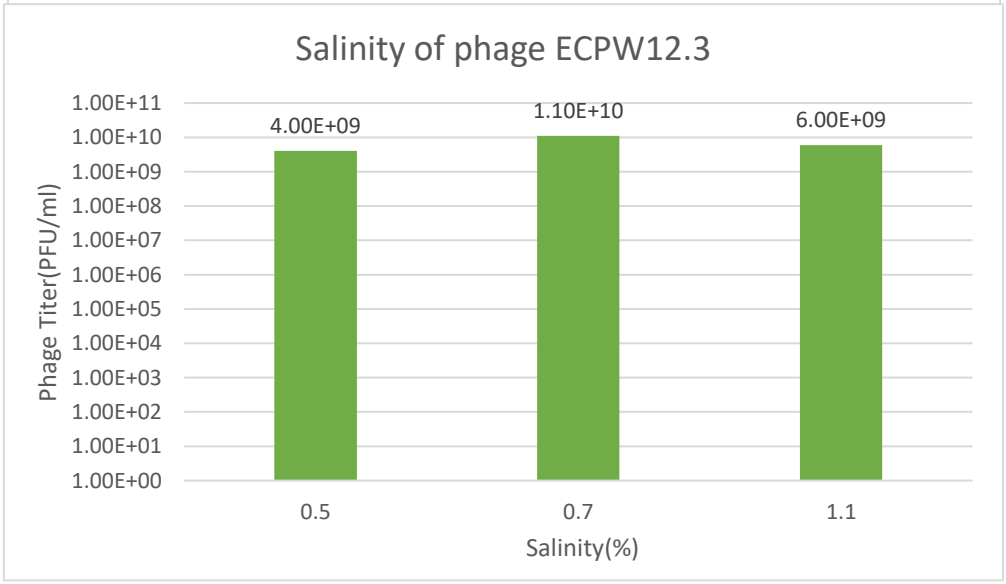
Salinity	**Phage ECPW4.4**	**Phage ECPW5.1**	**Phage ECPW12.3**
0.5%	$2.5x10^{7}$ PFU/ml	$1x10^{7}$ PFU/ml	$4x10^{9}$ PFU/ml
0.7%	$9x10^{6}$ PFU/ml	$7x10^{5}$ PFU/ml	$1.1x10^{10}$ PFU/ml
1.1%	$1.2x10^{7}$ PFU/ml	$1x10^{6}$ PFU/ml	$6x10^{9}$ PFU/ml

Table 6: Phage titer of phage ECPW4.4, ECPW5.1 & ECPW12.3 in 0.5%, 0.7% & 1.1% salinity.

The charts of salinity test and the DLA results of phages ECPW4.4, ECPW5.1 and ECPW12.3 are shown below;


Salinity of phage ECPW4.4
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Phage Titer(PFU/ml)
2.50E+07
9.00E+06
1.20E+07
0.5
0.7
1.1
Salinity(%)


Salinity of phage ECPW5.1
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Phage Titer(PFU/ml)
1.00E+06
7.00E+05
1.00E+06
0.5
0.7
1.1
Salinity(%)


Salinity of phage ECPW12.3
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Phage Titer(PFU/ml)
4.00E+09
1.10E+10
6.00E+09
0.5
0.7
1.1
Salinity(%)

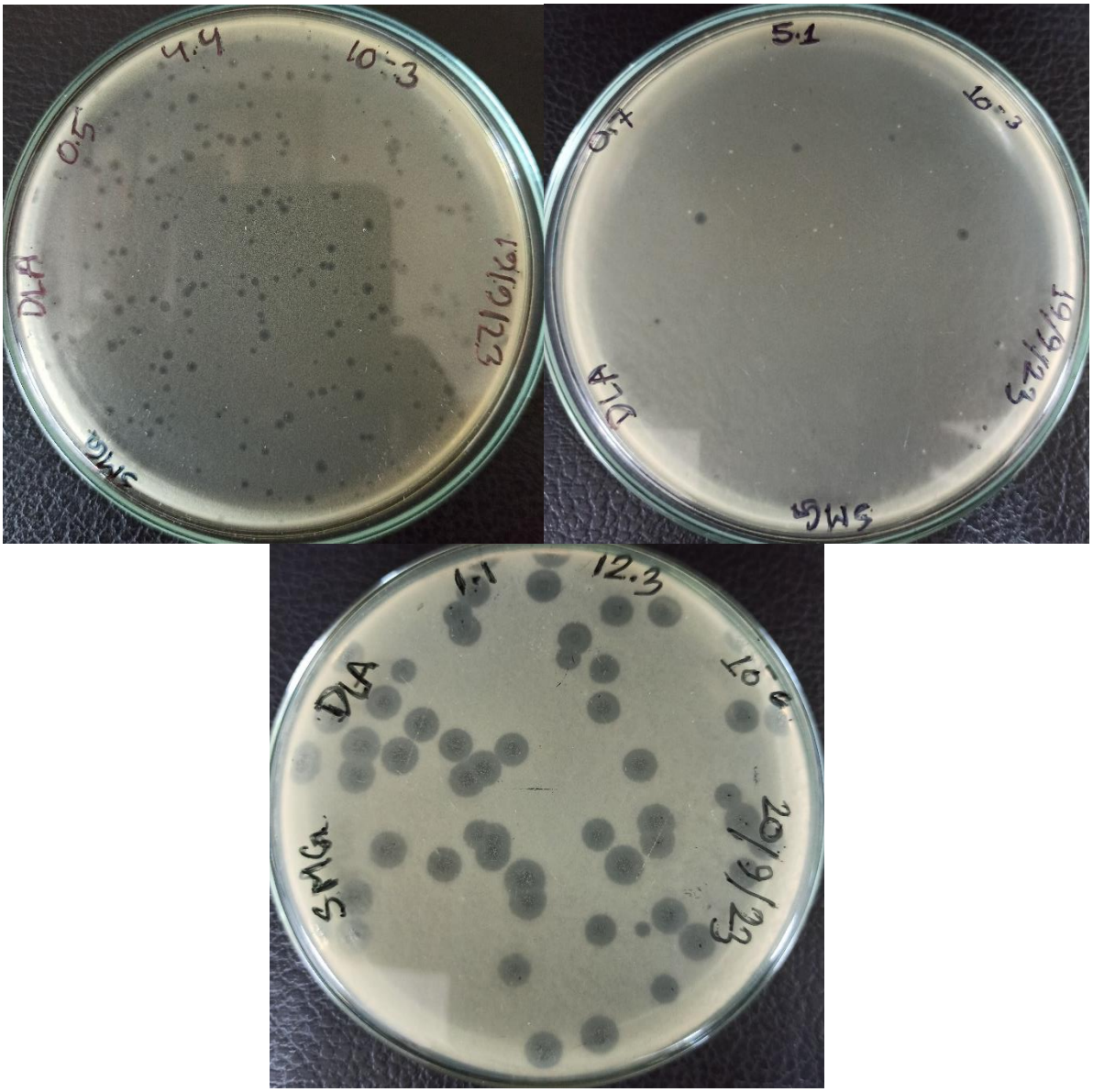


Figure 11: DLA of Salinity test of phages ECPW4.4, ECPW5.1 & ECPW12.3

Here, it was observed that, all three phages had a slight change of phage titer due to the change of salinity. Therefore, it could be said that all three phages are tolerable against the salinity test.

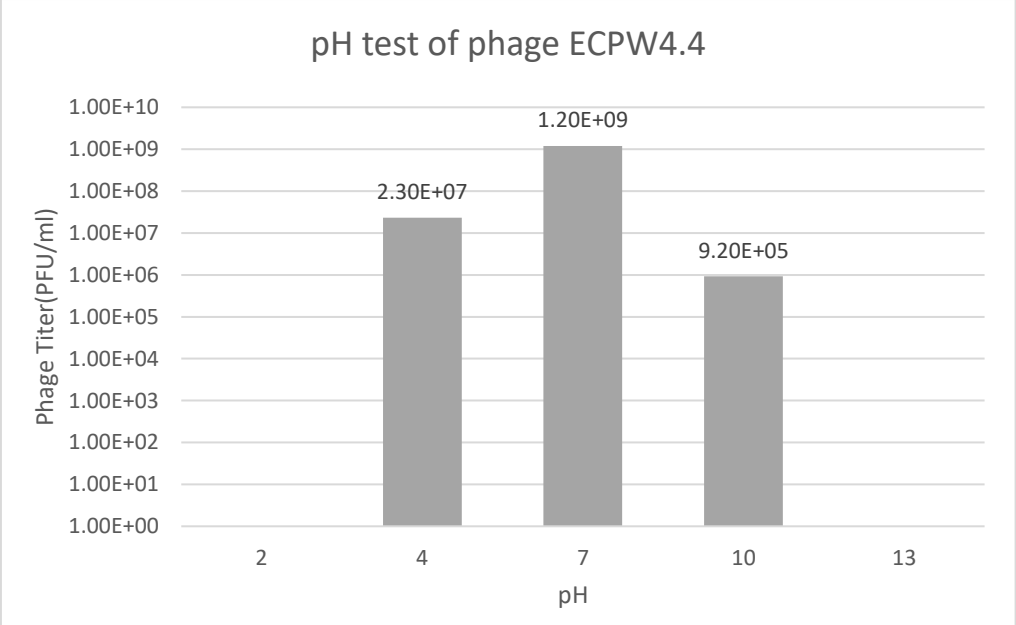
3.3.3 pH test

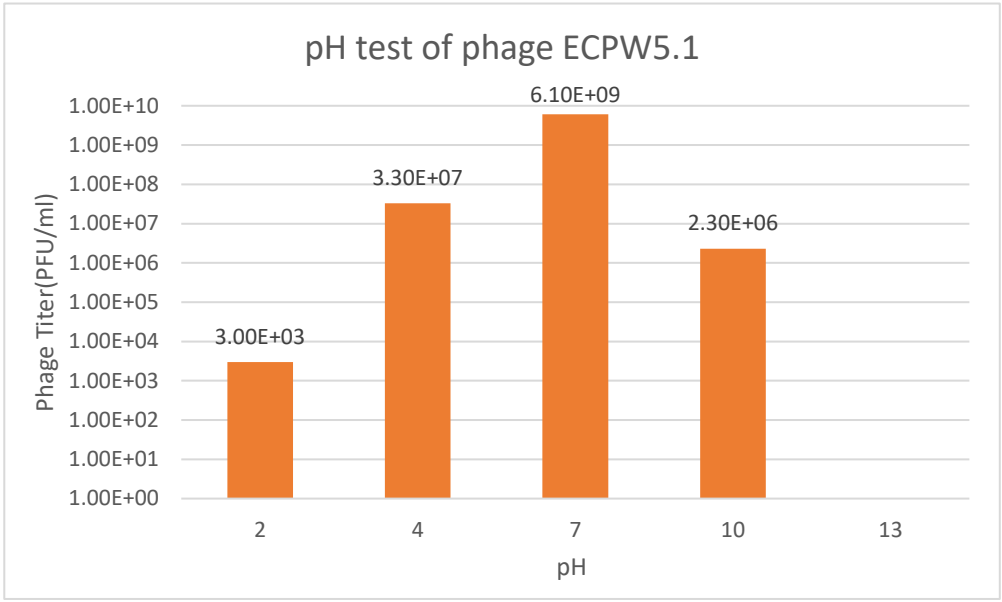
pH tests are conducted to determine the pH stability of a bacteriophage. From high acidic to high basic, 5 pH values are taken in this test, which are; pH2, pH4, pH7, pH10 and pH13. The phage titer of the phages ECPW4.4, ECPW5.1 and ECPW12.3 in pH2, pH4, pH7, pH10 and pH13 are shown below;

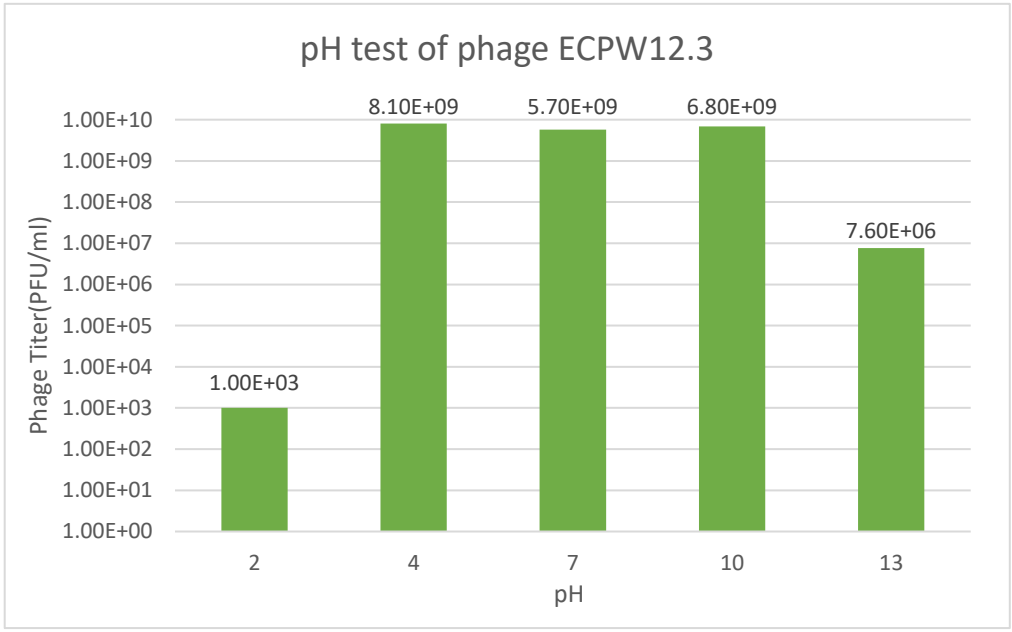
pH	Phage ECPW4.4	Phage ECPW5.1	Phage ECPW12.3
2	-	$3x10^3$ PFU/ml	$1x10^3$ PFU/ml
4	$2.3x10^7$ PFU/ml	$3.3x10^7$ PFU/ml	$8.1x10^9$ PFU/ml
7	$1.2x10^9$ PFU/ml	$6.1x10^9$ PFU/ml	$5.7x10^9$ PFU/ml
10	$9.2x10^5$ PFU/ml	$2.3x10^6$ PFU/ml	$6.8x10^9$ PFU/ml
13	-	-	$7.6x10^6$ PFU/ml

Table 7: Phage titer of phages ECPW4.4, ECPW5.1 & ECPW12.3 for pH2, pH4, pH7, pH10, pH13.

The charts of pH test and DLA result of the pH test of the phages ECPW4.4, ECPW5.1 and ECPW12.3 are shown below;




pH test of phage ECPW5.1
Phage Titer(PFU/ml)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
3.00E+03
3.30E+07
6.10E+09
2.30E+06
2
4
7
10
13
pH


pH test of phage ECPW12.3
Phage Titer(PFU/ml)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E+03
8.10E+09
5.70E+09
6.80E+09
7.60E+06
2
4
7
10
13
pH

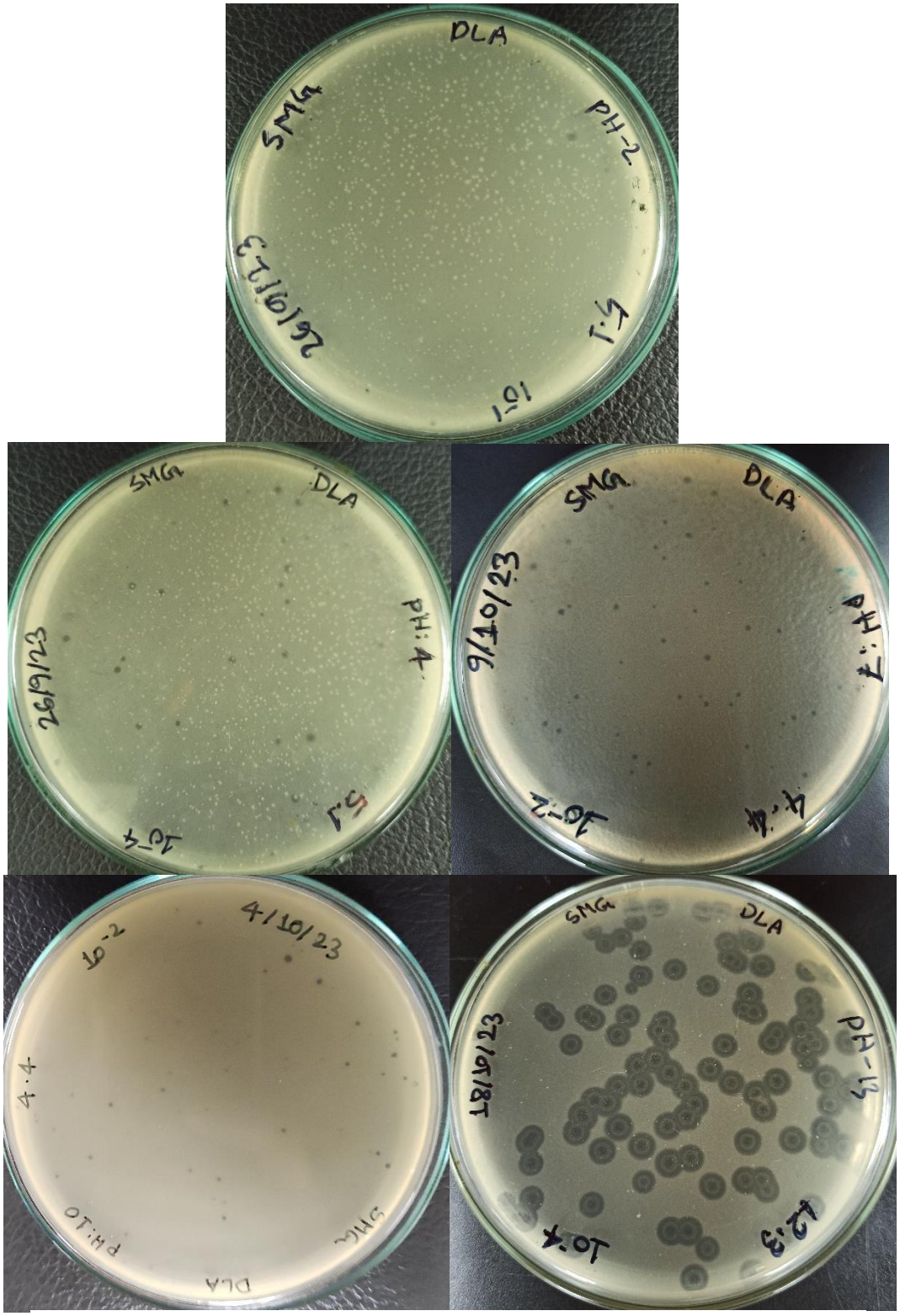


Figure 12: DLA result of pH test for phages ECPW4.4, ECPW5.1 & ECPW12.3

Here, it was observed that the phage ECPW4.4 doesn't have the pH stability in high acidic and high basic condition, even though it showed a significant phage titre on weak acidic, neutral and weak basic condition. Phage ECPW5.1 showed good phage titer result in high acidic, weak

acidic, neutral and weak basic condition but it lost its stability on the high basic condition. Lastly, phage ECPW12.3 was the overall pH stability because it showed phage titer growth in all the section from high acidic to high basic condition.

Overall, from all the characterization of the three phages, they were resistant, tolerable and stable in all conditions.

Chapter 4

Discussion

The aim of research was to isolate bacteriophage which had *E. coli* (isolated from raw meat) as their host and to characterize them in a way so that it can treated as ideal phage for further research.

4.1 Bacteriophage Isolation

Bacteriophage isolation was the hardest part of this research as it was too much difficult to isolate bacteriophage from meat sample. From 13 meat samples, only one bacteriophage was isolated from the 12th sample (ECPM12.2.1). Since only one bacteriophage was isolated from raw meat, more phages were required for continuing the research. Therefore, four more phages were isolated from Hatirjheel and Gudaraghat (ECPW4.4, ECPW5.1, ECPW12.2.1, ECPW12.3). Firstly, a little hazy zone had been occurred through the spot test from our phages. For this reason, regular enrichment was important to get the clear zone of our phages and also, to keep a good number of phages through routine enrichment. On the other hand, in order to make the phage count to maximum, regular phage enrichment was important. Moreover, for viral decay in aquatic environment, most important factor is sunlight, especially UV light (ultraviolet light) which can harm the genomic materials of phage to a limit which cannot be repaired. For this reason, phages which are stored in aquatic environment should be stored around 4°C.

4.2 Temperature stability

Another significant element that affects the stability of bacteriophages is temperature. The entire replication process, including attachment, penetration, and multiplication, is influenced by temperature, which is a sensitive factor. Phages participate less in the process of phage replication at suboptimal temperatures because fewer phages are able to integrate their genetic

material into the host, leading to an increasing number of phages being created. Furthermore, temperature controls bacteriophage viability, occurrence, and storage**.**

After conducting our experiment and 12 hours of incubation, Bacteriophage ECPW4.4 maintained at 25℃ demonstrated normal growth. On the other hand, the phage titer dropped by 62.5% after 12 hours of incubation at 50°C. So, we can say that ECPW4.4 phage is resistant in normal temperature whereas it is not resistant in higher temperature. Moreover, Bacteriophage ECPW5.1 is also resistant at 25°C whereas it is susceptible at 50°C, and phage titer has decreased 89.79%. Lastly, Bacteriophage ECPWW 12.3 also shows resistance at 25°C and susceptible at 50°C. But it has drastically decreased and the percentage is 199.99%

4.3 Salinity test

The main objective of Salinity test for bacteriophage is to assess the effect of salt concentration on the stability and activity of bacteriophage. Salt concentration have direct effect on bacteriophage. At higher salinity concentration, the bacteriophage activity appeared to be severely inhibited which suggests a salinity dependent effect on phage-bacteria interaction.

In the salinity test for bacteriophage ECPW4.4, it was observed that a clear dependence of bacteriophage activity on salt concentration. In 0.5% salinity, it was observed that, robust plaque formation indicating efficient infection and lysis of the bacterial lawn. However, as the salinity increased to 0.7% salinity, a significant reduction in plaque formation was observed which suggested higher salt concentration inhibited bacteriophage growth. But at 1.1% salt concentration, ECPW4.4 showed efficient infection.

In the salinity test for bacteriophage ECPW5.1, we observed the same result as ECPW4.4. However, in the salinity test for bacteriophage ECPW12. 3 a completely different result had been observed. In the control group (0.5% & 1.1% salinity) less bacteriophage plaque formation was observed compared to 0.7% saline concentration. At 0.7% salinity, it showed

robust plaque formation indicating efficient infection and lysis of the bacterial lawn. At 0.5% and 1.1% salinity it decreased to accordingly 63.63% and 45.45%.

4.4 pH test

The survivability of a phage, among many factors is also dependent on the acidity or alkalinity of the environment. The isolated phage was shown to have resistance and susceptibility from a pH range of 2 to 13.

Bacteriophage ECPW4.1 is only resistant at pH7. It shows a 99.99% survival rate. On the other hand, the survival rate was significantly decreased at pH4 and 10. But it could not survive in highly acidic and alkaline environments. (pH2 and pH13).

Bacteriophage ECPW5.1 is also resistant at pH7. The survival rate started decreasing in pH2, pH4 and pH10. The higher survival chance among these is pH4. But it could not survive in pH13. That means bacteriophage ECPW5.1 is susceptible in highly alkaline environments (pH13).

Bacteriophage ECPW12.3 can survive not only acidic but also alkaline environments. It has a 99.99% survival rate at pH4, pH7, pH10 but the phage titer decreased in pH2 and pH13.

4.5 Limitations

- The isolation of the phage should be in summer season but sudden weather change can be meant as a hindrance in the study.
- Keeping the bacteriophages active through enrichment is a lengthy process.
- It is important to use tips carefully while performing double layer assay.
- The usage of soft agar could be difficult sometimes as temperature should be maintained of soft agar for double layer assay as the temperature can kill both bacteria and phage.

- Due to the closure of BRAC University laboratory, further analysis and characterization for the bacteriophages were not possible. More tests like pathogenicity and PCR weren't performed and because of that, it wasn't proved that the phages are ideal for killing pathogenic *E. coli* bacteria strains.

4.6 Future aspects

- To understand the identity of the phages, the research of these isolated phages could be used to make phylogenetic tree of the phages.
- For phage therapy research, the isolated phages could give a high assurance of the phage therapy research as it can give multi drug resistance bacteria which could be on a high emergence.
- These phages could be useful for the research of biofilm degradation.

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