Bacteriophages isolation from different water samples

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
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Declaration

This has been declared that

1. The thesis submitted is my 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. I have acknowledged all main sources of help.

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Approval

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Ethics Statement

All human and animal rights were preserved and Laboratory safety manual was followed during this experiment.

Abstract

Bacteriophages are found almost everywhere in environment such as water, soil, air etc. Water is a necessity in daily life for different purposes and bacteriophages can be found plenty in number in daily used water. Isolation of bacteriophages has great impact on biotechnology, medicine industries as it can be used for identification of strains of bacteria which show counteraction against antibiotic drugs. Bacteriophage is a pack of viruses which as potential for antimicrobial treatment yet it is in clinical trial phage, it has made progress in medicine science. This project work on the isolation of bacteriophage from collected water samples.

Keywords: Bacteriophage; Antibiotic resistant; Anti-microbial treatment; Bacteria; Isolation; Biotechnology

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PT Phage Treatment

AMR Anti-Microbial Resistance

JAMA The Journal of the American Medical Association

Chapter 1

Introduction

1.1 Bacteriophage

Bacteriophages are common and found plenty in number in nature. They are known as natural predator of bacterial species, which are used vastly in biotechnology industry, petroleum industry, agricultural industry etc. These bacterial virus have potential for using in vaccine to detect antibiotic resistant bacterial strains for therapeutic and experimental reasons (Ul Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012). Moreover, Bacteriophage therapy may describe on the point of a potential weapon against Anti-microbial Resistance (AMR) that can be applied for treating diseases caused by antibiotic resistant bacteria. Furthermore, currently, *JAMA, The Lancet* has conferred about the advancement in PT (Phage Treatment) which has drawn attention in expanding Phage Treatment in similar with growing AMR threat in humankind; when phage treatment through Intravenous administration in independent samples became successful in USA and Belgium (Górski et al., 2018).

1.2 Structure of Bacteriophage

Bacteriophages are bacterial viruses with either DNA or RNA inside their encapsidated body, which is coated with protein. Phages have polyhedral capsid except filamentous phages. Bacteriophage like T_2phage contains a tail, which has no DNA or RNA inside; is used for the attachment with rector of bacterial cell and with tail they insert their genetic material into bacterial cells (Ul Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012). Coat of the virus not only protects it's nucleic acid from surrounding environment but also performs various roles such as; prevents pentosnucleic acid formation, avoid sebsequence infection created by other viruses etc (Roger, Am, & Barlow, 1957). Moreover, there is body part called collar found below the capsid or upper part of the body and base plates , tail fibres are found in lower part of the body of bacteriophages (Hossain, Azmol, Rahman & Islam, 2012).Bacteriophage is used as a model for studying virus genome, replication cycle and so on which significantlly helps to enrich molecular biology studies. For example; identification of genetic component that helps to code differnt genetic components found in virus etc. In addition, more than 10^{31} phages are found on earth which are commonly used they the scientists to indicate the importance of bacteriophage study(Navarro & Muniesa, 2017).

1.3 Isolation

Bacteriophages are bacterial virus, which can only proliferate inside bacterial cells; thus they are found lots of in number in sewage, animal wastes, rivers etc. Moreover, different types of bacteriophages are found in environment. According to experimental protocols, certain bacteriophages are isolated from their representative resources using possible

methods(Shende, Hirpurkar, Sannat, Rawat, & Pandey, 2017). For isolation purpose, in this experiment specific bacterial strain and its reference bacterial strain are cultured in different medium and bacteriophages are isolated from river or pond water samples through using various methods which will be described in methodology (Pelzek, Schuch, Schmitz, & Fischetti, 2013)

1.4 Classification of Bacteriophage

Bacteriophages have nucleic acid capsidated with protein coat and a tail to stick on bacterial cell. Classification of bacteriophages depends on its morphology, nucleic acid such as dsDNA(most common) ssDNA, ssRNA etc (Ackermann, 2009). Classifications of Bacteriophages are showed below:

Table 1: Classification of bacteriophages -

Shape	Nucleic acid	Family	Characteristics	Example
Tailed	**dsDNA (L)**	***Myoviridae***	**Tail, contractile**	**T4**
		Siphoviridae	**Tail, long, noncontractile**	**Lambda**
		Podoviridae	**Tail, short**	**T7**
Polyhedral	**ssDNA (C)**	***Microviridae***	**Conspicuous copsomers**	**Ø-X174**
	dsDNA (C,S)	***Corticoviridae***	**Complex double-capsid, lipids**	**PM2**
	dsDNA (L)	***Tectiviridae***	**Double-capsid, pseudo-tail, lipids**	**PRD1**
	dsDNA (L)	**SH1**	**Double-capsid, lipids**	**SHI**
	dsDNA (C)	**STIV**	**Turret Shaped Protrusions**	**STIV**
	ssRNA(L) dssRNA	***Leviviridae***	**Poliovirus-like**	**MS2**

	(L,M)	***Cystroviridae***	**Envelope, lipids**	**Ø-6**
Filamentous	**ssDNA (C)** **dsDNA (L)** **dsDNA (L)**	***Inoviridae*** ***Lipothrixviridae*** ***Rudiviridae***	**Long filaments or short rodes** **Envelope, lipids** **Stiff rods, no envelop, no lipids**	**M13** **TTV1** **SIRv-!**
Pleomorphic	**dsDNA (C,S)** **dsDNA (C,S)** **dsDNA (L,S)** **dsDNA (C,S)** **dsDNA (L)** **dsDNA (C)** **dsDNA (L)**	***Plasmaviridae*** ***Fusselloviridae*** **No name, genus *Salterprovirus*** ***Guttaviridae*** ***Ampullaviridae*** ***Bicaudaviridae*** ***Globuloviridae***	**Envelope, no capsid, lipids** **Lemon-shaped, envelope, lipids (?)** **Lemon-shaped, envelope** **Droplet-shaped** **Bottle-shaped, helical NC** **Two-tailed, helical NC** **Envelope, helical NC, spherical**	**L2** **SSV-1** **His1** **SNDV** **ABV** **ATV** **PSV**

(Pelzek et al., 2013).

1.5 *Pseudomonas putida* and Resistant *Pseudomonus putida*

*Pseudomonus pudtida*is a gram-negative bacteria of Pseudomonus species of fluorescent group found everywhere in environment for example-*Acinetobacter*, *Stenotrophomonus* species etc. In past, this bacteria was considered to have low pathogenicity. Nevertheless, in past few years it has been reported that *Pseudomonus putida* has caused nosocomical infection among the patients specially immunosuppressant patients and patients who use medical machineries by absorbing moist from environment and colonized on various hospital exterior. This bacteria is multi-drug resistant bacteria which can outbreaks in body fluid along with polluted liquid in human (Kim, Park, Park, Park, & Kim, 2012). Furthermore, majority of *Pseudomonus putida*are vulnerable to carbapenems, fluroquilones, aminoglycosides; unlike *Pseudomonu sputida,* which results in metallo β-lactameses, shows resistance to carbanems, ciprofloxacin, gentamycin etc. Moreover, various countries like Korea, Japan and Europe have reported that *Pseudomonus putida* release IMP- and VIM- type metallo β-lactameses which causes difficulty in treating infections and can also leads to nosocomical intection(Horii, Muramatsu, & Iinuma, 2005).

1.6 Significance of Bacteriophages

Bacteriophages are studied greatly by researchers around the world as they have large contribution in biotechnology industry and medical field. Bacteriophages can attack bacteria in vitro and cause lytic effect on it. Bacteriophages have host specificity which is related with the presence of specific bacterial antigen and phages can be neutralized by using antiserum, which leads information about vaccine designing(Significance, 1946). Bacteriophages can be used to identify bacteria (Conn, Bottcher and Randall, 1945). Strains of *Corynebacterium diphtheria* can be classified by using phages. Gravis type II strain can lyses by the phage

found in gravis type I strain. This phage plays a role in induction of trasmission of Diptheria bacillus (keogh, Simmons and Anderson, 1938).

1.7 lifecycle of Bacteriophages

Bacteriophages show two types of life cycle, such as;1) lytic, 2) lysogenic life cycle virulent bacteriophages undergo lytic life cycle. According to lytic life cycle, firstly bacteriophages attack on the cell wall of bacteria and insert genetic material into the host body. Afterthat, the DNA undergo lysogenic life cycle where genetic material of phages get together with genetic materials of bacteria, turn into prophage and attached to genetic components of bacteria. Afterwards, DNA Prophage induced into lytic cycle. Then, host DNA is cleaved by prophageDNase , production of capsid proteins and all genome join together. In the last stage, lysis of host cell happens and phages come out by disrupting host cell (Pelzek et al., 2013).

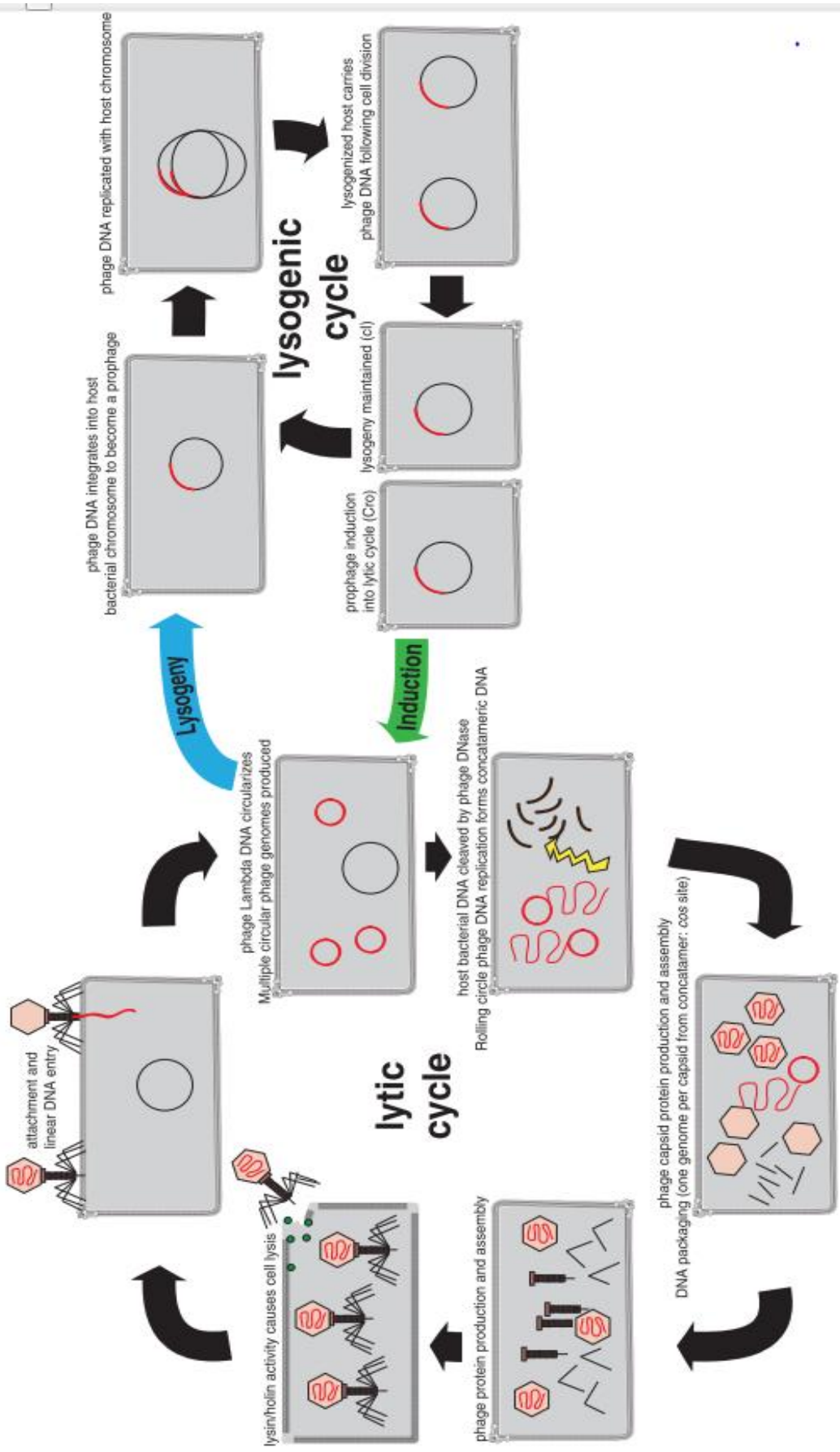

Figure 1.7: Life cycle of bacteriophage

(Pelzek et al., 2013).

Chapter 2

Methodology

2.1 Protocol: Titering of phage using Bacterial strains

Bacteriophages are found almost everywhere in environment; some bacteriophages are harmful are not. Plaque counting method is the most common method to detect infectious phage. This method was first modified by Felix d'herelle after he discovered phages. Plaque can be defined as clear zone where same kind of bacteria are terminated by phage lytic cycle (Pelzek, Schuch, Schmitz, & Fischetti, 2013).

2.2 Materials

- Bacterial strain *(Pseudomonus putida* and resistance *Pseudomonus pudita)*
- Water samples as source of bacteriophage (collected from different ponds, rivers)
- Nutrient Agar and nutrient broth (medium containing nutrients for bacterial growth)
- Autoclave (uses for sterile apparatus and agar medium)
- Incubator (uses for bacterial growth at temperature 37°C)
- Glass apparatus (petri dishes, conical flasks, beakers, glass rods)
- Dryer (dry agar medium)
- Hot bath (capable for heating more than 70°C)
- Laminar air flow (directs air in one-way and keeps sterile working environment)
- Streaking loops (for streaking bacteria strain on agar medium)
- Ethanol (for sterile purposes and as fuel for Bunsen burner)
- Cotton (for cleaning purpose)

- PBS (Phosphate Buffer Saline).

2.3 Bacterial culture and growth

Bacterial culture and growth is a crucial part of this experiment. For better result bacterial strains were cultured according to ATCC (American Type Culture Collection) website maintaining optimal temperature and conditions. Nutrient agar medium was prepared using autoclave machine and after pouring, petri dishes were left for drying. After that, this cultures were preserved in refrigerator (Pelzek, Schuch, Schmitz, & Fischetti, 2013).

.2.4 Phage dilution using tittering

Firstly, 10ml nutrient broth was prepared and two bacterial strains were added into the nutrient broth. After that, incubation of these test tubes were done inside incubator at room temperature for one day. Secondly, filtration of collected water was done by using 0.22µmmicro pipette filter to separate bacteria. Two test tubes were taken to pour 300µl of filtered water sample and 900µl of previous cultured broth in each test tube. Later, 200ml PBS was prepared. 900µl of PBS and 100µl of previously prepared filtered water and broth mixture were added into a beaker .later, serially dilute stock phage was tested from10^0dilute. Next, 20ml of hard agar medium and 20ml of soft agar medium were prepared. Furthermore, spot-tittering method was performed; bacterial strains were mixed with soft agar and poured it on solidified hard agar. Then, representative concentration were taken and poured it on soft agar prepared before and was kept those plates in incubator for 24 hours at room temperature. Finally, overlay-titer method was used. In this method, hard agar and soft agar were prepared. After that, hard agar was poured into four petri dishes and left it for solidified. When hard agar became solidified soft agar was

poured on it after mixing it with bacterial strains, Two test tubes were prepared with 3ml of soft agar, 100µlof dilute and 100µl of previously prepared broth for each test tubes and kept it in shaking incubator for 10-15 minutes at 37°C. Then, poured it on previously prepared petri dishes and kept it overnight. Next day, it had been checked that if there was any plaque formation or not. These plaque indicates that bacteriophages have bound with certain bacteria(Pelzek, Schuch, Schmitz, & Fischetti, 2013).

Chapter 3

Result

Ten water samples were collected from different location to perform this experiment and bacteriphages were isolated from this water samples**.**

3.1 Sample-1: Padma river water sample

First water sample was collected from Padma river,Rajshahi on 10th february,2019 . Latitude and longitude of Padma river are 24°00'63.55"N and 89°24'92.98'.

Figure 3.1.1: Padma river

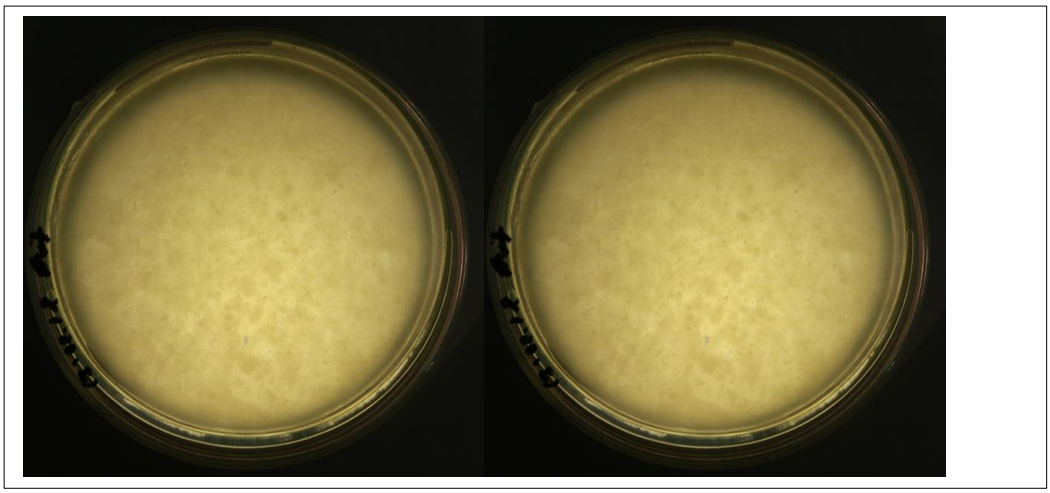

Figure 3.1.2: Bacteriophage isolation from sample-1

3.2 Sample-2: Mohakhali ICDDRB pond

Second water sample was collected from Mohakhali ICDDRB pond on 17th february,2019. Latitude and longitude of this pond is 23°93'o9.36"N and 90°72'11.68"E accordingly.

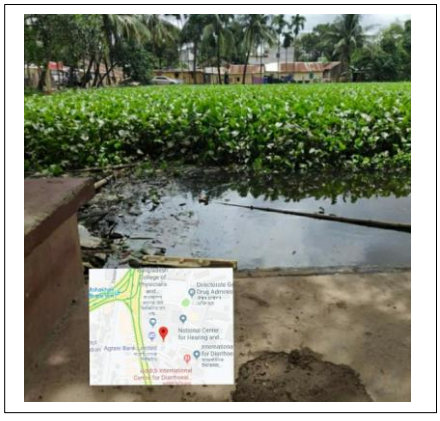

Figure 3.2.1: Mohakhali ICDDRB pond

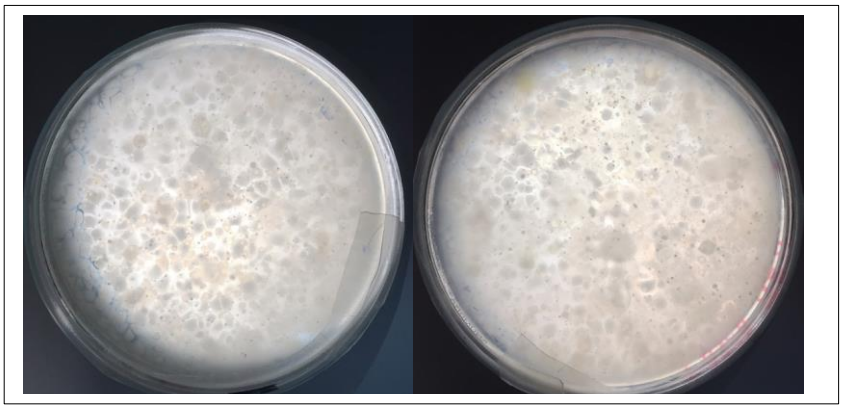

Figure 3.2.2: Bacteriophage isolation from sample-2

3.3 Sample-3: Arambagh water sample

Third sample was collected from Arambagh water lake on 22th February, 2019. The latitude and longitude of this pond are 23°58'75.95"N and 89°85'76.16"E accordingly.

Figure 3.3.1: Arambagh water sample

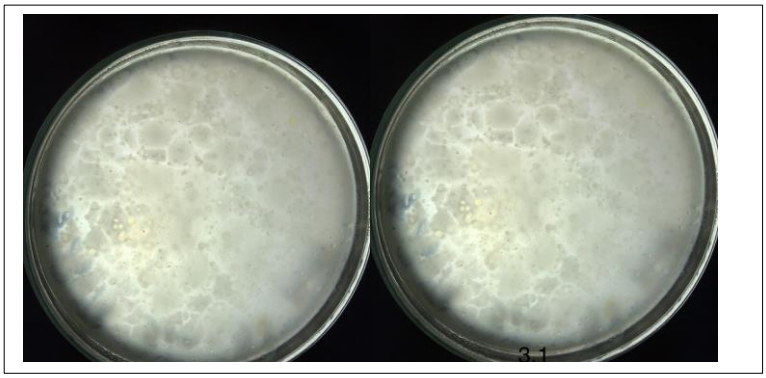

Figure 3.3.2: Bacteriophage isolation from sample-3

3.4 Sample-4: Narsingdi local pond water sample

Sample-4 was collected from Narsingdi local pond on10th March, 2019. The location of this pond is latitude 23°9'309.36"N and longitude is 90°72'11.68"E.

Figure 3.4.1: Narsingdi local pond

Figure 3.4.2: Bacteriophage isolation from sample-4

3.5 Sample-5: Jubliee lake water sample

This sample was collected from Jubliee tank, Faridpur on 15th May, 2019. Location of this sample is latitude 23°60'23N and longitude is 89°83'81E.

Figure 3.5.1: Jubliee water tank

Figure 3.5.2: Bacteriophage isolation of sample-5

3.6 Sample-6: Buriganga water sample

Buriganga water sample was collected on 25th June, 2019.latitude of this river is 23°37'59.99"N and longitude of this river is 90°37'59.99"E.

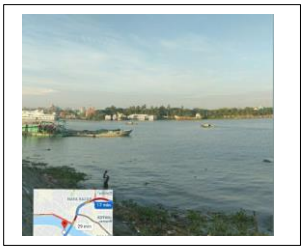

Figure 3.6.1: Buriganga river

Figure 3.6.2: Bacteriophage isolation from sample-6

3.7 Sample-7: Kyamch pond water sample

This water sample was collected from Kyamch pond, Sirajgonj on 13th July, 2019. The location of this pond is latitude 24°19'17.5"N and longitude is 89°41'51.7"E.

Figure 3.7.1: Kyamch pond

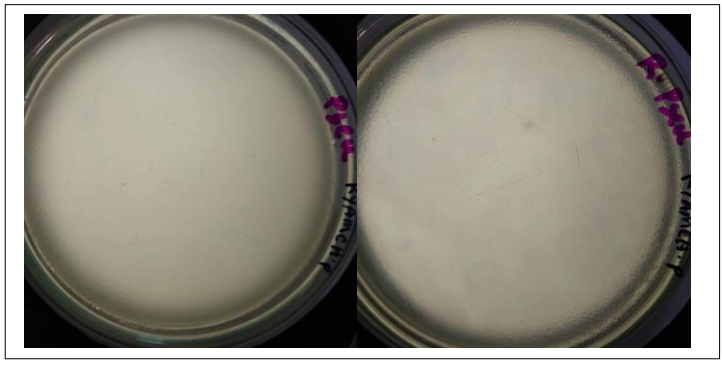

Figure 3.7.2: Bacteriophage isolation from sample-6

3.8 Sample-8: Hospital para pond water sample

Hospital para pond was collected on water sample was collected on 25 June,2019. Location of H.P.P is latitude found 24°19'17.5"N and longitude found 89°41'14.1"E.

Figure 3.8.1: Hospital para pond

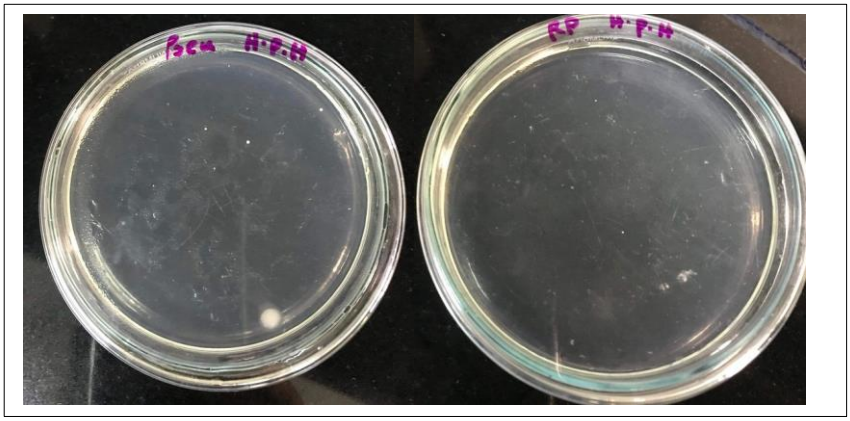

Figure 3.8.2: Bacteriophage isolation from sample-8

3.9 Sample- 9: Rani Dighi, Comilla

This sample was collected from rani dighi, Comilla on 6th July, 2019 and the location found latitude was 23°45'93.42"N and longitude was 91°18'30.57"E.

Figure 3.9.1: Rani Dighi

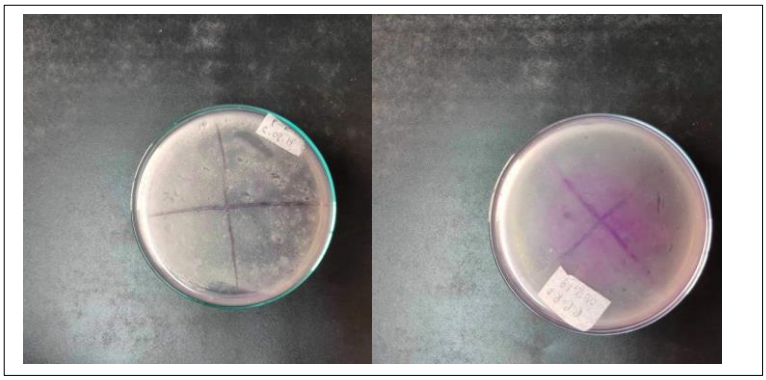

Figure 3.9.2: Bacteriophage isolation of sample-9

3.10 Sample-10; Nanua Dighi, Comilla

This sample was collected from nanua dighi of Comilla and location represented latitude of this dighi was 23°45'91.74"N and longitude of this dighi was 91°18'90.40"E.

Figure 3.10.1: Nanua Dighi

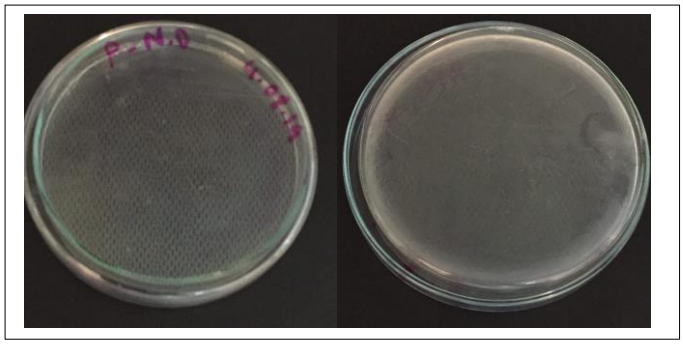

Figure 3.10.2: Bacteriophage isolation from sample-10

From ten water samples, only sample-8 (H.P.P) and sample-10 (Nanua dighi) showed positive result. In these two samples, bacteriophages were able to be isolated**.**

Chapter 4

Discussion

Bacteriophages can be isolated from water sample using representative bacterial strains. To perform this experiment, ten water samples were collected from different location and out of ten samples only two samples had showed positive outcomes. Figure 3.8.2 and figure 3.10.2 are showed plaque in the medium, which means bacteriophages were isolated from onlythis two samples. This result is not sufficient because most of the samples had showed negative results. In addition, specific dilution range was used for spot-tittering method, which is described in following table.

Table 2: Dilution factor of ten water samples

Sample No.	Name of water sounces	Dilution factor
01	Padma River	not done
02	Mohakhali ICDDRB pond	not done
03	Arambagh lake	not done
04	Narsingdi local pond	not done
05	Jubliee water tank	10^{-4}
06	Buriganga River	10^{-6}
07	Kyamch pond	10^{-6}
08	Hospital para pond	10^{-6}
09	Rani dighi	10^{-8}
10	Nanua dighi	10^{-6}

From this table, first four samples were not diluted because of time limitation and other six samples were diluted. However, only two samples showed plaque and other samples had showed clouded medium. Thus, it can be said that this experiment has showed negative outcome as most of the samples had negative results with no plaque formation in medium. There was no plaque formulation because specific bacteriophage for *Pseudomonus putida* was not present in water samples. *Pseudomonus putida* is rarely found bacteria, so

bacteriophages found in water samples did not bind with these bacteria, which result in no drug formulation. This problem can be resolved by collecting water from different locations and in different seasons. The more samples are collected, the more chance to found bacteriophages. So, in order to isolate bacteriophages, more water samples are needed to collect from different areas.

Chapter 5

Conclusion

Bacteriophages are bacterial virus, which has potential to bind with susceptive bacterial receptors and immobilize them. As a result, scientists are working on bacteriophages to use it against resistant bacteria which can cause life threating diseases (Ul Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012). In addition, for studying bacteriophages, isolation of bacteriaphages is necessary and phage tittering method is popular in this purpose. In this experiment, phage tittering method had been performed and result of this experiment has showed in this project. Ten water samples were presented with their collection and their outcomes were also shown. Eight samples showed no plaque formation, which means, it has negative results and two samples showed positive results, which have showed in figure3.8.2 and figure 3.10.2 with clear plaque formation. To conclude, most of the samples had cloud medium and no plaque, which indicates that this experiment has showed negative result.

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