Isolation of Bacteriophages from Aquatic Environments

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Isolation of Bacteriophages from Aquatic Environments" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Judith M. Noronha and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Isolation of Bacteriophages from aquatic environments"** is a bonafide work carried out by **Ms Sanju Shamboo Chauhan** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology in the Discipline Microbiology programme at the School of Biological Sciences and Biotechnology, Goa University

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Sanju S. Chauhan

LIST OF ABBREVIATIONS

UV Ultraviolet

DNA Deoxyribonucleic acid

RNA Ribonucleic acid

MPN Most probable number

PFUPlaque-forming units

dsDNA Double stranded DNA

SDS Sodium dodecyl sulphate

CTAB Cetyl trimethyl ammonium bromide

TBETris borate EDTA

PCR Polymerase chain reaction

OD Optical density

CONTENTS

INTRODUCTION AND REVIEW OF LITERATURE

Viruses are ubiquitous biological entities. They are obligate intracellular parasites having the potential to infect all types of living cells – prokaryotic and eukaryotic. They utilize the host's cellular machinery and energy for their replication and propagation. (Patzer et al., 1979) They are the smallest life forms, their size ranging from 20-400 nm, representing the greatest reservoir of uncharacterized genetic diversity on earth. (Nair et al., 2022)

1.1 Structure and life cycle

Viral genomes consist of single or double stranded DNA or RNA. The genome is surrounded by a protein coat (Patzer et al., 1979). Viruses are classified as enveloped or non-enveloped, depending on the presence or absence of a lipid-rich envelope surrounding the protein-nucleic acid core (Ono, 2010).

Among the diverse classes of viruses, a class of virus that has held a lot of interest for the scientific community is bacteriophages and cyanophages often referred to as "phages". Bacteria are the most abundant microorganisms in aquatic habitats, followed by cyanobacteria and microalgae. Bacteriophages specifically infect bacteria.

On entry into the specific host organisms, viruses may undergo any of the following kinds of life cycle which culminates in their reproduction.

(i) Lytic Cycle

Typically, the lytic cycle of DNA phages is characterized by the lysis of the infected bacterial host at the end of the phage replication cycle. Lytic phages are mostly tailed, double-stranded DNA phages. The lytic cycle of DNA phages is carried out by diffusion of the phage to the bacterial cell surface entry with the help of specific bacterial receptors and injection of the phage DNA into the bacterial cytoplasm, followed by replication and transcription of the phage genome. This results in synthesizes of viral proteins which help in the assembly of the virus and packaging of the viral genome into the capsids to form virions. (Fortier et al., 2013) A number of progeny viruses are then produced and released into the environment by the fatal rupture of the host cell (Sime-ngando, 2014) These can then re-infect susceptible bacteria nearby, repeating and the cycle. (Fortier et al., 2013).

(ii) Temperate or lysogenic cycle

Temperate bacteriophages either integrate into the host genome or remain as independent prophages until some external stimulus results in a switch to lytic cycle. Both the provirus and the host cell benefit from this process, called lysogeny. It provides a mode of persistence for viruses when the count of the host cells is very low. Lysogeny helps the provirus to acquire new phenotypic characteristics such as antibiotic resistance, antigenic changes, and virulence factors, this increase its fitness. Lysogeny also maintains the flow of genetic information within the environment. In some cases Pseudolysogeny may also occur where the viral genome is not integrated with the host genome but rather remains in an inactive state within the host cell. This may occur in very poor nutrient conditions where host cells are undergoing starvation and cannot offer the energy necessary for viral gene expression. (Sime-ngando, 2014)

1.2 Factors affecting viral community composition

There are many factors responsible for influencing the viral community composition in freshwater environments. These include anthropogenic factors, seasonal variations, and ecological factors.

(i) Ecological Factors: The major factors shown to influence the viral communities in freshwater ecosystems include the host species composition, diversity, and abundances of the potential hosts, which is known to vary with the trophic status, depth, and physiochemical conditions.

(ii) Anthropogenic Factors: Several human activities such as pollution, coastal engineering, urban development, oil extraction, refinery, mining, and agriculture have a significant influence on the diversity and composition of microbial organisms in different freshwater ecosystems which could subsequently change the viral community composition

(iii) Seasonal Variations: Viral abundance also follows seasonal variations: in summer there is high viral abundance consequent to higher host abundance, while in winter the host and viral abundance and activity is generally low (Munang, 2016).

Viral production rates are dependent on the host's metabolic rate, hence productivity is highest in eutrophic waters and least in oligotrophic waters. Decay rates on the other hand, are strongly

dependent on the habitat type in which viruses occur. The mechanisms, of decay include enzymatic degradation of viral nucleic acids, adsorption to surfaces and sinking, direct consumption by heterotrophic nanoflagellates, or ultra- violet (UV) and other light irradiation. (Hewson & York, 2010)

Environmental viruses are a key factor in microbial mortality. Viral lysis of microbial cells recycles intracellular materials as dissolved organic matter Thus, viral lysis maintains organic matter in surface waters, which further results in the release of small quantities of free amino acids, peptidoglycan and other cell wall components of organisms. These nutrients sustain the high rates of photosynthesis of planktons, ultimately sustaining much of life on earth. As a result, viruses are important drivers of global biogeochemical cycles. They play crucial roles in aquatic food webs as active constituents of the microbial loop and in the population ecology of both prokaryotic and eukaryotic microorganisms. (Winsley et al., 2014) They also contribute in biogeochemical and ecological processes, including nutrient cycling, system respiration, particle size-distributions and sinking rates, bacterial and algal biodiversity and species distributions, algal bloom control, dimethyl sulphide formation and genetic transfer. (Fuhrman, 1999)

There are various methods involved in isolations of phages from environment, including traditional culture-based methods, electron microscopy, and molecular methods such as polymerase chain reaction (PCR) and quantitative PCR (qPCR). Each method has its advantages and limitations, and the choice of method depends on the purpose of the study(Winsley et al., 2014) But the basic principles of virus isolation has remained same since it was developed by Felixd Herelle.(Hyman, 2019) .This is usually referred to as the “double agar overlay or plaque assay”.

Typically, two different ways have been used to enumerate viruses: the indirect, ‘viable’counts and the direct total counts. Viable counts are obtained as plaque-forming units (PFU) on a lawn of host cells on an agar plate (plaque assay) or in liquid medium as most-probable-number (MPN) assay. (Weinbauer, 2004)

1.3 Culture based techniques to study microbial viruses

(i) Spot test / Direct plating

Spot test involves plating of environmental samples directly with isolated host samples, without enriching them and looking for spot clearing or plaque formation.Bacteriophage detection and isolation by this method requires sufficient concentration of phages in the sample. At least 10–100 bacteriophages per ml must be present for visibility on the plate, because if the phage concentration is too low, there may be very few phages that can form visible zone of clearance on the plate. This method has been useful in isolating novel bacteriophages from sources like sewage effluents, stool samples, saliva (Bhetwal et al., 2017). This method has limited utility. (Nair et al., 2022)(Hyman, 2019).One advantage of direct plating is that, any biases introduced during sampleprocessing can be avoided. Thereforeit can be readily used for isolation and characterization of individual phages from viral communities.(Hyman, 2019)

(ii) Plaque Assay

The plaque assay, the gold standard technique for isolation of bacteriophages, was first developed by D'Herelle (Katz, 2016). The protocol was subsequently modified by a number of scientists. The most widely used variation today, is the double agar overlay plaque assay. The purpose of the plaque assay method is to determine the bacteriophage concentration in the sample, in order to get isolated plaques of phage particles within a lawn of bacteria. The basic idea of this method involves dilution of the phage particle to the point where individual phage particles can infect individual bacterial cells. These infected cells then undergo lysis, releasing more phage particles, which go on to infect neighboring bacterial cells. This process creates a visible "plaque" on a layer of bacterial lawn, where the bacterial growth is inhibited by the presence of the phage particles.(Katz, 2016) This method has greatly increased the yield of bacteriophages and is useful for different kinds of water samples including sewage. This method detects the viruses even at very low concentration. (Grabow & Coubrough, 1986)

Though culture-based methods are useful for isolating only a small proportion of existing viruses, they remain indispensable for isolation and purification of virus-host systems. it has been found that viable counts, obtained using culture methods, significantly underestimate the number of bacteria in the sample, and the same holds true for cultured phages (Clokie et al., 2009)

1.4 Culture independent techniques to study microbial viruses

A. Enumeration methods

Direct total viral counts can be determined using three different methods, by using Transmission Electron Microscopy (TEM), epifluorescence microscopy (EFM) and flow cytometry.

1) Transmission electron microscopy

Transmission electron microscopy has been used to visualize phages and characterize their morphology, particularly after the negative staining technique was introduced in 1959. The first evidence of high viral abundances was also obtained by using TEM (Zawrah & Abd El-Moez, 2011). Before total counts of viruses can be obtained using TEM, they have to be concentrated. Typically this is done either by ultracentrifugation onto TEM grids or by filtration onto 0.02 μm pore- size filters. Ultrafiltration may be used to concentrate viruses for counting, in samples where the viral abundance is low. (Ács et al., 2020)

2) Epifluorescence microscopy (EFM)

Viruses are often an order of magnitude more abundant than bacteria in aquatic environments. (Weinbauer, 2004) Their size ranges from 20-200 nm, and hence they cannot be visualized by light microscopy. Instead, epifluorescence microscopy combined with the use of nucleic acid stains, is carried out. Viruses are generally stained using the cyanine-based fluorescent dye SYBR Green I, that binds to double-stranded DNA and stains virus particles brightly. (Patel et al., 2007) Epifluorescence microscopy is an accurate and convenient technique and has been used to determine the abundance of viruses in samples from marine and freshwater environments.(Hermes & Curtis, 1995).

3) Flow cytometry

Flow cytometry, like epifluorescence microscopy, utilizes the nucleic acid staining properties of SYBR Green I. It is a high-throughput technique, capable of analysing a larger number of samples in a shorter time than the other two enumeration techniques. Flow cytometry can also distinguish between two and sometimes three virus populations in natural samples. (Noronha et al., 2022)(Marie et al., 1999)

B) Metagenomic methods

The term metagenomics was first coined in 1998 and is defined as the direct sequencing and analysis of all genetic material isolated from an environmental sample.(Kim et al., 2013) It enables a researcher to sequence and identify all the genetic material present in a sample, thus generating community-level data on viral diversity and functions, rather than focusing on individual viruses. Further, metagenomics is the only route to identify unculturable microbes and viruses present in an ecosystem. Information obtained through metagenomics can help us to understand the relationships between viruses and the biotic and abiotic components of the ecosystem.(Munang, 2016)

1.5Studies on bacteriophage isolation from aquatic ecosystems

Over the years, various studies have shown that viruses play a significant role in the aquatic environment. They can help control the spread of diseases and improve the quality of life for humans and other living organisms. They can also assist in the creation of new habitats for beneficial species.

(Grabow & Coubrough, 1986) provides a detailed description of the methods used to accuratelycount viruses collected from various sources like river, tertiary treated wastewater, seawater, and drinking water supplies using single-agar-layer plaque assay for the direct detection of coliphages in 100-ml samples of water. The samples were collected from Port of Elizabeth and East London. The assay was found to be more sensitive, reliable, and accurate than various other methods and proved to be rapid, simple, and economic.

Studies carried out in China demonstrated the efficacy of bacteriophage in controlling the populations of pathogenic bacteria. *Elizabethkingia anopheles* an opportunistic pathogen that caused severe infections in immune compromised patients, and the emergence of multi-drug resistant strains of this bacterium was major public health concern. Poluri and co-workers isolated and characterized the phage TCUEAP, from various sources of freshwater in China, and by electron microscopy and genome sequencing, demonstrated its ability to effectively lyse *Elizabethkingia anopheles* in vitro.(Poluri et al., 2021)

Several other studies have been carried out in order to isolate novel bacteriophage, for their therapeutic effectiveness as an alternative antimicrobial therapy for infectious diseases Researchers carried out studies in Katmandu valley by collecting water sample from different locations and using double agar overlay method, isolated 67 different bacteriophages. These isolated bacteriophages showed potential therapeutic effects against clinical strains of bacteria, including multidrug-resistant pathogens.(Bhetwal et al., 2017)

These novel bacteriophages, play very important role in microbial loop and in the population ecology of microorganisms in both freshwater and marine environments. They also regulate biogeochemical cycles, such as photosystem gene expression, and have been shown to actively participate in marine virioplankton assemblages. Therefore, research on aquatic viruses has provided a more comprehensive understanding of their importance in aquatic ecosystems.(Hewson & York, 2010)

Moon et al isolated and characterized two novel phages, P26059A and P26059B, from Lake Soyang in South Korea that caused infection to member of the family Comamonadaceae, which in turn control the population size of this bacterial group. Overall, the studies highlights the importance of studying phages in freshwater environments, and provides valuable resources for future research on freshwater viruses.(Moon et al., 2018).

Shaburova et al. isolated a lytic phage named vB_EfaS_IME207 from a soil sample in Russia, which has the potential to control infections caused by *Enterococcus faecalis*, a common human pathogen that is often resistant to multiple antibiotics.(Nair et al., 2022)

Similarly, Lekshmi et al. isolated several phages from the gut of chickens in India, which has the ability to lyse *Salmonella enterica*, a major foodborne pathogen. (krishna et al., 2021). Isolation and characterization of a novel bacteriophage, Phage vB_XooS_NR08, belonging to the Siphoviridae family, which shows antibacterial activity against *Xanthomonas oryzae* pv. Oryzae (Xoo), the causative agent of bacterial leaf blight (BLB) disease in rice was carried out. This isolated bacteriophage has the potential of phage therapy as a biocontrol agent for the management of BLB disease in rice.(Jain et al., 2023)

Overall, the isolation and characterization of bacteriophages from different environmental niches highlights their potential as a natural and sustainable alternative to antibiotics for the treatment of bacterial infections.(Poluri et al., 2021)

Objectives of the present study

The present study was consequently undertaken with the following objectives:

1. Isolation, culturing and identification of freshwater aquatic bacteria as hosts for bacteriophage isolation;
2. Isolation and characterization of bacteriophages from freshwater aquatic systems.

MATERIALS AND METHODS

Materials

Instruments and chemicals : Membrane filtration unit, incubator, autoclave, hot air oven, weighing machine, micro pipette (10µl, 100 µl, 1000 µl), centrifuge, electrophoresis unit. nutrient agar, nutrient broth, agar powder, sodium chloride, SM buffer, lysis buffer (SDS,Tris Cl (1M), EDTA), phenol, chlorophenol, isoamylalcohol, 70% ethanol, 10mM Tris Cl buffer and isopropanol.

Methods

2.1 Sample collection and processing:

Water samples were collected from Quellosim field area of Goa. Water was collected from pond, stream, and paddy field (**Table 1**). Samples were collected in sterile plastic bottles, bought to the laboratory, filtered through a 0.22µm membrane filter and stored in sterile screw cap bottles in refrigerator.

S. no	Sampling sources	Location
1.	Pond	15.40478, 73.95159
2.	Stream	15.38991, 73.93763
3.	Paddy field	15.38881, 73.93933

Table1. Sample collection site, for isolation of bacterial cultures andviruses

2.2 Isolation and purification of host cultures

Serial dilutions upto 10^{-8} of all the collected water sample was carried, 0.1ml serially diluted water sample from each tube was spread plated on nutrient agar medium to isolate colonies, which were re-streaked to obtain pure culture. The pure cultures were Gram stained and their colony characteristics were studied.

2.3Phage Detection by Spot test

A pure culture of a bacterial host was diluted in a molten 0.8% agar matrix as a top agar and dispersed evenly in a Petri dish onto solid agar medium; bottom agar 1.5% so that it can grow into a bacterial lawn. The plates were kept stationary for 1 hour. After the top agar matrix was solidified, 10 µl of 0.22µm-filtered water samplewas inoculated on top of the agar. The plates were kept stationary for 1 to 1.5 hour, then inverted and incubated at 37^0C for 24 hours. The plates were observed for a zone of clearance.

2.4 Phage Detection by Double Agar Overlay Plaque Assay

The double agar overlay plaque assay is a quantitative method on solid medium where a densely growing culture of bacteria is exposed to phage.

A loopfull of bacterial host was inoculated in 25ml of nutrient broth and kept for incubation on shaker at 37^0C overnight. Next day, 125µl of 0.22µm filtered water sample was mixed with 250µl inoculated bacterial host in a molten 0.8% agar matrix and was poured evenly onto solid 1.5% agar medium. The plates were kept stationary for 1 hour, then inverted and incubated at 37^0C for 24 hours. After overnight incubation, the host bacterium forms a lawn on the solid medium, except where infectious phage particles lyse the bacterial cells, forming what is known as a "plaque", a zone without bacterial growth caused by the propagation of one phage particle. A plaque develops on the plate wherever a single virion initially infects a single cell.

2.5 Lysis of bacteria in liquid broth to optimize plaque formation.

Loopful of bacterial culture was inoculated in 50ml nutrient broth and incubated for overnight. OD was measured at 600nm, on showing enough growth (OD 0.4-0.6) the broth content was later divide into two sterile flasks. One was maintained as control and another was inoculated with the 0.22µm filtered water sample containing viruses and was kept stationary at room temperature for seven days.

After seven days again OD was measured at 600nm, the flask containing the virus sample showed less OD in comparison with the control. The broth containing the virus sample was later centrifuged at 8000 rpm for 5 minutes, and the supernatant was later filtered using 0.22 µm membrane filter and was stored at 4^0C for further use. This preliminary infection in liquid

medium increases the concentration of viruses, which was later used in plaque assay technique.

2.6 DNA isolation of susceptible host bacteria

DNA isolation of the bacterial cultures showing positive result for plaque assay was carried out. Loopfull of bacterial isolates was inoculated in 30 ml of nutrient broth and incubated at 37^0C for overnight on shaker. On showing enough turbidity

2ml of each cultures were taken in a separate 2ml eppendorf tubes and was centrifuged at 7000 rpm for 3 minutes. Supernatant was discarded and pellet was resuspended in 600µl lysis buffer and was vortexed and incubated for 45 minute at 37^0C. Tubes containing sample and lysis buffer was thawed and 30µl 10% SDS and 4µl of proteinase k was added and mixed well, then Incubated at 45^0C in water bath for 1 hour. After incubation 100µl of 5M NaCl and 80µl of CTAB/NaCl solution was added and mixed well and tubes were incubated for 10 minutes at 65^0C. Later, Equal volume of Phenol : Chloroform : Isoamyl alcohol (25 : 24 : 1) was added and mixed by inverting the tubes and centrifuged for 10 minutes at 10,000 rpm, upper aqueous phase was carefully transferred to a new tube using a pipette leaving behind the organic phase. To this aqueous phaseequal volume of Chloroform : Isoamyl alcohol (24 : 1) was added and mixed well and centrifuged the tubes for 10 minutes at 10,000 rpm.The aqueous phase was then again transferred to a new tube (1.5 ml microfuge tube) and 0.7 volumes of isopropanol were added to the precipitated DNA.The tubes were incubated for 1 hour at room temperature and were then centrifuged for 15 minutes at 10,000 rpm. Supernatant from the tubes was discarded and pellets was rinsed with 1ml of 70% ethanol. After rinsing the isolated DNA. It was re-suspended in 15µl 10mM tris cl buffer, and was later subjected to agarose gel electrophoresis.

2.7 Agarose gel preparation

0.4g of agarose powder was dissolved in 50 ml 1X TBE buffer and digested in microwave for 5 minutes. It was allowed to cool and 1.3µl of ethidium bromide was added. The electrophoresis unit was set placing the comb and agarose was allowed to set for 1-1.5 hours. On solidification comb was removed. 6X gel loading dye was added to 10µl of DNA sample and loaded along with the ladder onto the gel. The gel was allowed to run at 50 V. Separated bands of DNA in agarose gel was observed under UV radiation.

2.8 PCR amplification of the isolated DNA sample

Sr. no.	Reagents	Vol.in µl
1	Mili Q water	36.6
2	PCR buffer	5
3	dNTP mix	1
4	Forward primer	2.5
5	Reverse primer	2.5
6	Template DNA	2
7	Taq polymerase	0.8
	Total	50

Table 2. PCR reagents

Primers used were the universal primers for amplification of the bacterial 16S rRNA gene, i.e. 27F and 1492R.

All the required reagents listed in the above table was added in PCR tubes following the same order, gently mixed using pipette. The thermocycler was programmed for the PCR reaction to take place.30 cycles were carried out. The PCR products were checked by agarose gel electrophoresis.

PCR parameters		
	Temperature	Time
Initial denaturation	94^0C	5 min
Denaturation	94^0C	30 sec
Annealing	55^0C	45 sec
Extension	72^0C	1 min
Final extension	72^0C	5 min

Table 3. PCR reaction conditions

2.9 Identification of host bacteria based on 16S rRNA gene sequences:

Purified fragments were sequenced by automated Sanger sequencing. Forward and reverse sequences obtained were combined to generate a contig, using BioEdit software. The contig was analysed by BLAST against existing sequences in the NCBI-GenBank database, to find the closest match. A phylogenetic tree was generated in MEGA-X, to further confirm the identification of isolates.

RESULTS AND DISCUSSION

Figure1. Locations of sample collection sites.

3.1 Isolation of bacteria as hosts for bacteriophages

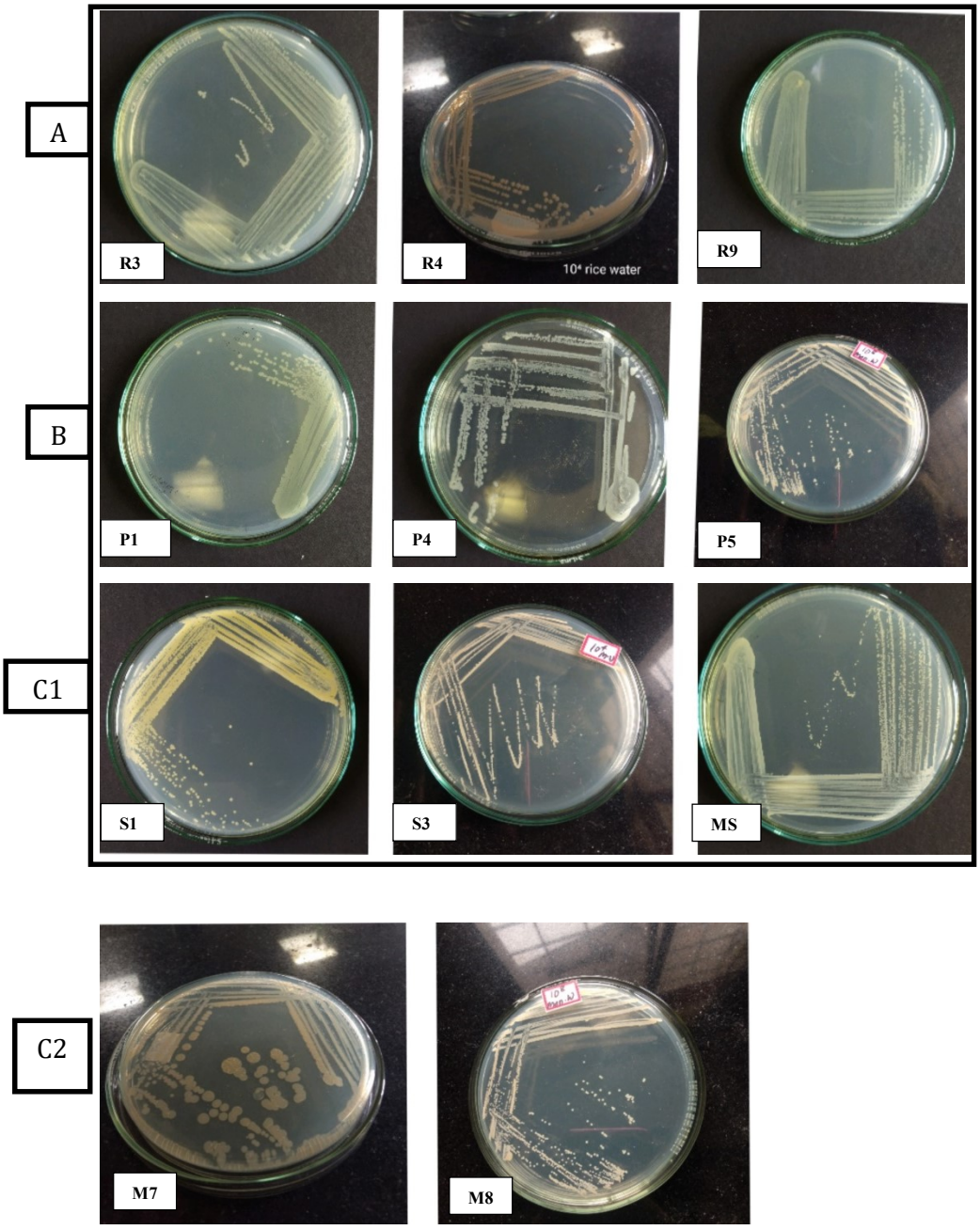


Figure2. Isolation of pure bacterial cultures from: A) Rice field water; B) Pond water; C1 and C2) Mangrove-associated water

The host cultures were isolated from various aquatic ecosystems and maintained on nutrient agar medium. In total, 11 bacterial cultures were isolated from three different aquatic environments.

Out of these, three cultures were from rice water sample, three from pond water, two from stream water and the remaining three from mangrove water source.

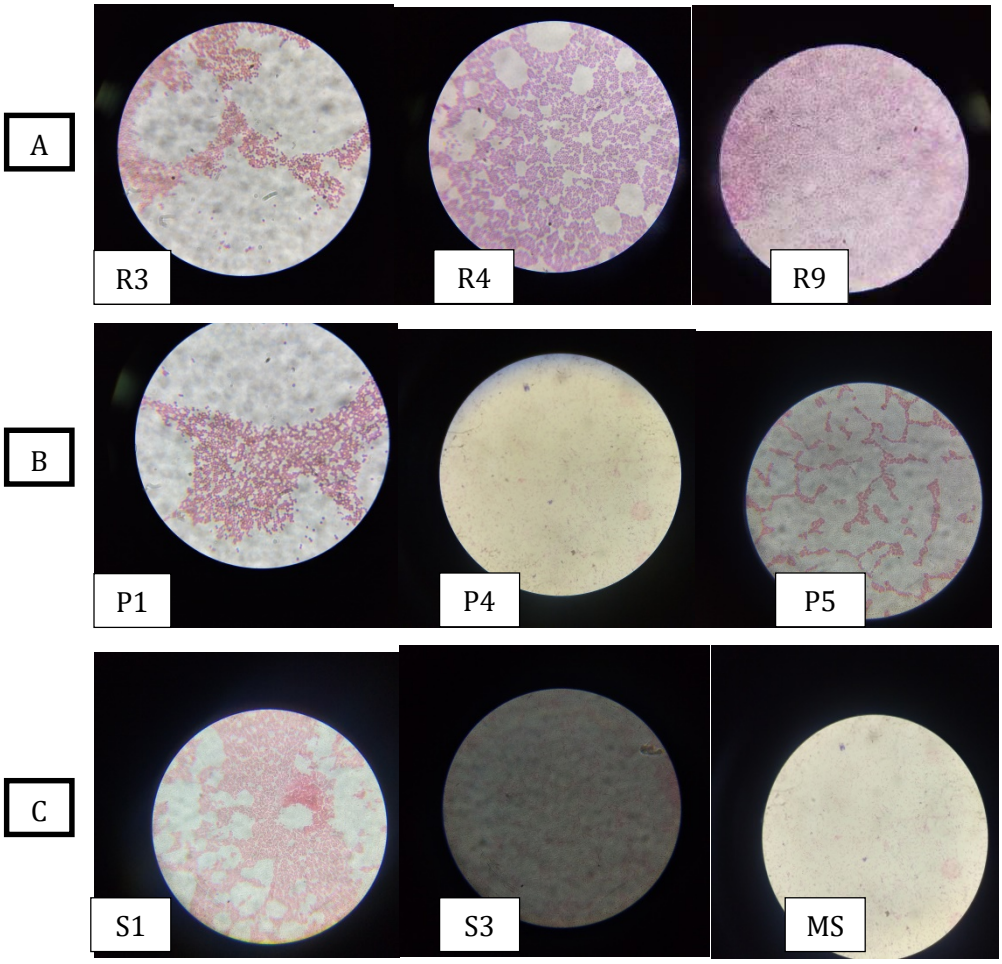
3.2 Morphological characterization of bacterial isolates

The colony characteristics of each isolate are listed in Table 4. The Gram characteristics are depicted in Figure 3.

Table 4. Colony morphology of bacterial isolates

Colony characteristics	**Bacterial cultures**				
	R3	**R9**	**R4**	**P1**	**P3**
Size	1mm	0.5mm	1mm	1-2mm	2mm
Shape	Circular	Circular	Circular	Circular	Circular
Color	Yellow	Light brown	Off white	Light yellow	White
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Raised	Raised	Raised	Raised
Opacity	Translucent	Translucent	Translucent	Translucent	Translucent
Gram character	Gram –ve	Gram –ve	Gram +ve	Gram –ve	Gram –ve

Colony characteristics	**Bacterial cultures**				
	P5	**S1**	**S3**	**M7**	**M8**
Size	1mm	1mm	2mm	2-3mm	1mm
Shape	Circular	Circular	Circular	Circular	Circular
Color	Off white	Yellow	Off white	Off white	Yellow
Margin	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Convex	Convex	Raised	Convex
Opacity	translucent	Translucent	Translucent	Translucent	Translucent
Gram chact.	Gram –ve	Gram –ve	Gram -ve	Gram –ve	Gram +ve


A
R3
R4
R9
B
P1
P4
P5
C
S1
S3
MS

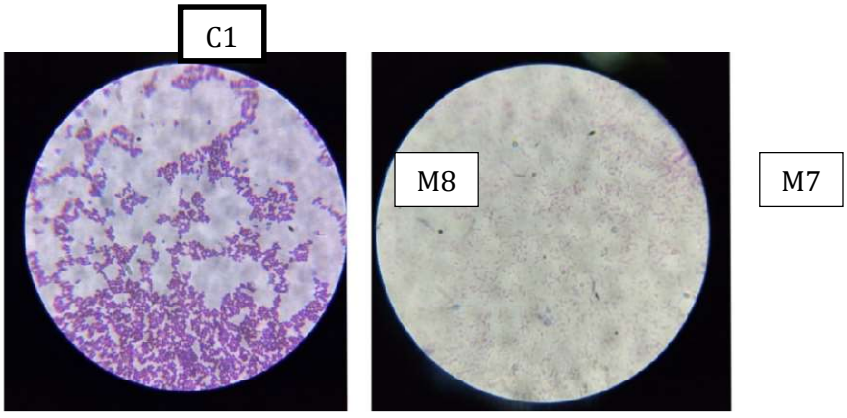

C1
M8
M7

Figure3. Gram staining of bacterial isolates

A: R3, R4, R9

B: P1, P4, P5

C:S1, S3, MS

C1: M8, M7

On gram staining, it was observed that among cultures of rice field water:

- R3 and R9 weregram negative, cocci in clusters and short rods shape
- R4: gram positive cocci in bunches.

All three isolates of pond water sample were gram negative:

- P1: cocci in bunches
- P5: cocci in chainand
- P4:short scattered rods.

Among bacterial isolates obtained from stream water sample:

- S1 and S3 appeared to be gram negative, cocci in bunches and short rods.

Among the isolates from mangrove water:

- M8 consisted of gram positive cocci in bunches,
- M7: gramnegativeshort rods.

3.3 Phage detection by spot test

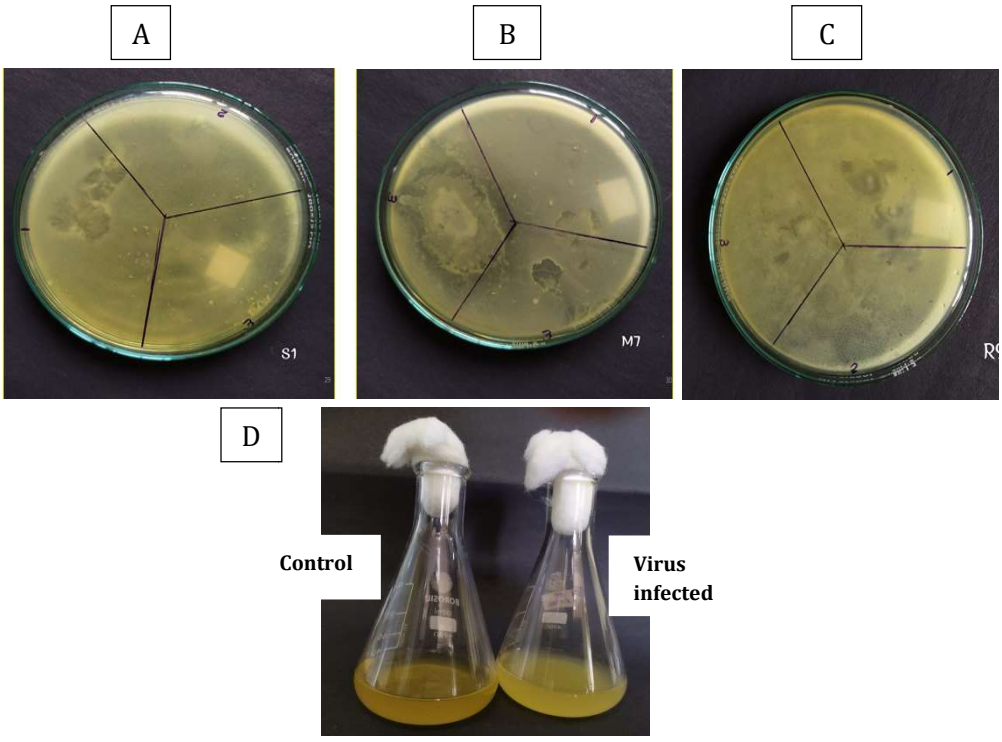


Figure 4.(A,B,C) Lysis of bacteriophage detected via spot test on solid media(D) Growth of control and virus-infected hostin liquid medium

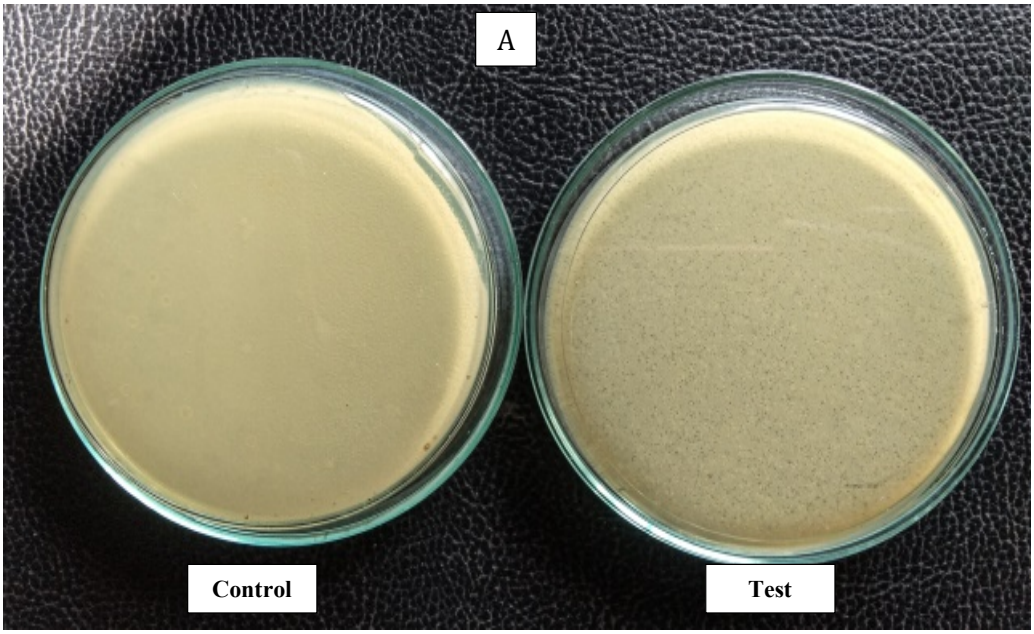
The water samples were screened against isolated bacterial hosts, by the spot test and plaque assay respectively, for the presence of lytic bacteriophages. Clearing of host growth, indicating lysis, was observed in the case of the following hosts, inoculated with respective water samples:

S1 – with pond water

M7 – with stream water

R9 – with pond water.

3.4 Phage detection by plaque assay method.



B

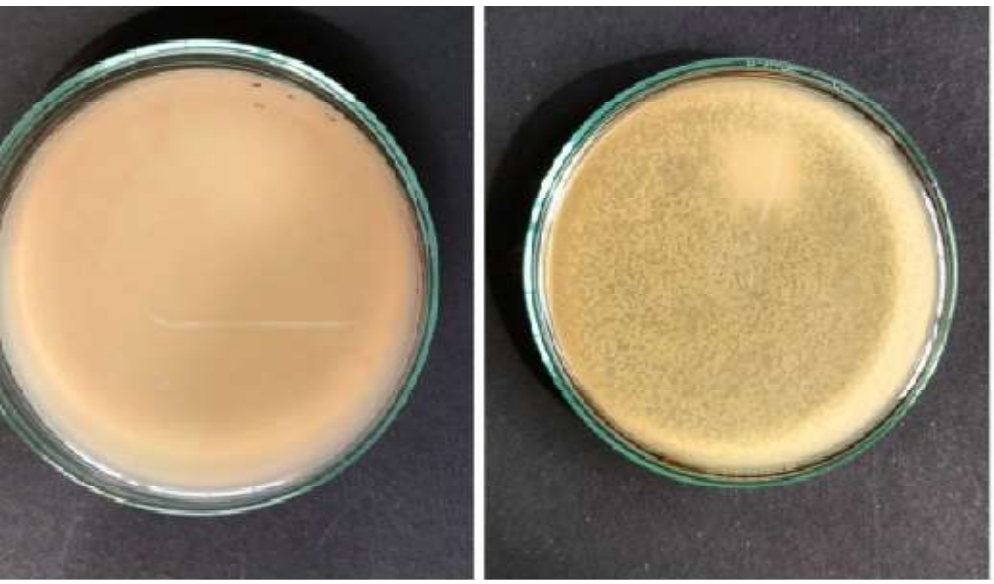


Figure 5. Detection of lytic bacteriophages via the plaque assay:

A: Host: **M7**;plaques formed upon plating pond water sample

B: Host: **M8**; plaques formed upon plating paddy field water sample

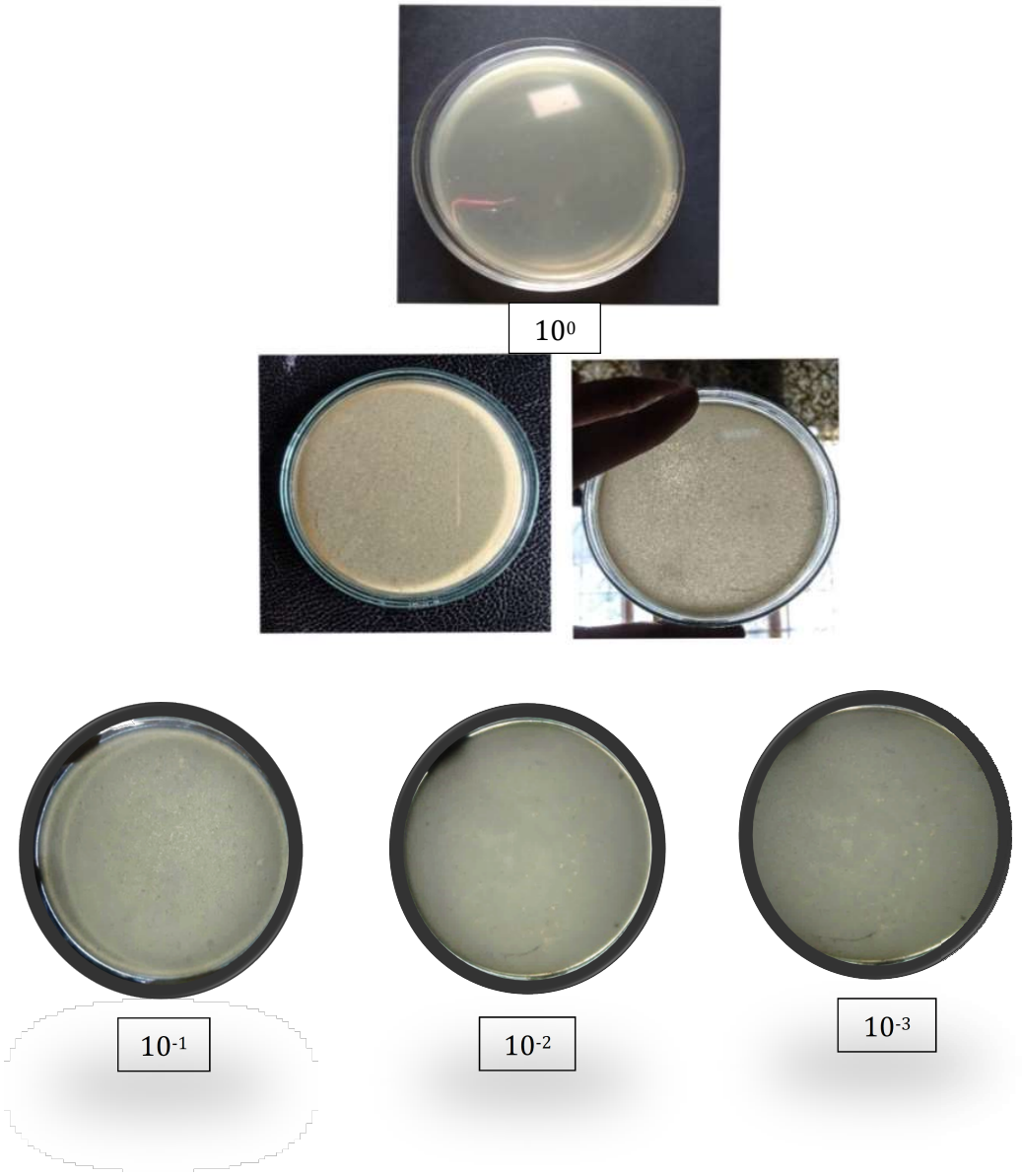


Figure 6: Infection of susceptible host culture M7 with various dilutions of phage stock, to optimize plaque count.

The results of plaque assay for detection of lytic bacteriophages were as follows.

Hosts M7 and M8 showed clear, visible plaques upon inoculating with water samples(**Fig. 5**)Plaque count observed on initial isolation on M7 plate was 256 and on M8 plate 364. The clear plaques in both cases indicated the isolation of lytic phages against M7 and M8 respectively.

The phage of host M8 could not be propagated further. Hence, further propagation of the phage of host M7 was carried out. In order to optimize plaque formation, various combinations of host volume were combined with a constant volume of phage stock (Table 5).

	Volume of host (µl)	Volume of phage stock (µl)
Test 1	100	10
Test 2	200	10
Test 3	300	10
Control	100	--

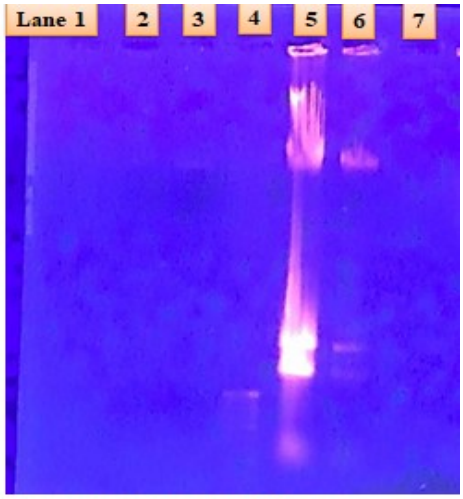
Table 5. Varying concentration of host M7 for plaque assay

On observation it was seen that M7 showed plaques with all the different concentration.

It was concluded that, the filtered broth had high concentration of virus particles,

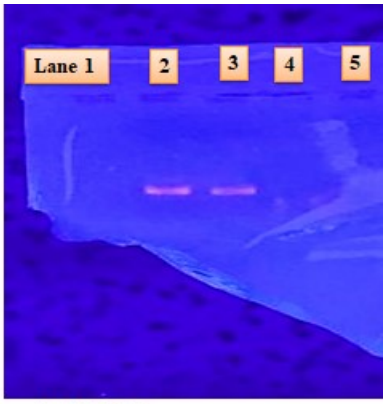
Therefore serial dilution was carried upto 10^{-3} of phage sample keeping the host concentration same, plaque assay was carried out. Dilutions upto 10^{-2} showed plaque formation, except 10^{-3} which showed very few plaques.

3.5Molecular identification of the susceptible hosts M7 and M8



Lane 4- ladder 5µl (100bp)
Lane 5- M7 10 µl
Lane 6 – M8 10 µl

Figure 7. Genomic DNA of M7 and M8



Lane 2- M8 10 µl
Lane 3- M7 10 µl

Figure8. PCR-amplified 16S rRNA gene of M7 and M8

A

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Staphylococcus succinus subsp. succinus strain DSM 14617 16S ribosomal …	Staphyloco…	769	1538	95%	0.0	99.07%	4326	MF678913.1
Staphylococcus succinus subsp. casei strain DSM 15096 16S ribosomal RN…	Staphyloco…	769	1538	95%	0.0	99.07%	4327	MF678912.1
Staphylococcus gallinarum strain DSM 20610 16S ribosomal RNA gene, part…	Staphyloco…	769	1538	95%	0.0	99.07%	4322	MF678881.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC 7…	Staphyloco…	769	1538	95%	0.0	99.07%	1511	MN077137.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC 7…	Staphyloco…	769	9231	95%	0.0	99.07%	2499507	CP035294.1
Staphylococcus pseudoxylosus 16S ribosomal RNA gene, partial sequence	Staphyloco…	769	1538	95%	0.0	99.07%	1500	MH643903.1
Staphylococcus gallinarum strain VIII1 16S ribosomal RNA gene, partial seq…	Staphyloco…	769	1538	95%	0.0	99.07%	1505	MG988386.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC 7…	Staphyloco…	769	1538	95%	0.0	99.07%	1506	MG988385.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC 7…	Staphyloco…	769	1538	95%	0.0	99.07%	1553	NR_074999.2
Staphylococcus edaphicus strain CCM 8730 16S ribosomal RNA, partial seq…	Staphyloco…	769	1538	95%	0.0	99.07%	1541	NR_156818.1
Staphylococcus succinus strain DSM 14617 chromosome, complete genome	Staphyloco…	769	13814	95%	0.0	99.07%	2757983	CP118976.1
Staphylococcus pseudoxylosus strain S04009 16S ribosomal RNA, partial se…	Staphyloco…	769	1538	95%	0.0	99.07%	1500	NR_180150.1
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 = NCTC 7…	Staphyloco…	769	1538	95%	0.0	99.07%	1100	MW012658.1
Staphylococcus xylosus strain JCM 2418 16S ribosomal RNA gene, partial s…	Staphyloco…	769	1538	95%	0.0	99.07%	1270	MW012656.1

B

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stutzerimonas stutzeri ATCC 17588 = LMG 11199 16S ribosomal RNA gene…	Stutzerimo…	765	765	99%	0.0	100.00%	1472	MT027239.1
Stutzerimonas stutzeri ATCC 17588 = LMG 11199 16S ribosomal RNA, com…	Stutzerimo…	765	765	99%	0.0	100.00%	1537	NR_103934.2
Pseudomonas oligotrophica strain JM10B5a 16S ribosomal RNA, complete …	Pseudomo…	765	765	99%	0.0	100.00%	1537	NR_181924.1
Pseudomonas songnenensis strain NEAU-ST5-5 16S ribosomal RNA, parti…	Pseudomo…	765	765	99%	0.0	100.00%	1458	NR_148295.1
Pseudomonas sp. strain JM10B5a 16S ribosomal RNA gene, partial sequence	Pseudomo…	765	765	99%	0.0	100.00%	1537	OM341414.1
Stutzerimonas kunmingensis strain HL22-2 16S ribosomal RNA gene, partia…	Stutzerimo…	765	765	99%	0.0	100.00%	1435	OL875289.1
Stutzerimonas balearica DSM 6083 16S ribosomal RNA gene, partial seque…	Stutzerimo…	765	765	99%	0.0	100.00%	1435	MZ276325.1
Stutzerimonas frequens strain FDAARGOS_877 chromosome, complete ge…	Stutzerimo…	765	3062	99%	0.0	100.00%	4394686	CP065720.1
Stutzerimonas stutzeri strain FDAARGOS_875 chromosome, complete gen…	Stutzerimo…	765	3062	99%	0.0	100.00%	4535380	CP065750.1
Stutzerimonas stutzeri strain NBRC 14165 16S ribosomal RNA, partial sequ…	Stutzerimo…	765	765	99%	0.0	100.00%	1462	NR_113652.1
Stutzerimonas stutzeri strain CGMCC 1.1803 chromosome, complete genome	Stutzerimo…	765	3062	99%	0.0	100.00%	4547930	CP002881.1
Pseudomonas nitritolerans strain GL14 16S ribosomal RNA, partial sequence	Pseudomo…	765	765	99%	0.0	100.00%	1459	NR_169495.1
Pseudomonas knackmussii B13 16S ribosomal RNA, partial sequence	Pseudomo…	765	765	99%	0.0	100.00%	1441	NR_117756.1
Stutzerimonas stutzeri strain VKM B-975 16S ribosomal RNA, partial seque…	Stutzerimo…	765	765	99%	0.0	100.00%	1495	NR_116489.1

Figure 9. Sequences showing identity to the query sequence.

A: M8; **B:** M7

Culture M8 Showed 99.07% identity with *Staphylococcus succinus* subsp. succinusstrain DSM 14617

Culture M7 Showed 100% identity with *Stutzerimonas stutzeri* ATCC 17588 = LMG 11199

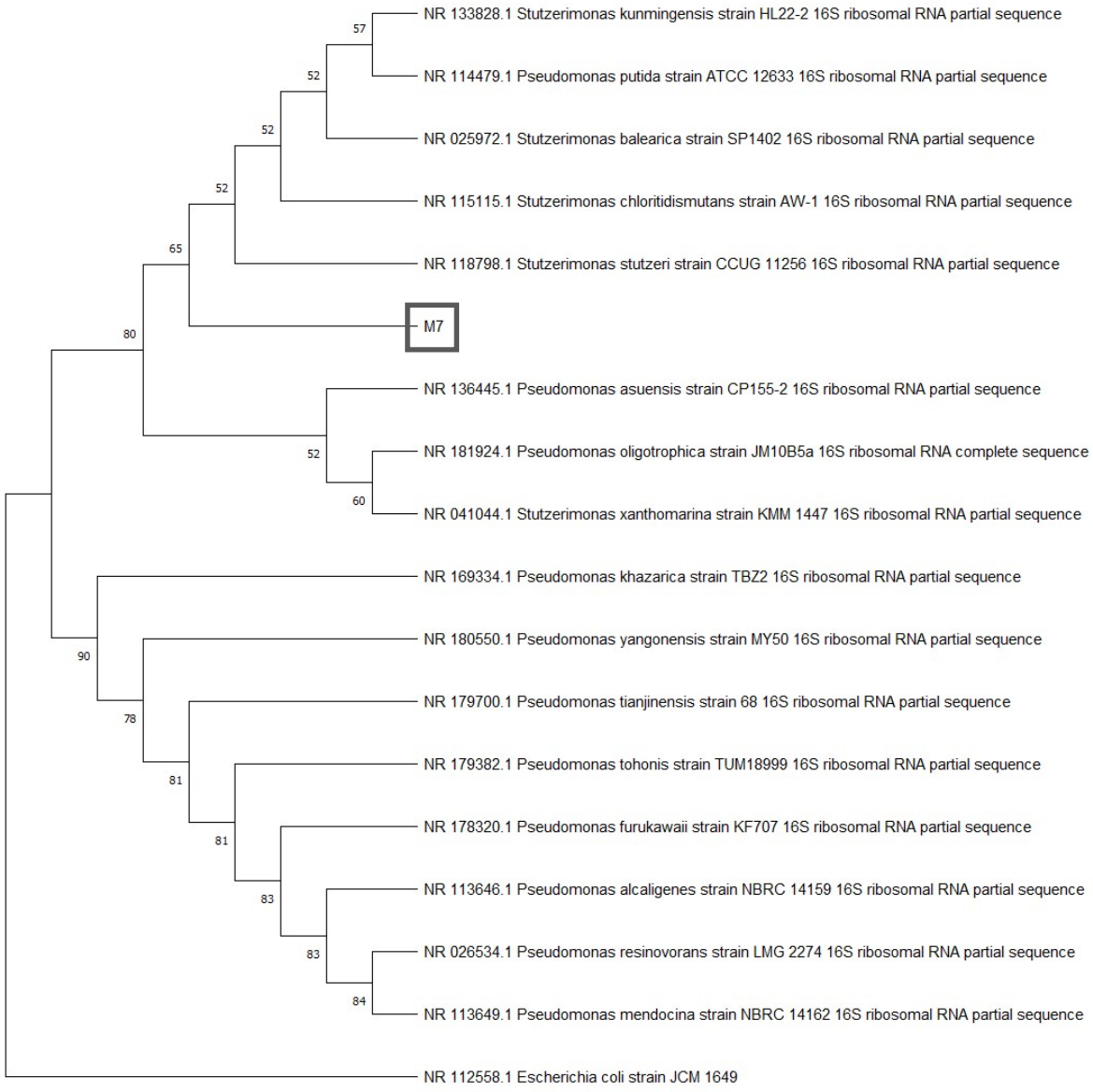


Figure 10 A. Phylogenetic tree constructed using 16S rDNA sequence of M7 (highlighted)with sequences showing identity >97% and with*Escherichia coli* as the outgroup

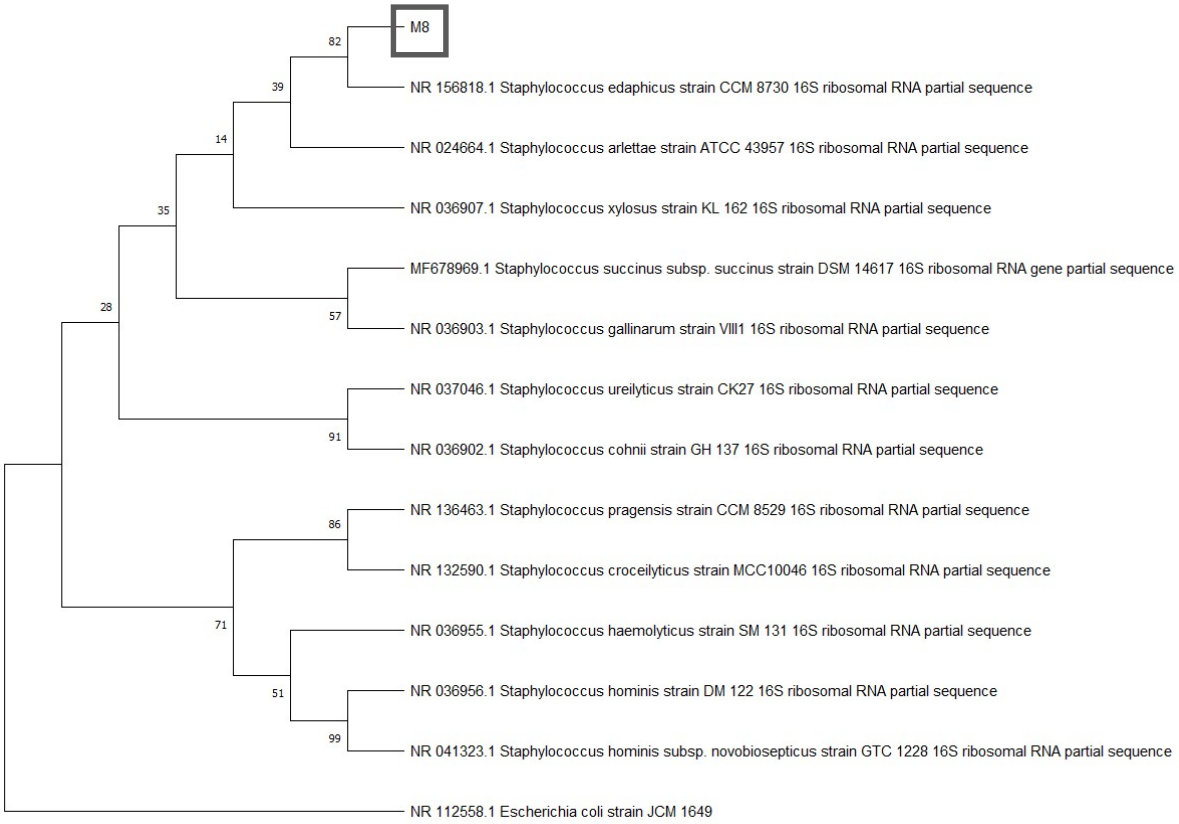


Figure 10 B. Phylogenetic tree constructed using 16S rDNA sequence of M8 (highlighted)with sequences showing identity >97% and with*Escherichia coli* as the outgroup

Bacterial host M7 was tentatively identified as *Stutzerimonas* sp. It is a gram-negative bacillus, resident to aquatic and soil habitats.(Lalucat et al., 2006).*Stutzerimonas* is a recently proposed genus within the Pseudomonadaceae comprising strains in the former phylogenetic group of *Pseudomonas stutzeri*(Gomila et al., 2022)

Host M8 was tentatively identified as *Staphylococcus succinus*, which is a gram positive coccoid bacterium(Lambert et al., 1998)

Both organisms are known to play important roles in manyenvironmental processes,including nutrient cycling and bioremediation(Mulet et al., 2023)(Huang et al., 2020).*Staphylococcus succinus* also produces certain compounds, such as bacteriocins, which helps to inhibit the growth of other bacteria. (Newstead et al., 2020)

Discussion

3.6 Pathogenicity of *Staphylococcus sp.* and *Stutzerimonas stutzeri*

Staphylococcus sp. areorganisms commonly found on the skin and other diverse mucosal membranes of healthy humans, and it is considered a commensal organism. However, they can also cause infections in humans, ranging from mild skin infections to more serious infections such as pneumonia, endocarditis, and sepsis(Haag et al., 2019)

Pseudomonasstutzeri is a Gram negative, aerobic bacterium found in soil and water environments, and is a rare cause of infective endocarditis, which is an infection of the inner lining of the heart and heart valves. Endocarditis caused by *Pseudomonas stutzeri* is typically seen in individuals with underlying medical conditions, such as immunosuppression, heart valve disease.(Alwazzeh et al., 2020)

3.7 Bacteriophages previously isolated against *Stutzerimonas* sp.and *Staphylococcus sp.*

Bacteriophage SA2 infecting*Staphylococcus* sp. was isolated, which led to the discovery of a new genus within the Siphoviridae family. Phage SA2 has unique characteristics which sets it apart from other phages, like its genome structure, the proteins it produces that distinguish it from other bacteriophages. SA2 also has a double-stranded DNA genome with a length of 89,055 bp and a G + C content of 31.9%, and has relatively short latent period and a high burst size.(Wang et al., 2019)

Another, novel bacteriophage named 94 infecting *Staphylococcus* sp. has been discovered. This phage have multi-species host range i.e they can infect multiple species of *Staphylococcus*.(Göller et al., 2021)

Bacteriophage 8P has been isolated upon infecting *Pseudomonas stutzeri* host found in soil and aquatic environment. The size of the phage genome is larger than 63 kb, which is relatively large compared to other phages. On identification it was observed that phage 8P is a member of Hollowayvirus within the family Podoviridae. It also shares some common similarities with F116 like phages. (Liu et al., 2021)

3.8 Significance of isolated phages infecting *Stutzerimonas* sp.and *Staphylococcus sp.* in phage therapy

Phages isolated against*Staphylococcus*sp. such as phage SA2 are used in phage therapy, due to its short latent period and a high burst size, which favours its use as a therapeutic agent, and also the fact that it has no identified virulence genes makes it an attractive candidate for phage therapy for bacterial infections.(Wang et al., 2019)

In addition to straight-forward structural proteins in phage 94, they also have the presence of enzymes such DNA polymerase, helicases, recombinases, exonucleases, and RNA ligase polymer, which they inject into the bacteria during the period of infection along with the genomic DNA to take over the host metabolism as soon as possible after infection. Due to their varied host range, among Staphylococcus sp. it is advisable that they could be used in cocktail formulations for targeting a broader range of bacteria.(Skurnik, 2020)

Bacteriophage 8P could be used as an alternative to antibiotics in the treatment of bacterial infections caused by *Pseudomonas stutzeri* species, and so could be effectively used for phage therapy. It also inhibit biofilm development in *Pseudomonas stutzeri* species. (Liu et al., 2021).

In the light of the above studies on phages of *Stutzerimonas* sp. *and Staphylococcus sp.*, the isolation of lytic phages against hosts M7 and M8 is significant. These phages hold the potential to be used as anti-bacterial agents in cases where their hosts are pathogenic.

SUMMARY

Bacterial cultures as host organisms were isolated from freshwater ecosystems, colony character and gram character of the same was noted, the cultures were purified and maintained.Water samples from freshwater sources were processed for the isolation of bacteriophages against bacterial isolates. Isolation was attempted in both solid and liquid medium, by spot test, plaque assay and lysis of bacteria in liquid broth. Two phages were isolated, against hosts M7 and M8 respectively. The isolation of the lytic phages was confirmed by the formation of clear plaques on lawns of host bacteria. Further, DNA isolation of the bacterial hosts susceptible to the bacteriophage was carried out, followed by PCR amplification of the 16S rRNA gene, for the purpose of molecular identification. BLAST alignment and phylogenetic analysis was carried out. The susceptible hosts were identified as *Stutzerimonas* sp.and *Staphylococcus* sp. respectively.

CONCLUSION AND FUTURE PROSPECTS

Conclusion

In the era of microbiome and virome-level studies, the traditional isolation and cultivation of bacteriophages from environmental samples has not lost significance. Culture-based studies of phages and their respective hosts further contributes to an in depth understanding of the role of viruses in aquatic environments. In the present study, two lytic bacteriophages infecting species of Stutzerimonas and Staphylococcus respectively, have been isolated from aquatic environments. The existence of these phages is significant in the light of their potential use in phage therapy against the pathogenic hosts.

Future Prospects

1. Growth studies and genomic characterization of the isolated bacteriophages.
2. Host range analysis against other strains of *Stutzerimonas* sp. and *Staphylococcus* sp., to ascertain the significance of the isolated phages.
3. Further isolation of aquatic bacteriophages having lytic potential against pathogenic bacteria.

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APPENDIX

Nutrient agar

Ingredient	**1 L**	**500 mL**
Distilled water	1L	500 ml
beef extract	1g	0.5g
Yeast extract	2g	1g
Peptone	5g	2.5g
Sodium chloride (NaCl)	5g	2.5g
Agar	15g	7.5g

Nutrient broth

Ingredient	**1 L**	**100 mL**
Distilled water	1L	100 ml
beef extract	0.3g	3g
Peptone	0. 5g	5g
Sodium chloride (NaCl)	0.5g	5g

SM buffer

Ingredient	**100 mL**
Tris cl (1M pH 7.5)	5 ml
NaCl	0.58 g
$MgSO4.7H_2O$	0.2 g

H_2O	100 ml

Extraction buffer

Ingredient	**100 mL**
Tris HCI (pH 8.0)	10mM
EDTA (Ph 8.0)	2.5 mM
SDS	5%
Nacl	1 M