ISOLATION OF CLINICALLY SIGNIFICANT BACTERIOPHAGES FROM WETLANDS

A dissertation report for

Course code and course title: MIC -651 Discipline Specific Dissertation

Credits: 16

Submitted in partial fulfilment of

Master of Science in Microbiology

By

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This is to certify that the dissertation report **"Isolation of clinically significant bacteriophages from wetlands"** is a bona fide work carried out by **Miss. Sampada Purushottam Nadkarni** 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, at the School of Biological Sciences and Biotechnology, Goa University.

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I hereby declare that the data presented in this Dissertation report entitled, "Isolation of clinically significant bacteriophages from wetlands" 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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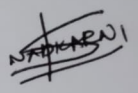

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PREFACE

Isolation of bacteriophage holds immense importance nowadays since microorganisms including bacteria have developed resistance mechanisms against the drugs that are designed to kill them. Properties such as host specificity, abundance in nature and so on make bacteriophage promising tools in combating infectious diseases. Isolating and identifying bacteria as host for bacteriophage from wetland ecosystem which could have various applications such as phage therapy, as diagnostic tools, biocontrol agents etc. Isolation is carried out by using simple techniques such as plaque assay, virus enrichment etc. Also identification of host bacteria was done using biochemical and molecular methods such as DNA extraction and PCR.

ACKNOWLEDGMENT

It gives me immense pleasure to bring out this dissertation thesis on **“Isolation of clinically significant bacteriophages from wetlands"**. Carrying out this research project has indeed been a fulfilling and enjoyable experience. Hence, I thank each and every one who has lent a helping hand in my work, and without whose assistance this project would have been impossible.

I would like to express my deep and sincere gratitude to my guide, Dr. Judith M. Noronha, who gave me excellent and constant guidance, encouragement and suggestions, with patience at all times.

I express my sincere gratitude to the Programme Director, Microbiology, Dr. Lakshangy Charya, and all the teachers for their constant encouragement and support.

I gladly acknowledge the assistance rendered by the non-teaching staff.

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LIST OF ABBREVATIONS

Entity	Abbreviation
International Committee on Taxonomy of Viruses	ICTV
Deoxyribonucleic acid	DNA
Ribonucleic acid	RNA
Double stranded deoxyribonucleic acid	dsDNA
Single stranded deoxyribonucleic acid	ssDNA
Double stranded ribonucleic acid	dsRNA
Single stranded ribonucleic acid	ssRNA
Messenger ribonucleic acid	mRNA
Food and drug administration	FDA
Plaque forming units	PFU
Ultraviolet	UV
Sodium dodecyl sulphate	SDS
Ethylene diamine tetra acetic acid	EDTA
Cetyl trimethyl ammonium bromide	CTAB
Tris borate EDTA	TBE
Deoxyribonucleotide triphosphate	dNTP
optical density	OD
Polymerase chain reaction	PCR

ABSTRACT

Isolation of bacteriophages for phage therapy involves isolating and identifying specific bacteriophages. It involves sampling, screening, isolation, purification and characterization. Bacterial cultures were isolated as host for the bacteriophage from Nanda Lake and Sarzora Lake, which are wetland ecosystems. The water was processed using membrane filtration method and processed to isolate bacteriophages against bacterial cultures isolated from wetlands and against previously maintained pathogenic cultures. The gram character and colony characters of all cultures were noted. Screening for phages was carried out in liquid medium (virus enrichment) and in solid medium (plaque assay). Isolation of bacteriophage was unsuccessful as there was no plaque formation.

Keywords

Bacteriophage, plaque assay, phage therapy, wetland

CHAPTER 1

INTRODUCTION

Wetlands

Wetlands are defined as “areas where water covers the soil, or is present either at or near the surface of the soil all year or for varying periods of time during the year, including during the growing season” (EPA, 2023). Wetlands are ecosystems rich in biodiversity, and combine land and water traits to provide various ecological functions. Wetlands differ from other aquatic ecosystems due to changes in groundwater levels, surface inflow, and tidal fluctuations, leading to recurrent flooding. (Brinson and Lugo.,1981). This encompasses a wide variety of wetland types, from bogs and fens, to tropical wooded wetlands, and from coastal salt marshes to mangroves at lower latitudes. While wetlands range in appearance and function, the majority have two basic traits that are crucial to understanding wetland microbial and viral communities: (i) shallow water and (ii) aquatic plants. Thus, benthic and connected microbial communities (biofilms) are expected to be far more important in wetlands than planktonic ecosystems. (EF Jackson et al., 2008)

Wetlands, acting as interfaces between land and water, feature shallow water close to the soil surface, nurturing distinct plant communities and micro flora. (Vepraskas.,2016 , Mitsch and Anderson.,2009) They serve as essential hubs for biogeochemical processes like pollutant degradation, nitrification, denitrification, methanogenesis, methanotrophy, and iron and sulfate reduction. These processes are largely facilitated by microbial activity (Davidsson and Stepanauskas.,1997, Gutknecht and Goodman., 2006)

Nanda Lake

Nanda Lake is a Ramsar designated wetland in the state of Goa, located in Curchorem. It covers 0.42 square kilometers. The Indian government announced the lake as a wetland in 2021 under the Wetland (Conservation and Management) Rules, 2017. On August 3, 2022, it was designated as a Ramsar wetland site under the 2022 Ramsar Convention. The marsh is home to red-wattled lapwing, black-headed ibis, bronze-winged jacana, common kingfisher, wire-tailed swallow, intermediate egret, tiny cormorant, and lesser whistling duck, among others.

Nanda Lake consists of intermittent freshwater wetlands close to one of the Zuari River's major tributaries. It allows the marshes to be flooded when it is closed. The site allows local communities to conserve water during the off-monsoon season, as well as to cultivate rice and carry out fishing activities. Further, the lake protects downstream areas from monsoon floods. The wetland ecosystem has been threatened by invasive non-native species, waste disposal, overfishing, and aquatic resource harvesting. The Union Environment Ministry stated that the Nanda Lake in Curchorem is one of ten new wetlands in India to receive the title of “Wetlands of international importance”. (Malkarnekar.,2022)

Sarzora Lake

Sarzora Lake in South Goa is a pristine freshwater lake, located about 8 kilometers away from Margao. It is surrounded by dense green forest. The water from this lake is used for irrigating the crops as well as for drinking purposes. It is a beautiful place to visit but has been closed to public permanently since 2020.

Bacteriophages

Bacteriophages or “phages” are viruses that infect bacteria. They hijack the host cell machinery for their replication and ultimately destroy it. Bacteriophages generally have a specific host range, implying that a specific bacteriophage will usually infect and kill only bacteria belonging to a particular genus or even species. Thus, it is safe to assume that as many bacteriophages exist, as their host bacteria. Bacteriophages are found everywhere in nature, including water bodies, soil and domestic waste (Nikolich and Filippov.,2020 , Jun-hang ye et al.,2023). They are the most numerous group of viruses present in aquatic and soil ecosystems. The estimated global population of phages is massive. They are found in areas ranging from extremely hot environments like hot springs, the Sahara, to extremely cold environments like polar inland waters. The overall number of phages in aquatic systems is estimated to be above 10^{31}, whereas land ecosystems have 10^{7} phage particles per 1 gram of soil and $10^{8\text{-}10}$ phages per 1 mL of sewage. Phages have been found to surpass bacteria in all investigated conditions, potentially making them the dominant form of life in the biosphere.(Ashelford et al.,2003). Phages are as diverse as their hosts, with some capable of surviving severe temperatures (up to 95°C) and pH levels as low as 1. They are classified by their host specificity, morphology, nucleic acid type, mechanism of infection, morphogenesis, phylogeny, serology, susceptibility to physical and chemical stimuli, and environment. (Abeles and Snyder.,1984) Phages can infect all bacterial genera, including cyanobacteria, archaea, and mycoplasmas.(Gabiatti et al.,2018, Stanton and Rose.,2019,Sharp.,2001,)

Scientists are interested in phages for their role in molecular biology, horizontal gene transfer, bacterial evolution, diagnostic tools, and potential therapeutic agents. (Clokie et al.,2011). Bacteriophages that inhibit bacterial growth and that depict visible lytic activity in the form of plaques can be considered to have further therapeutic potential. (Doub et al.,2020).

Bacteriophage structure and life cycle

The International Committee on Taxonomy of Viruses (ICTV) has classified bacteriophages based on their nucleic acid morphology (dsDNA, ssDNA, dsRNA, and ssRNA). (Rohwer and Edwards.,2002, Ackermann and Slater.,2006). Phages are made up of nucleic acid i.e., DNA and RNA, a highly symmetrical protein capsid, and sometimes accessory proteins and enzymes (Sanz-gaitero et al.,2021). Depending on presence and absence of lipid rich envelope surrounding the protein nucleic acid core, they are classified into enveloped and non-enveloped. (Ono.,2010)

Phages come in various shapes, including spherical, icosahedral, and filamentous. The length of phages varies widely and usually ranges from 24 to 200 nm. There are two types of phages: virulent (lytic) and temperate (lysogenic). Thus, there are broadly two types of phage life cycles, although a spectrum of variations in life cycle do exist.

Lytic cycle

Virulent phages are those in the lytic phase. During the lytic cycle, the phage multiplies and kills the host bacteria. It then ruptures the host cell and releases additional phage particles. According to (Karlsson and Borrebaeck.,2003), the lytic cycle begins when the phage attaches to bacteria via a protein complex. After attaching the viral particle, the bacteriophage inserts its genetic material into the bacterial host cell. The phage uses bacterial metabolic machinery to replicate its genetic material (DNA or RNA) after penetration. DNA viruses directly transcribe into messenger RNA (mRNA) that are subsequently employed to control the host cell's ribosomes. Retroviruses use reverse transcriptase, a special enzyme that transcribes viral RNA into DNA and then follows the DNA virus's path for transcription. During the later stages of translation, freshly translated proteins form the capsid and tail of phages, which break out of the host cell and

rupture its membrane. As additional particles arise, they infect and multiply in host cells. During phage replication, the host chromosome may be packed into the capsid instead of the phage genome, resulting in horizontal gene transfer within the bacterial population by transduction (Madigan and Martinko.,2006). Phages employing solely the lytic cycle can effectively combat antibiotic-resistant pathogens.

Lysogenic cycle

In comparison to the lytic cycle, temperate phages exhibit the lysogenic phase (Campbell and Reece.,2005), which results in the integration of the viral genetic material with the bacterial genome (called prophage), ensuring continued replication of the viral genetic material without causing death to the infected host (Inal.,2003). Infected bacteria often modify their phenotypic after incorporating viral genetic material into their host. Bacterial pathogenicity may be induced by this change, as seen with common strains (Brussow and Canchaya.,2004 ,Keen.,2012). Hydrogen peroxide can prevent lysogenic conversion by producing glutathione and overexpressing transcriptional repressors (Wagner and Acheson.,2001).Due to various stimuli, the DNA of the bacteriophage gets excised and then it follows lytic cycle. (Froissart and Brives.,2021). A known example is the gene associated with *Vibrio cholerae* that cause cholera symptoms due to the toxins it encodes. (Clokie et al.,2011)

Functions of bacteriophages

Phages mediate lateral gene transfer, affect host metabolism, and transfer bacterially-derived substances via cell lysis, playing a vital role in the ecosystem. Phages thrive and evolve alongside bacteria, developing multiple anti defense mechanisms in reaction to bacterial defense

methods against them. Phages owe their existence to their bacterial hosts, thereby they cause modifications to their host genomes by transferring resistance genes and toxin-encoding genes in order to boost the hosts' fitness. (Naureen and Dautaj.,2020)

Bacteriophages are naturally occurring antimicrobial agents that combat antibiotic-resistant bacteria that cause diseases in animals, humans, or agricultural crops. Phages play a crucial role in shaping bacterial evolution. They influence bacterial genome evolution through mechanisms like general and specialized transduction, which have been extensively researched in lab settings. (Chevallereau and Pons.,2022, Chiang and Penadés.,2019). Phages can adapt to specific hosts within a microbial community, favoring abundant and high-quality hosts while avoiding poor ones. Evolving the ability to bind to new receptors is a key step in expanding their host range, often requiring multiple mutations in genes controlling phage tail proteins. The presence of intermediate hosts can facilitate this adaptation. When exposed to various receptors, a phage population may develop multiple genotypes with distinct host ranges that do not overlap. (Heineman and Springman.,2008, Sordi and Khanna.,2017)

Antimicrobial efficacy of bacteriophages

Multidrug resistance is a global health issue since bacteria have developed resistance mechanisms against the antibiotics that are used to kill them. Phages have demonstrated potential to be effective and natural substitutes for antibiotics (Pang and Raudonis.,2019, Santos and Azeredo.,2019). The alternative therapy that utilizes the antibacterial activity of lytic phages is known as phage therapy. Even in small doses the phage particles are effective and they are safe for use in humans and animals. New biotechnological methods use recombinant phage and

purified phage lytic protein to combat multidrug-resistant bacterial development. (Lin and Koskella.,2017)

AIM:

To isolate clinically significant bacteriophages from wetland ecosystems.

OBJECTIVES:

1. Isolation of bacteria as hosts for bacteriophages.
2. Isolation of bacteriophage(s) and their characterization.
3. Determining the efficacy of the isolated bacteriophage(s) against the pathogenic bacteria.

HYPOTHESIS:

Various approaches are being researched, to tackle the problem of multidrug resistance in bacteria, and consequent growing ineffectiveness of conventional antimicrobial agents. One of the promising avenues for a solution is phage therapy. Bacteriophages are abundantly found in environments where their hosts are present. Thus, the present study proposes to isolate bacteria as well as bacteriophages from selected wetland ecosystems. Screening for bacteriophages against host bacteria is expected to reveal the presence of lytic phages. Further purification and characterization of these phage isolates may result in the establishment of promising candidates for phage therapy and other applications.

CHAPTER 2

LITERATURE REVIEW

Phage therapy

Bacteriophages offer several advantages as therapeutic agents. Firstly, they are highly specific and effective against their target bacteria, avoiding the disruption of natural micro flora in host organisms, unlike most antibiotics (Jamal and Bukhari.,2019, Marks and Sharp.,2000). Phages exclusively target bacteria and don't infect human or animal cells, and they can be administered through various methods. They replicate within susceptible bacteria for as long as they are present. (Clark and March.,2006; Skurnik and Strauch.,2006). Their mode of action differs from antibiotics, making them effective against multi- drug resistant bacteria. Phage production is straightforward, rapid, and relatively cost-effective. They have a history of successful use in treating infectious diseases in both plants (Fox.,2000) and animals (Barrow and lovell.,1998).

Phage therapy has been applied in humans to treat various diseases such as dysentery, skin infections, pulmonary infections, meningitis, infected wounds, or myelitis, caused by a wide range of organisms including *Staphylococcus spp.*, *Streptococcus spp.*, *E. coli*, *P. aeruginosa*, *Shigella spp.*, and *Salmonella spp.*(Shabalova and Karpanov.,1995, Weber-Dabrowska and Górski.,2001, Stroj and Weber-Dabrowska.,1999, Abdul-Hassan and El-Tahan.,1990)

Bacteriophage remedy has surfaced as a promising treatment option(Duckworth and Gulig.,2002, Wright and Hawkins.,2009,Sulakvelidzeetal et al.,2001) reported their operation in treating abscesses, septic injuries, vaginitis, mastoid infections, and respiratory tract infections. Recent studies on phage combinations have shown promising results for treating open septic injuries and burn injuries, supporting their remedial eventuality. (Abdul-Hassan and El-Tahan.,1990)

Other applications of bacteriophages

Biotechnological applications: Recently, phages have proved to be an important tool for biotechnologists. They are being utilized for various purposes like drug designing, emulsion of new proteins, delivery of protein and DNA vaccines, control of pathogenic bacteria, and netting of protein libraries, peptides, or antibodies (Sperinde et al.,2001,Clark and March.,2006). Phages are employed to treat antibiotic- resistant bacteria (Donnelly et al. 2015) and as bio control agents in husbandry, monoculture, and the oil painting oil and petroleum industries. (Gao et al. 2015, Haq and Chaudhry.,2012).

Agriculture and food safety.

Phage application in agricultural and food material is an emerging area of development. Bacteria such as *Salmonella*, *Campylobacter*, *Listeria*, and *E. coli* frequently cause food-borne illnesses (Herbert and DuPont.,2007) Bacterial infection in crops is a serious concern that decreases production. To prevent infection, a number of reports of phage spray on diverse crops (such as tomato, citrus, and onion) exist (Jones and Vallad.,2012, Balogh and Momol.,2003). Phage therapy can reduce bacterial contamination on numerous food items, including chicken flesh, beef, fruits, and vegetables. (Guenther and Huwyler.,2009, Marcó and Moineau.,2012.)

Aquaculture industries

Uncontrolled microbial infections pose a threat to the development and sustainability of aquaculture sectors, causing economic losses (Almeida and Gomes.,2009, Silva and Mateus.,2014). Fish-based products are gaining popularity worldwide as a low-cost protein source. Aquaculture products contaminated with pathogenic bacteria such as *Flavobacterium psychrophilum*, *Photobacterium damselae*, *Vibrio anguillarum*, *Vibrio vulnificus*, *Aeromonas*

hydrophila, and *Aeromonas salmonicida* are causing significant loss and disease in humans (Subasinghe and McGladdery.,2001, Nakai and Park.,2002,Flagel.,2006,). Phages have shown efficacy in treating *V. anguillarum* infection in fish larvae, as antibiotic therapies are less successful against multidrug-resistant bacteria (Silva et al.,2014). The effectiveness of aquaculture phage therapy is primarily determined by two factors: the number of phages produced by each host cell following lysis, and the time it takes for new phage particles to infect a new host.

Culture based technique used for isolation.

Plaque assay

The plaque assay, which was developed shortly after the discovery of bacteriophage by Felix de Herelle, is the earliest but still the most frequently employed and most effective method for enumerating bacteriophage. (Duckworth.,1976) Determining the phage titer is mostly done using the agar overlay method. It is the current "gold standard" for quantitating phage titers (Anderson and Rashid.,2011). The indicator bacteria grow by using the nutrients present in the bottom layer whereas there are fewer nutrients in the top soft agar. The identification of bacterial host is done mostly by checking the ability of bacterial host to form plaques calculated as PFU. (Lillehaug.,1997). After incubation period, the bacterial cells surrounding the phage are lysed forming a zone of clearance called plaque. (Panec and Katz.,2006)

Aliquots of diluted bacteriophage are combined with host bacterial cells in several ml of soft agar prior being disseminated onto media-containing agar plates. The use of soft agar assists the phage readily propagates throughout the media, leading to more consistent plaque development. The technique eliminates uneven bacterial-phage solution absorption into hard agar, that can lead

to uneven formation of plaque on the plate (Ellis and Delbrück.,1939). Bacteriophages infect and lyse host bacterial cells before spreading to additional cells nearby. After 6-24 hours, plaques can be seen in the bacterial growth on the plate. According to (Jones and Krueger.,1951) bacteriophage growth depends on the physical and chemical features of the system.

Using the double-agar-layer method, plaque-forming units (PFU) of *Bacteroides* phages related to the host bacterium was determined wherein using the *B. thetaiotaomicron strain GA17* human pollution and using *B. fragilis* strain PG76 porcine pollution was detected.(Balleste and Blanch.,2021).Using plaque assay technique, from litter sample of broiler 3 phages were isolated. Phage LestyG, Tamron, Kbmars were isolated from 3 different soil sample. (Eid and Tolba.,2022, Manu and Wolyniak.,2021)

Virus enrichment.

Initially phages are enhanced by incubating the sample with the necessary host and nutritive media, followed by isolation and amplification of the host and related bacteriophages in large quantities. This method identifies and amplifies host-specific phages from the primary sample. (Ghugare and Nimkande.,2018). The densities of bacteria and bacteriophages required for phage replication and eventual host cell killing are far lower than the numbers of bacteria attained in enrichment cultures .In addition, the physiological condition of enriched bacteria which are actively developing is ideal for bacteriophage replication. Thus, if certain phages are present in the sample, the densities of host bacteria achieved throughout the enrichment period may be sufficient to cause phage infection and reproduction, even if the starting phage count is low. (Muniesa and Blanch.,2005)

CHAPTER 3

METHODOLOGY

Requirements: Sterile plastic can, screw cap bottles, membrane filter, falcon tubes, Eppendorf tube, petri-plates, spreader, beaker, boiling tubes, conical flasks, wash bottle, tips, cuvettes, syringe filter, syringes, nichrome loop,

Instruments: Membrane filtration unit, vaccum pump, centrifuge, autoclave, hot air oven, incubator, laminar air flow, biosafety cabinet, UV spectrophotometer, Eppendorf centrifuge, weighing balance, electrophoresis unit, micro pipette (10µl, 100 µl, 1000 µl), refrigerator, Ph meter, refractometer,

Chemicals: Nutrient broth, nutrient agar, , Gram stains (crystal violet, grams iodine, safranin) ethanol, lysis buffer, proteinase k, SDS, phenol, chloroform, isoamylalcohol, isopropanol, triscl, EDTA, CTAB, NaCl ,agarose , ethanol, TBE, sterile milli q water ,DNTPS, buffer, reverse primer ,forward primer, Taq polymerase, glucose, sucrose, dextrose ,lactose, peptone , tryptone, O Meara reagent, Simmon citrate, Kovacs reagent, methyl red, hydrogen peroxide ,

Sample collection and processing

Water samples were collected from Nanda Lake and Sarzora Lake of Goa during wet and dry seasons respectively (Table 1). Samples were collected in sterile plastic cans, bought to the laboratory, filtered through a 0.22µm membrane filter and stored in sterile screw capped bottles in refrigerator until further use. The salinity and pH of both the water samples was checked during both the seasons.

Table1: Source of water sample, location co-ordinates, pH and salinity of the lakes during wet and dry season

	Sampling site	Location co-ordinates	pH (wet season)	pH (dry season)	Salinity wet season	Salinity dry season
07/07/2023	Nanda lake	15.241323,74.105460	6.48	6.38	0	0
28/11/2023	Sarzora lake	15.218624,74.004720	6.7	6.64	0	0

Isolation and purification of the host cultures.

The water sample was taken in a sterile tube and 0.1ml of it was spread plated on nutrient agar plates. After 24 hours the colonies from the plate were picked and re-streaked to get pure cultures. This pure culture was then Gram stained and their colony characteristics were noted.

Phage detection by double agar overlay plaque assay

10 ml of nutrient broth was prepared and autoclaved. Then loopful of culture was inoculated in this flask and then they were incubated at 37°C on the shaker incubator overnight. Nutrient agar plates were prepared having base layer containing 1.5% agar. The molten agar matrix containing 0.8% agar was mixed with 0.5ml of water sample and 1ml of bacterial host and poured on the solid agar medium (basal layer containing 1.5%agar). In a modification of the direct plaque assay, the host culture along with filtered water sample was inoculated in nutrient broth and the absorbance at 600nm measured for 6 days, in comparison with a control uninoculated culture flask. The culture that showed reduction in growth, as measured by absorbance values, was centrifuged and the supernatant filtered through a 0.22 μm syringe filter and used for plaque assay. The plates were kept stationary for one hour for pre- incubation and then inverted and incubated at 37°C for 24 - 48 hours. After incubation lawn of host bacterium formed over solid medium, clearance is seen where infectious phage particles have lysed the cells.

Growth measurement by absorbance at 600 nm.

A loopful of host culture was inoculated in 50 ml nutrient broth and incubated for overnight growth at 37°C. Then, 25 ml of it was transferred to another flask and used as control and the other half was inoculated with 1 ml of water sample (test). The growth of bacterial culture in test and control flasks was measured for 6 days in terms of absorbance at 600 nm. The test culture which showed decrease in absorbance, compared to control, was centrifuged at 10,000 rpm for 10 minutes at 4 °C and was then filtered through the 0.22 μm syringe filter and used for plaque assay.

Virus enrichment

Nutrient broth was prepared and autoclaved. 5 ml of water sample and 1 ml of host sample was taken and added to 10 ml of nutrient broth and, a control was kept containing 10 ml media and loopful of bacterial host. The flasks were checked visually for 4-5 days, to observe for any decrease in growth, compared to the control. If any change was observed, the contents of the respective flask would be filtered and assayed against the respective host culture for the presence of bacteriophages.

Identification of bacterial host cultures by molecular method

Genomic DNA isolation

2 ml of the bacterial culture was taken in an Eppendorf tube and was centrifuged at 10,000 rpm for 10 minutes. Th e supernatant was discarded and the pellet was resuspended in 565 µL of lysis buffer and incubated at 37°C for 45 minutes. After incubation, 30 µL of SDS and 3 µL of Proteinase k was added and was incubated at 37- 55°C for 1hour. Then 100µL of 5M NaCl was added followed by addition of 80µL of CTAB/NaCl mixture and incubated at 65°C for 10 minutes. Equal amount of Phenol: chloroform: isoamyl alcohol was added and centrifuged at 10000 rpm for 10 minutes. After centrifugation collect the aqueous layer and add equal amount of chloroform: isoamyl alcohol mixture and centrifuge at 10,000 rpm for 10 minutes. Collect the aqueous layer in a fresh Eppendorf tube and add 0.7 volume of isopropanol and incubated at room temperature for 1 hour. Then it was centrifuged at 10000 rpm for 15 minutes. Discard the supernatant and rinse the pellet in 1ml 70% ethanol. Allow the ethanol to evaporate and then resuspend the pellet in 15µL of 10 mM tris cl buffer and stored at -20°C for further use. The DNA that was isolated was loaded on a 0.8% agarose gel and observed under a UV transilluminator.

PCR amplification of 16S rRNA gene from bacterial genomic DNA

Table 2: Reagents used for PCR

Sr. No	Reagents	Vol.in µl
1	Water	29.5
2	PCR buffer	5
3	dNTP mix	5
4	Forward primer (27F)	2.5
5	Reverse primer (1492R)	2.5
6	DNA template	5
7	Taq polymerase	0.8
	Total	50

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

Table 3: PCR parameters

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

Identification of bacterial host cultures by biochemical method

Sugar fermentation test: Glucose peptone broth was prepared and dispensed 4.5 ml of it in each test tube with inverted Durham's tube and autoclaved .0.5% sugar solutions are prepared and autoclaved. After autoclaving under sterile conditions transfer 0.5 ml of sugar solution in each of the tube. Then Inoculated loopful of culture in each tube and incubated at 37 °C for 24- 48 hours. Note down the observations.

Catalase test: With the help of nichrome loop smear of actively growing 24-hour old culture is made on the surface of the slide. Add a drop of hydrogen peroxide and observe for bubble formation. Rapid formation of bubbles indicates positive result.

Indole test: Tube containing tryptone broth is inoculated with a small amount of a pure culture. The tubes were incubated at 37°C for 24 hours. Indole production is tested by adding 5 drops of Kovác's reagent directly to the tube. The test is positive if there is formation of pink to red color ring in reagent layer on top of medium after adding the reagent. If negative test then the reagent layer will remain yellow or be slightly cloudy.

Methyl red test: Glucose peptone broth is prepared and autoclaved. 5 ml of it is dispensed in each tube and then inoculated with the culture. The tubes were incubated at 37°C for 24 hours. After incubation the content is divided into 2 parts of which 1 part is used for Voges Proskauer test. Then methyl red indicator is added. Red color indicates positive result and yellow color indicates negative result.

Voges Proskauer test: Using sterile loop Glucose peptone broth is inoculated with 24-hour old bacterial sample and incubate the tube for 24 hours at 37°C. After incubation O'Meara reagent is added and the tube is kept for the reaction to complete for 30 minutes. Red color formation indicates positive result.

Citrate utilization test: Simmon citrate slants are made and inoculated with test organism by stab / streak method. Then the tubes are incubated for 24-48 hours at 37°C. Then Observed for color change to blue which indicates positive result and if color of media remains same.

Motility test: 0.5% nutrient agar is prepared and autoclaved and slants are made. Then stab the culture. Observe the tubes

CHAPTER 4

ANALYSIS AND CONCLUSIONS

Wetlands are teeming with life, including a rich abundance of bacteriophages, which are viruses that infect and replicate within bacteria. They play a crucial role in regulating bacterial populations, shaping microbial communities, and cycling nutrients within wetland ecosystems. In the dynamic balance of life within wetlands, bacteriophages are both players and spectators, influencing the intricate web of interactions that sustain these biodiversity habitats.

4.1.Sample collection and processing

The water samples were collected from wetland ecosystems, specifically Nanda Lake and Sarzora Lake, during wet and dry seasons.

Fig. 1.a

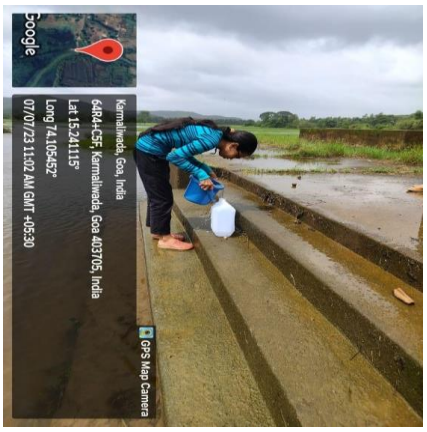


Fig. 1.b

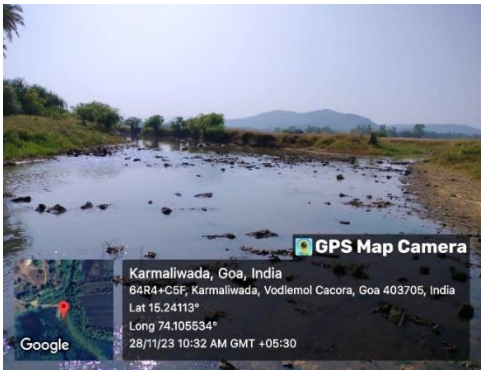


Fig1. c

Fig1. a- Location of sample collection (Nanda Lake)

Fig 1.b - Sample collected during wet season

Fig 1.c- Sample collected during dry season

Fig 1.d

Fig 1.e

Fig 1.f

Fig 1.d-Location of sample collection (Sarzora lake)

Fig 1.e- Sample collected during wet season

Fig 1.f- Sample collected during dry season

4.2.Isolation of bacterial host cultures from wetland sites

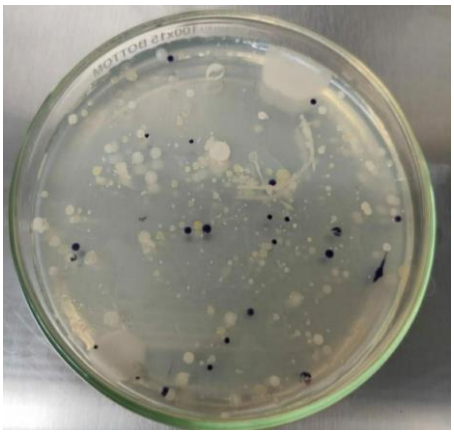


Fig 2.a

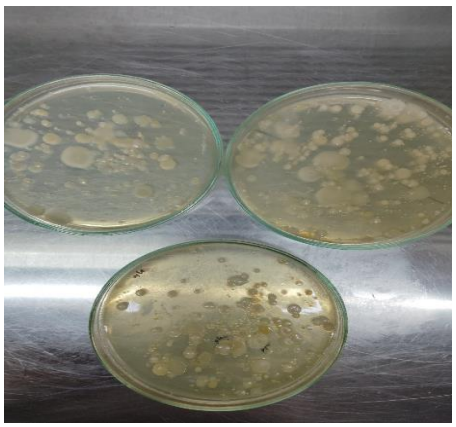


Fig 2.b

Nanda lake sample spread plated Fig 2.a- During dry season, Fig 2.b- During wet season.

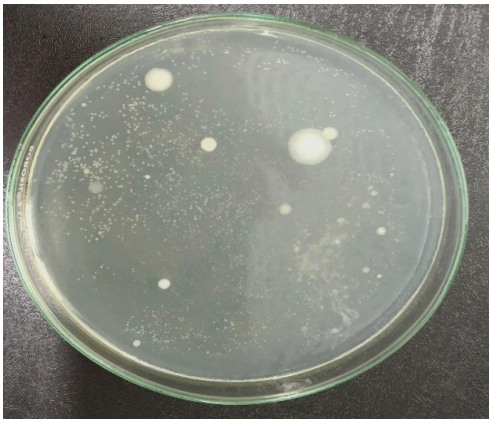


Fig 2.c

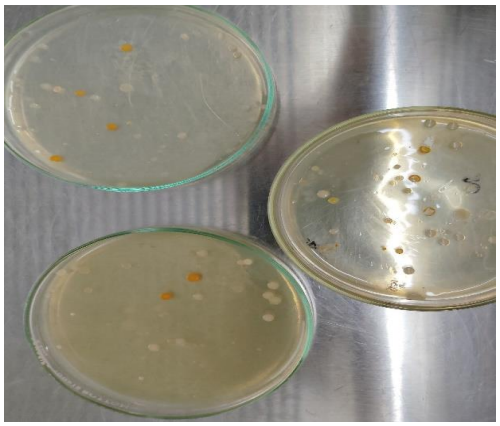


Fig 2.d

Sarzora lake sample spread plated Fig 2.c- During wet season, Fig 2.d- During dry season.

4.3.Pure cultures of bacterial isolates obtained from the wetland water sample.

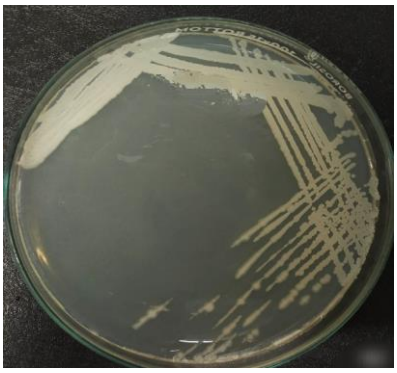


Fig 3.a

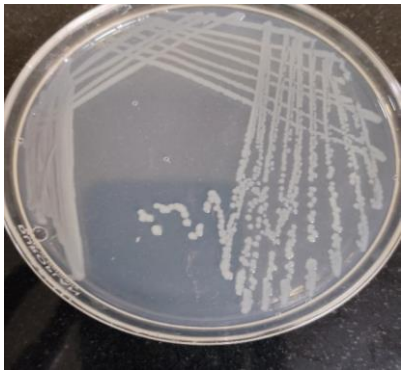


Fig 3.b

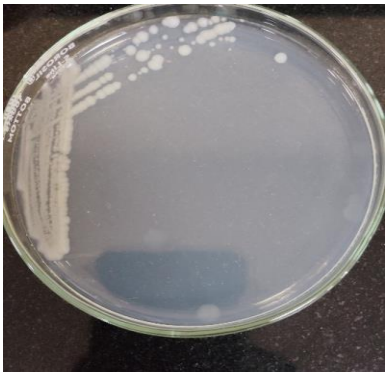


Fig 3.c

Fig 3.d

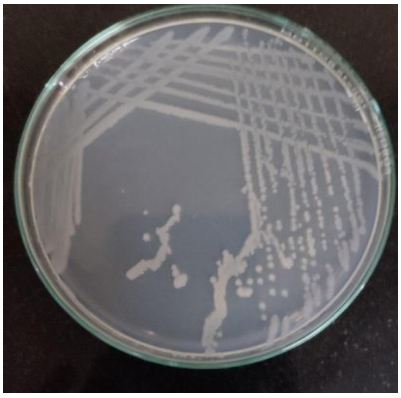


Fig 3.e

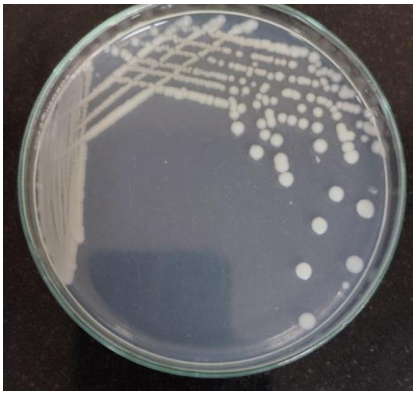


Fig 3.f

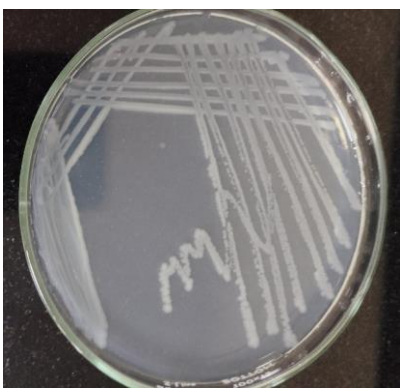


Fig 3.g

Fig 3.h

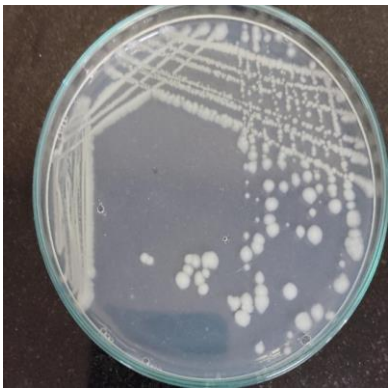


Fig 3.i

Fig 3. a. SNN1, b. SNN2, c. SNN3, d. SNN4, e. SNN5, f. SNN6, g. SNN7, h. SNN8, i. SNN9.

The host cultures were isolated from the aquatic ecosystem, Nanda Lake. In total, 9 bacterial cultures were isolated from the water sample which were maintained on nutrient agar plates. These cultures were re-streaked to get pure cultures.

Morphological characterization of the bacterial isolates

Table 4: Colony characteristics of bacterial cultures isolated from Nanda lake

Characters	SNN1	SNN2	SNN3	SNN4	SNN5	SNN6	SNN7	SNN8	SNN9
Size	0.3cm	0.4cm	0.6cm	0.5cm	0.1cm	0.1cm	0.2cm	0.4cm	0.6cm
Shape	Irregular	Circular	Circular	Irregular	Circular	Circular	Circular	Circular	Irregular
Color	Cream	White	Cream	Off-white	Cream	Off-white	White	White	Cream
Margin	Curled	Entire	Entire	Curled	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Crateriform	Flat	Flat	Flat	Raised	Flat	Flat	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Gram character	Gram positive	Gram negative	Gram negative	Gram positive	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative

Sarzora lake:

Fig 3.j

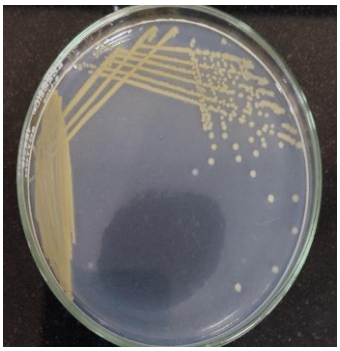


Fig 3.k

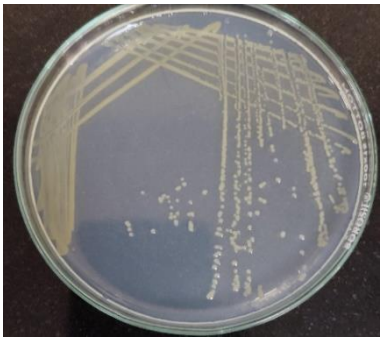


Fig 3.l

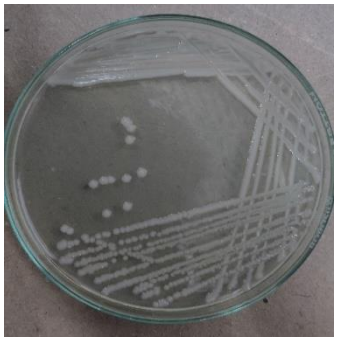


Fig 3.m

Fig 3.j - SNS1, fig3. k. SNS2, Fig 3.l. SNS3, Fig 4.m. SNS4

This four cultures were isolated from Sarzora lake and maintained on nutrient agar plates , they were re-streaked to get pure culture.

Table 5: Colony characteristics of bacterial cultures isolated from Sarzora lake.

Characters	SNS1	SNS2	SNS3	SNS4
Size	0.7cm	0.4cm	0.3cm	0.5cn
Shape	Irregular	Circular	Circular	Irregular
Color	Cream	Orange	Creamish orange	white
Margin	Lobate	Entire	Entire	Curled
Elevation	Flat	Flat	Flat	Flat
Surface texture	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Gram character	Gram positive	Gram positive	Gram negative	Gram positive

Pathogenic bacteria

Six pathogenic cultures, previously isolated from a clinical sample were also maintained.

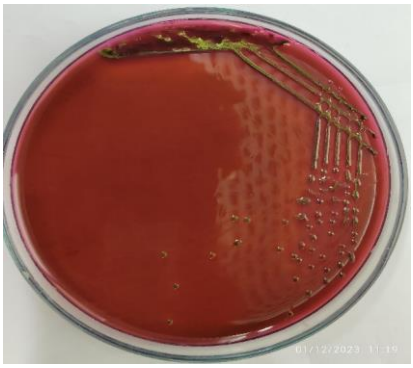


Fig 3 n.Emb1

Fig 3 o.Emb2

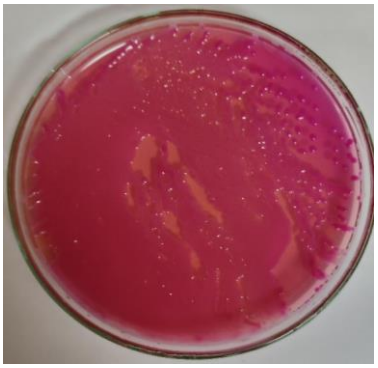


Fig 3 p. Mac

Fig 3q. SS

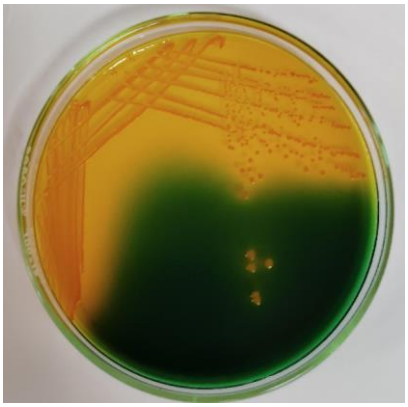


Fig 3 r.Tcbs1

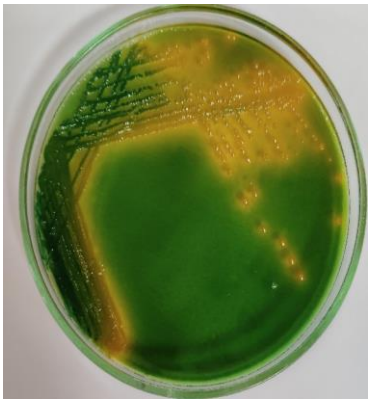


Fig 3s.Tcbs2

The 6 pathogens previously identified by selective media on which they were streaked

Emb1- Metallic sheen is seen indicating presence of *E. Coli*.

Emb2- Colorless colony.

Mac -Enterobacteriaceae family.

SS- presence of yellow color colonies indicate presence of Salmonella.

Tcbs1 and Tcbs2 both gave yellow-colored colonies indicating presence of *Vibrio cholerae* and *Vibrio alginolyticus*.

4.4. Gram staining of the isolates.

Nanda lake

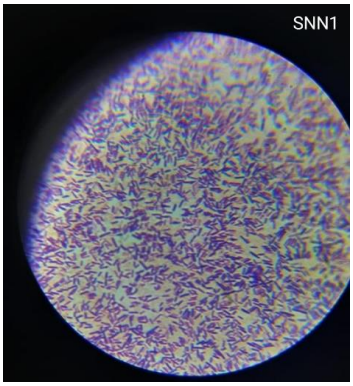


Fig 4.a

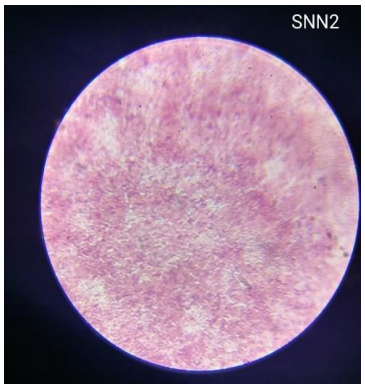


Fig 4.b

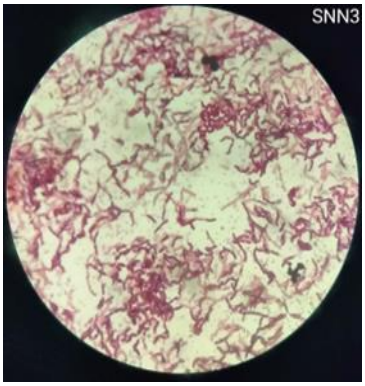


Fig 4.c

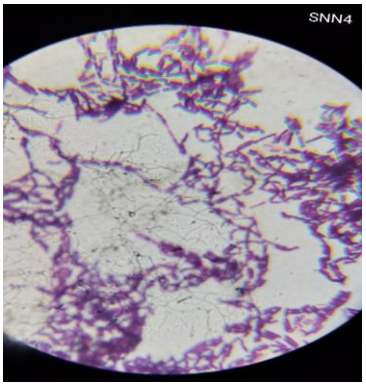


Fig 4.e

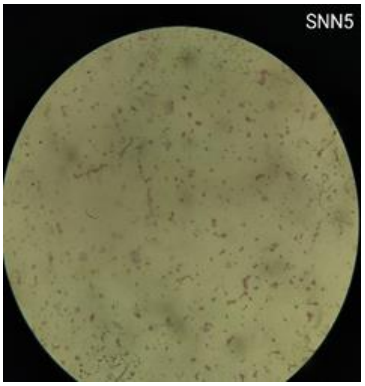


Fig 4.d

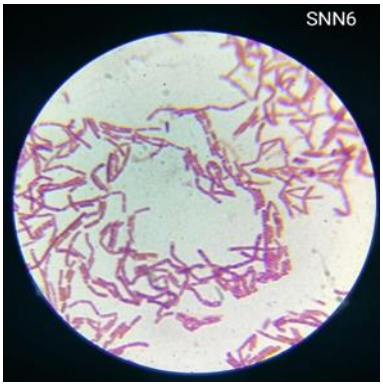


Fig 4.f

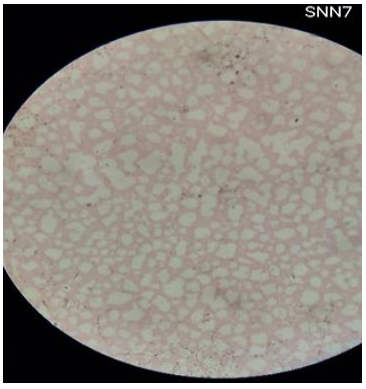


Fig 4.g

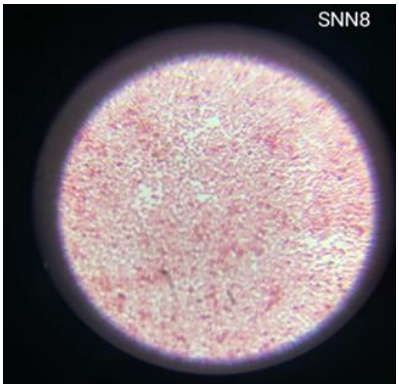


Fig 4.h

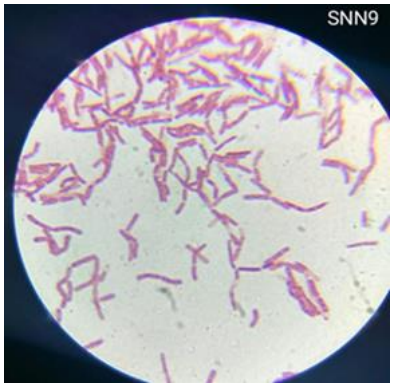


Fig 4.i

Fig 4.a,b,c,d,e,f,g,h,i -Pictures of gram staining of bacterial cultures isolated from Nanda lake.

Of the nine cultures isolated from the Nanda Lake, 2 were gram positive which are SNN1 and SNN4 and the rest seven were gram negative SNN2, SNN3, SNN5, SNN6, SNN7, SNN8, SNN9.

Sarzora lake

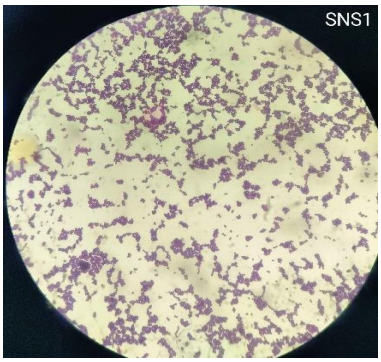


Fig 4.j

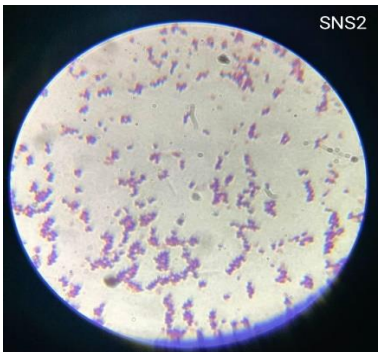


Fig 4.k

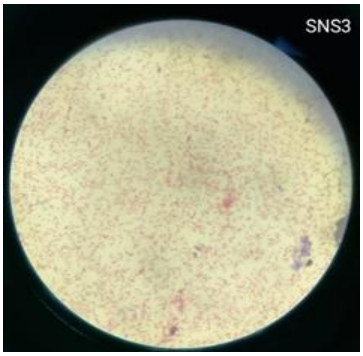


Fig 4.l

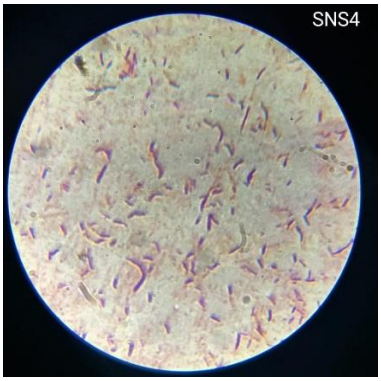


Fig 4.m

Of the 4 cultures isolated from Sarzora lake, 3 cultures were gram positive, i.e. SNS1, SNS2, SNS4 and 1 culture SNS3 was gram negative.

Pathogenic cultures.

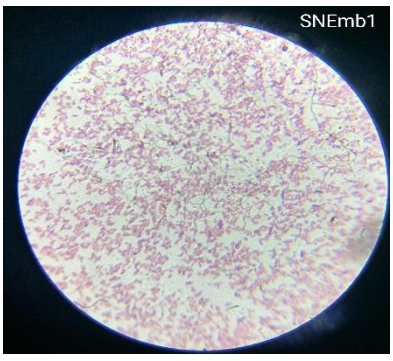


Fig 4.n

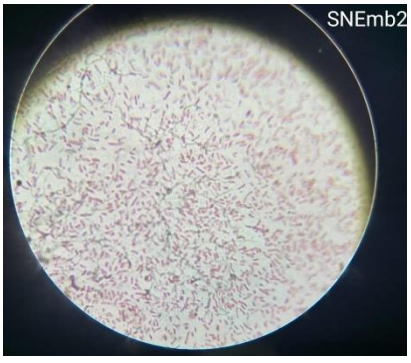


Fig 4.o

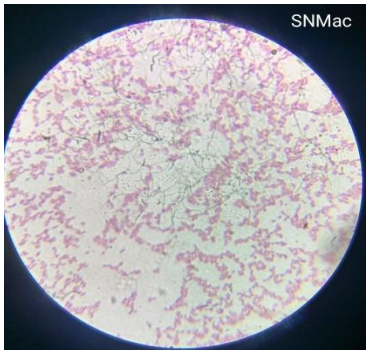
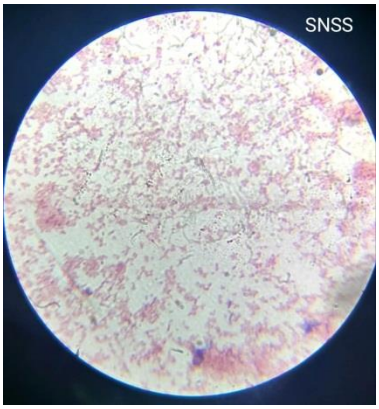
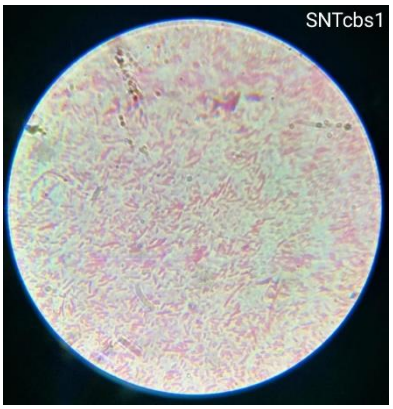


Fig 4.p





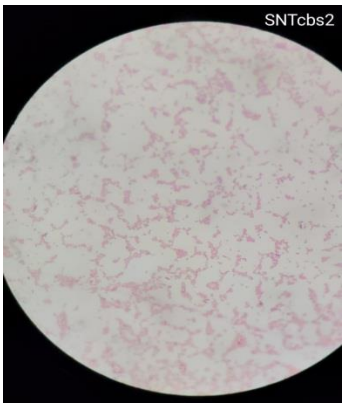


Fig 4.q Fig 4.r Fig 4.s

6 previously isolated pathogenic cultures were maintained and their gram staining was done. All the 6 cultures were gram negative.

4.5. Bacteriophage detection in liquid and solid media

Fig 5.a

Fig 5.b

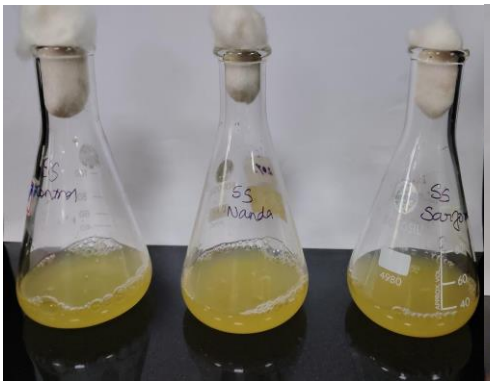


Fig 5.c

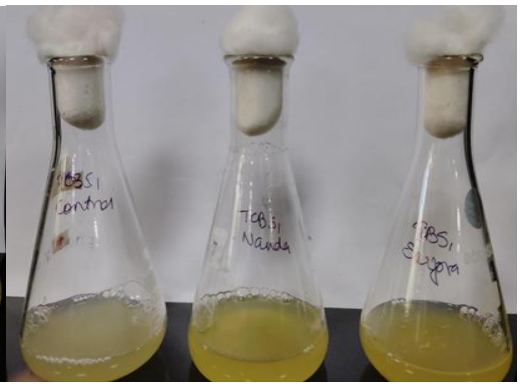


Fig 5 .d

Fig 5 .a, b, c, d : Cultures inoculated in liquid broth prior to plaque assay.

Table 6: Absorbance of bacterial cultures inoculated in nutrient broth with water sample from Nanda and Sarzora lake during wet season.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
SN Emb 1 control	1.852	1.955	2.069	2.119	2.285	2.309
SN Emb 1 Nanda - wet	1.949	2.075	2.144	1.943	1.916	1.702
SN Emb1- Sarzora wet	1.9	2.191	2.854	2.173	1.875	1.841
SN Emb2 control	2.078	2.391	2.228	2.344	1.87	1.833
SN Emb2- Nanda wet	2.045	2.092	2.236	1.76	1.432	1.365
SN Emb2- Sarzora wet	2.026	2.029	2.292	2.292	2.322	1.637
SN Mac control	1.972	2.091	2.225	1.925	1.177	1.083
SN Mac- Nanda wet	1.932	2.11	2.214	2.085	1.81	1.545
SN Mac -Sarzora wet	1.933	1.882	2.041	1.575	1.162	0.985
SN SS control	1.874	1.962	1.962	2.179	2.230	2.295
SN SS -Nanda wet	1.937	2.135	1.91	1.847	1.603	0.833
SN SS -Sarzora wet	1.875	1.905	2.114	2.245	2.231	1.252
SN Tcbs1 control	1.126	1.392	1.37	1.392	1.397	1.927
SN Tcbs1 -Nanda wet	0.087	0.964	1.86	1.572	1.195	1.172
SN Tcbs1 -Sarzora wet	0.924	1.351	1.894	1.351	1.266	1.064
SN Tcbs2 control	1.224	2.138	2.144	2.365	2.46	2.532
SN Tcbs 2- Nanda wet	1.831	2.1	2.206	2.178	2.028	2.022
SN Tcbs2- Sarzora wet	1.743	2.778	2.361	2.279	2.169	2.077

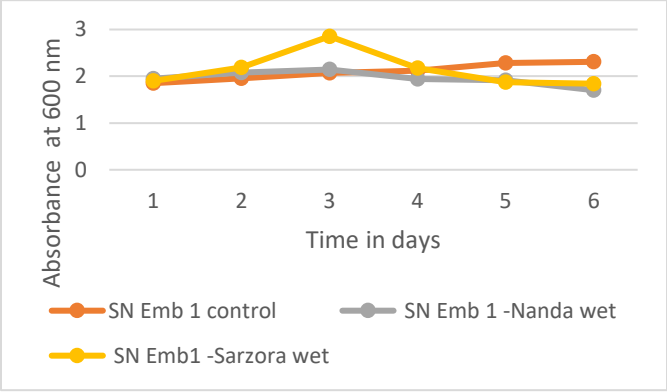


Fig 5.e

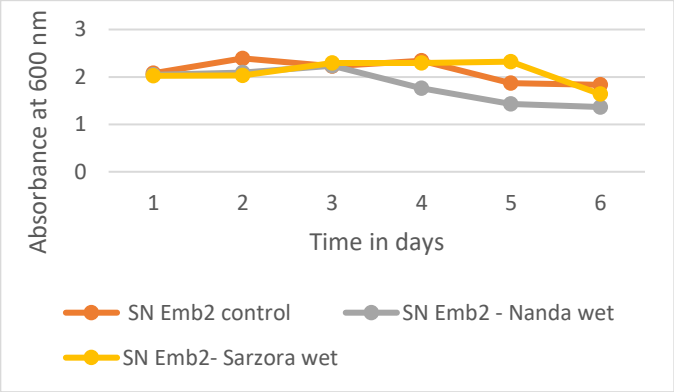


Fig 5.f

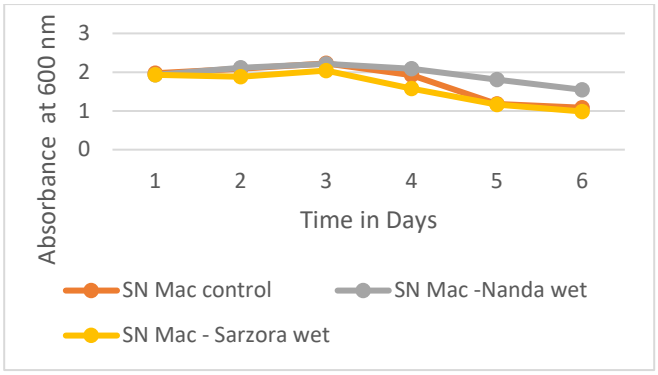


Fig 5.g

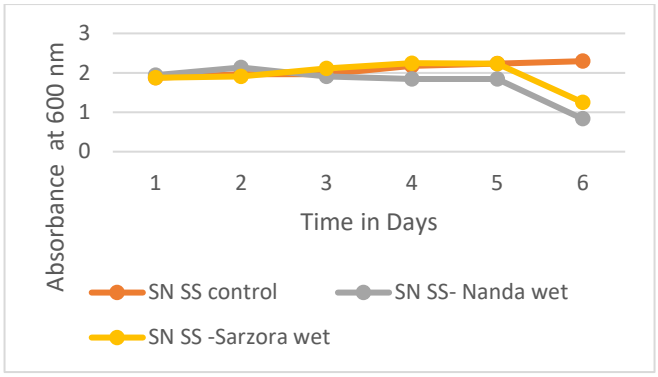


Fig 5.h

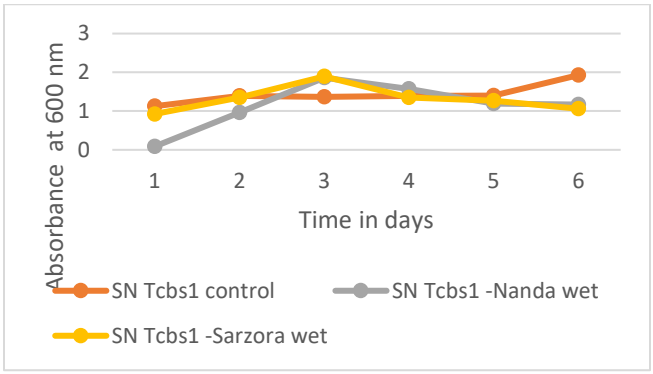


Fig 5.i

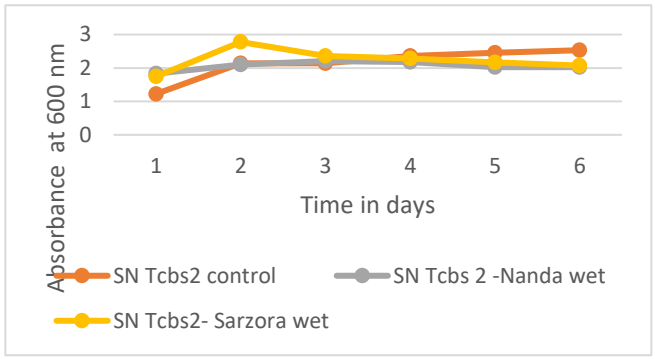


Fig 5.j

Fig 5.e-j: Growth of test and control cultures, measured in terms of absorbance at 600 nm, over a period of 6 days

The test flasks which showed decreased growth, compared to control flasks (reflected in the decreased absorbance) were Tcbs2 Nanda and Emb2 Sarzora. These were selected for further processing to perform the plaque assay, for detection of the presence of bacteriophages.

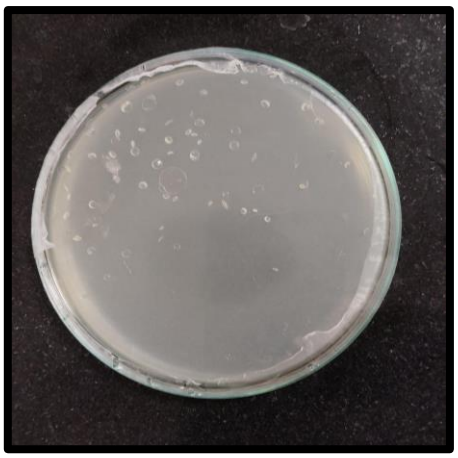


Fig 5.k

Fig 5.l

Fig 5. k, l - Plaque assay carried out by double agar overlay method

Table 7: Set 1: Absorbance of bacterial samples inoculated in nutrient broth with water sample from Sarzora lake

	1	2	3	4	5	6
SNEmb1 control	0.916	1.3	1.306	1.33	1.64	1.645
SNEmb1-Sarzora dry	1.325	1.726	1.593	1.373	0.809	0.728
SNEmb2 control	0.855	1.359	1.453	1.459	1.649	1.65
SNEmb2 -Sarzora dry	1.138	1.616	1.529	1.428	0.979	0.887
SNMac control	0.643	1.332	1.094	1.375	1.16	1.234
SNMac -Sarzora dry	1.311	1.621	1.535	1.342	0.832	0.61
SNSS control	0.975	1.42	1.636	1.841	1.969	0.863
SNSS-Sarzora dry	1.208	1.652	1.639	1.52	0.751	0.64
SNTcbs1 control	1.727	1.845	1.566	1.46	1.332	1.243
SNTcbs1- Sarzora dry	1.38	1.414	1.604	1.364	1.35	1.05
SNTcbs2 control	1.609	2.082	1.971	1.98	1.684	1.239
SNTcbs2- Sarzora dry	1.92	2.364	2.2	2.145	2.031	1.72

Table 7: Set 2: Absorbance of bacterial samples inoculated in nutrient broth with water sample from Sarzora lake

	1	2	3	4	5	6
SNEmb1Control	1.054	1.817	1.288	1.187	1.074	0.963
SNEmb1-Sarzora dry	1.259	2.108	1.576	1.26	1.22	0.913
SNEmb2 control	1.582	1.587	1.679	1.771	1.319	1.023
SNEmb2-Sarzora dry	1.58	1.569	1.654	1.268	1.156	0.848
SNMac control	2.256	1.926	1.551	1.299	1.203	0.872
SNMac-Sarzora dry	1.588	1.685	1.746	1.542	1.266	0.89
SNSS control	1.746	1.84	1.858	1.68	1.897	1.084
SNSS -Sarzora dry	1.267	1.262	1.656	1.454	1.444	1.225
SNTcbs1 control	2.126	1.755	1.324	1.164	0.944	0.89
SNTcbs1 -Sarzora dry	2.096	1.741	1.36	1.003	0.928	0.608
SNTcbs2 control	1.801	1.89	1.896	1.945	1.996	2.01
SNTcbs2- Sarzora dry	1.63	1.645	1.527	1.428	1.543	1.013

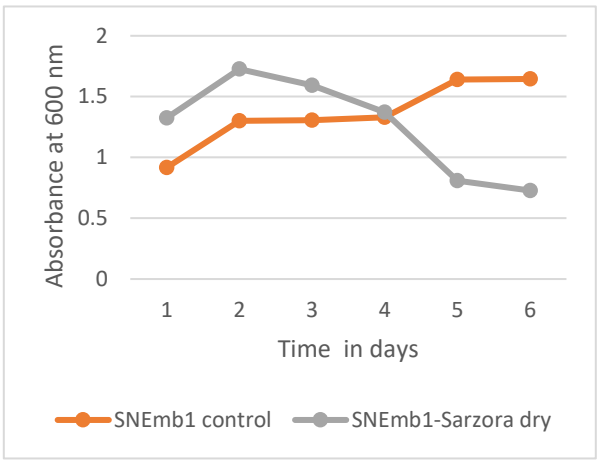


Fig 6.a

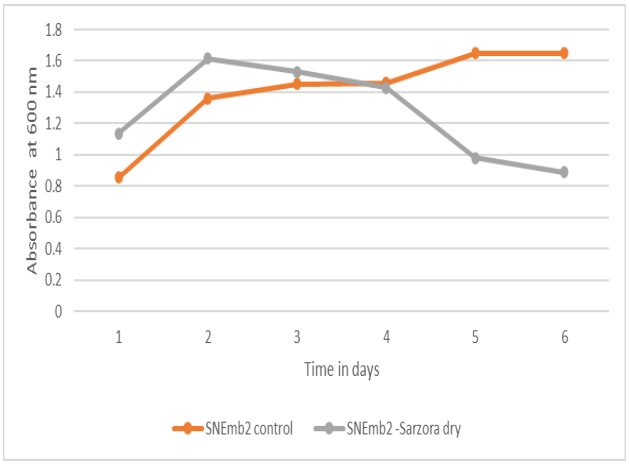


Fig 6.b

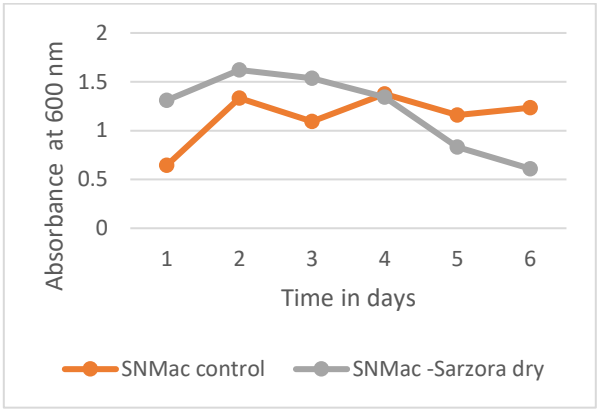


Fig 6.c

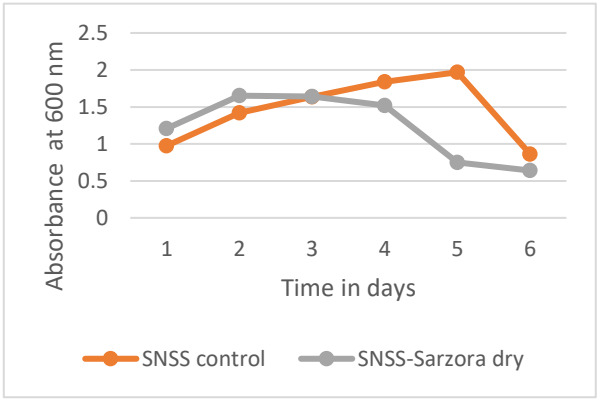


Fig 6.d

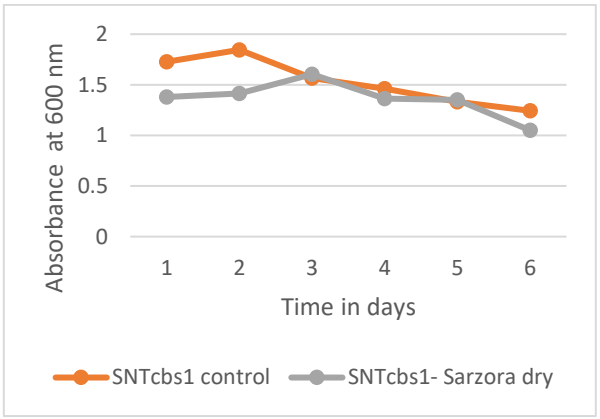


Fig 6.e

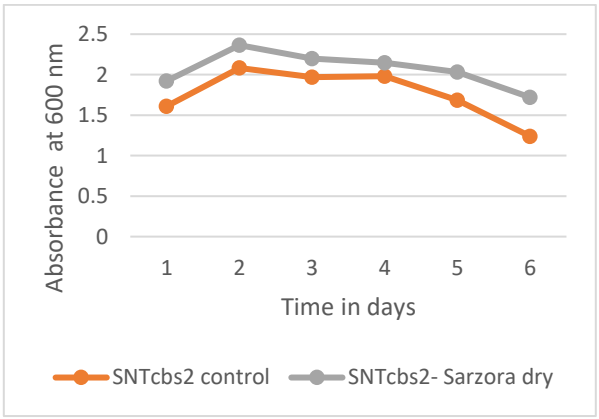


Fig 6.f

Fig 6.a-f – Growth of test and control cultures, measured in terms of absorbance at 600 nm, over a period of 6 days

Fig 6.g

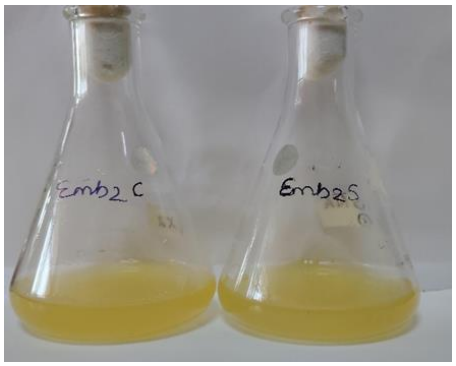


Fig 6.h

Fig 6.i

Fig 6.j

Fig 6.k

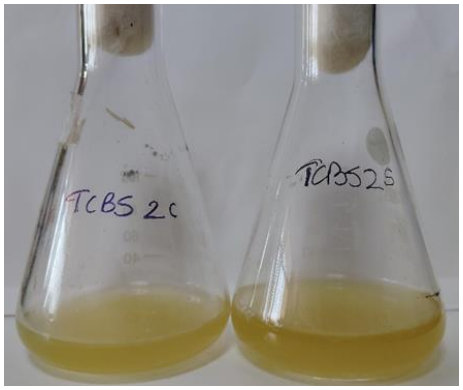


Fig 6.l

Fig 6g-l : flasks inoculated with bacterial culture to perform plaque assay

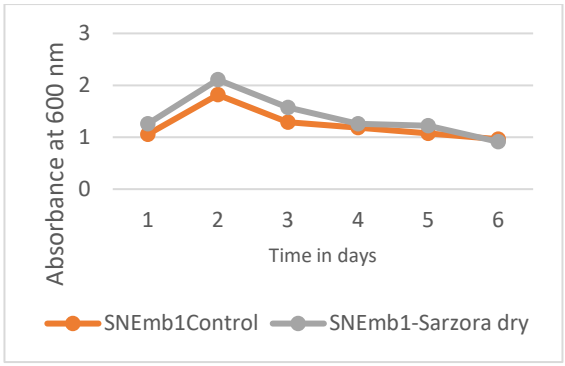


Fig 6.m

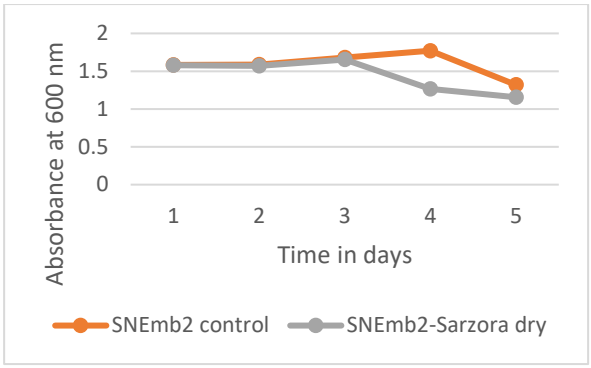


Fig 6.n

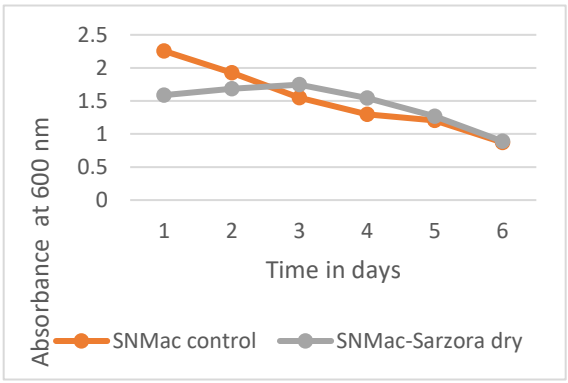


Fig 6.o

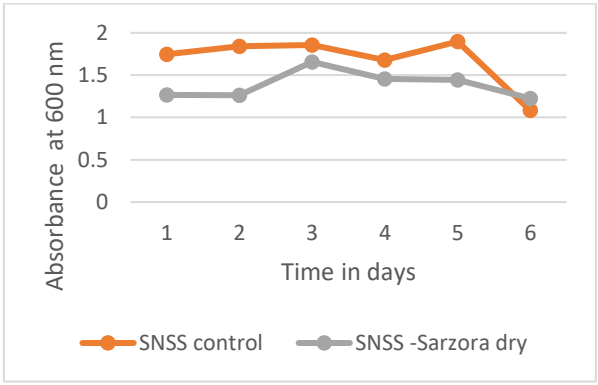


Fig 6.p

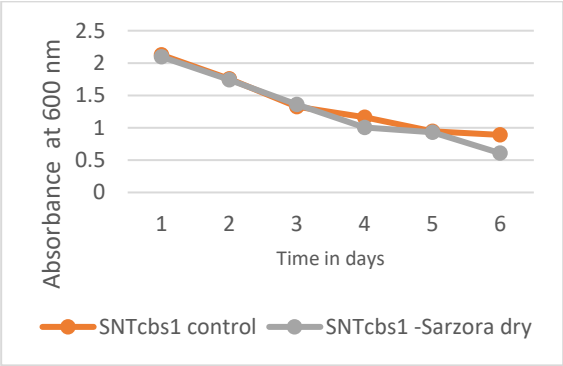


Fig 6.q

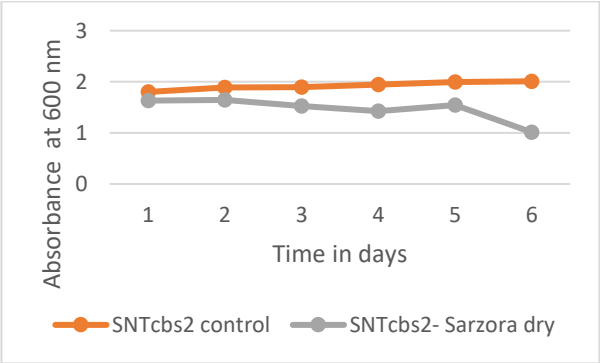


Fig6.r

Fig 6m-r: Growth of test and control cultures, measured in terms of absorbance at 600 nm, over a period of 6 days

Table 8: Absorbance of bacterial samples inoculated in nutrient broth with water sample from Sarzora lake

	1	2	3	4	5	6
SNN2 control	0.243	0.437	0.531	0.661	0.789	0.89
SNN2 -Sarzora dry	0.509	0.982	1.066	1.33	1.316	1.123
SNN3 control	2.301	1.705	1.872	2.205	2.245	2.386
SNN3- Sarzora dry	2.62	2.194	2.133	1.84	1.876	1.474
SNN4 control	1.056	1.702	1.796	1.941	1.197	0.746
SNN4 -Sarzora dry	0.838	1.763	1.77	1.506	0.952	0.753
SNN5 control	2.146	1.857	1.896	2.092	2.22	2.232
SNN5- Sarzora dry	2.064	2.159	2.011	1.827	1.82	1.478
SNN6 control	2.172	2.198	2.273	2.269	2.85	2.289
SNN6-Sarzora dry	2.326	2.193	2.14	1.765	1.76	1.402
SNN7 control	0.799	1.421	1.625	1.761	1.776	1.812
SNN7- Sarzora dry	0.932	1.714	2.215	1.45	1.209	0.981
SNN9 control	0.979	1.702	1.802	1.868	1.96	1.969
SNN9 -Sarzora dry	1.514	1.876	1.716	1.619	1.173	0.947
SNN11 control	1.385	1.844	1.86	1.868	1.92	1.931
SNN11- Sarzora dry	1.351	1.943	1.475	1.376	0.949	0.626
SNS1 control	0.699	1.74	1.804	1.879	2.32	2.341
SNS1- Sarzora dry	0.852	2.275	1.975	1.756	1.2	1.144
SNS2 control	0.821	1.607	1.619	1.693	1.42	1.022
SNS2- Sarzora dry	0.941	1.735	1.708	1.62	1.156	0.935

Fig 7.a

Fig 7.b

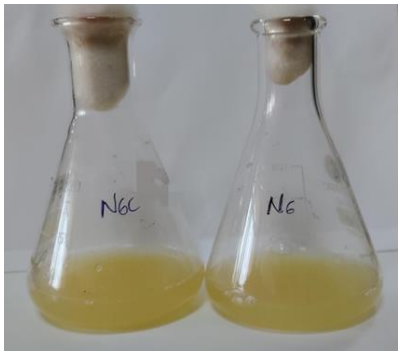


Fig 7.c

Fig 7a-c: flasks inoculated with bacterial culture to perform plaque assay

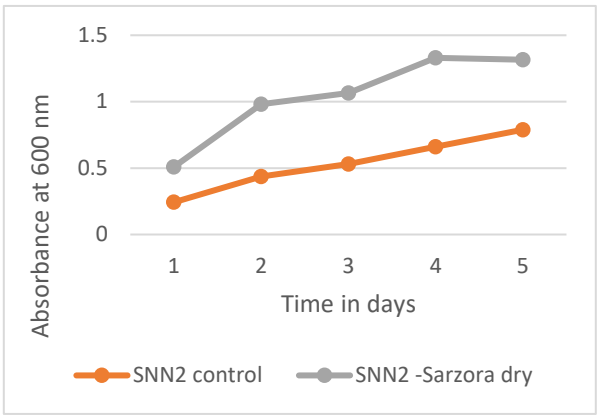


Fig 7.d

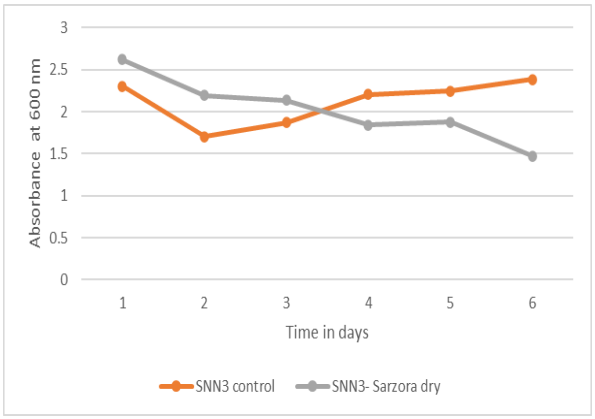


Fig 7.e

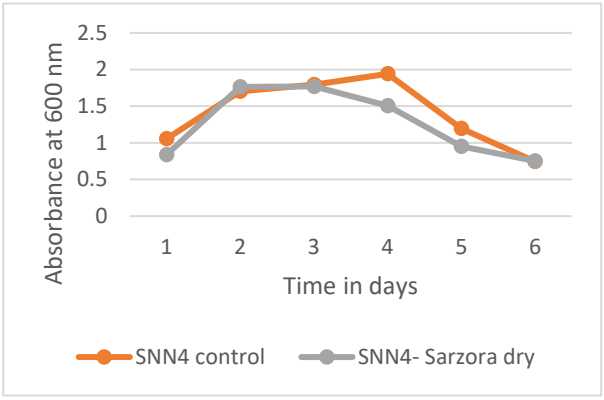


Fig 7.f

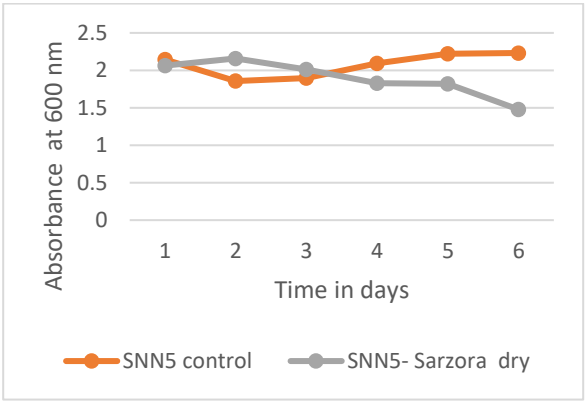


Fig 7.g

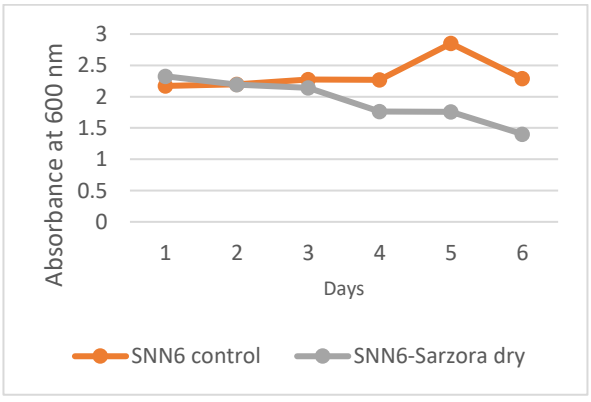


Fig7.h

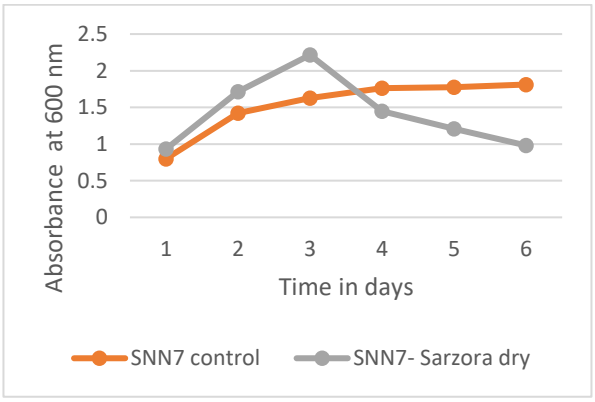


Fig 7.i

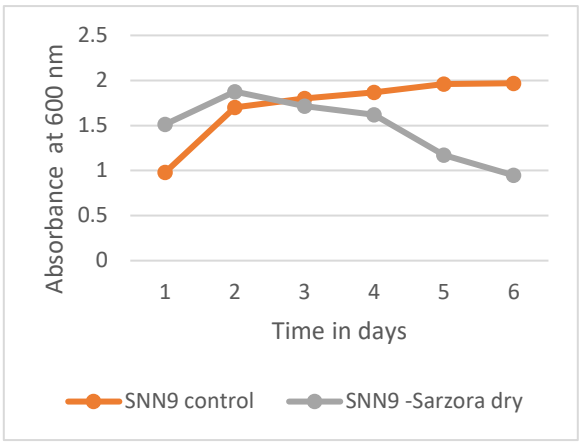


Fig 7.j

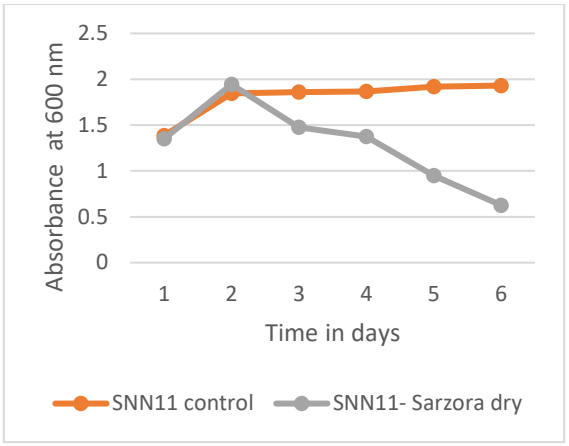


Fig7.k

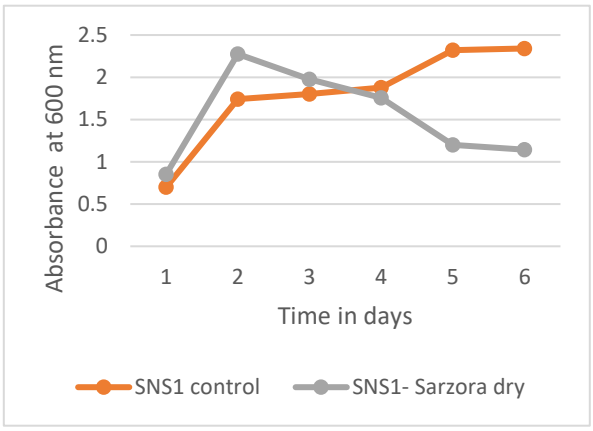


Fig7.l

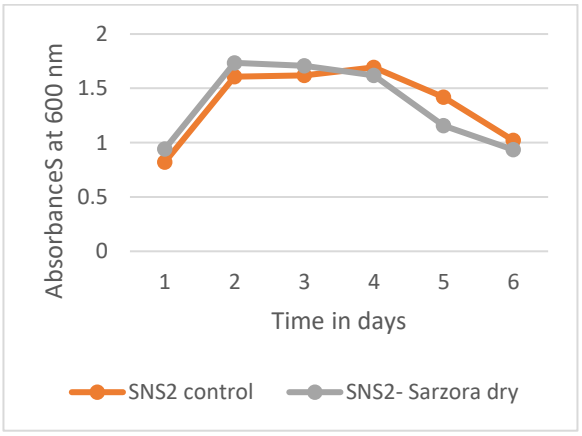


Fig.7.m

Fig 7.d-m - Growth of test and control cultures, measured in terms of absorbance at 600 nm, over a period of 6 days

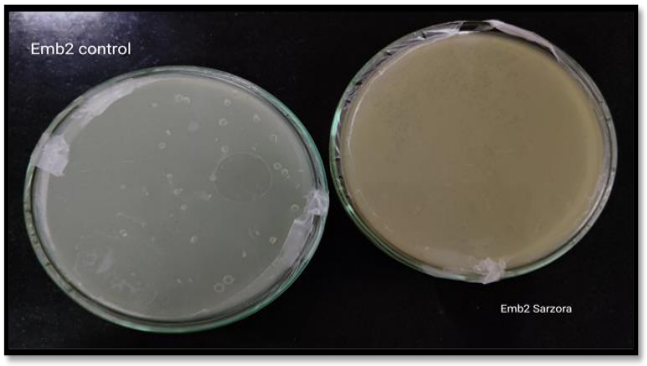


Fig 7.n

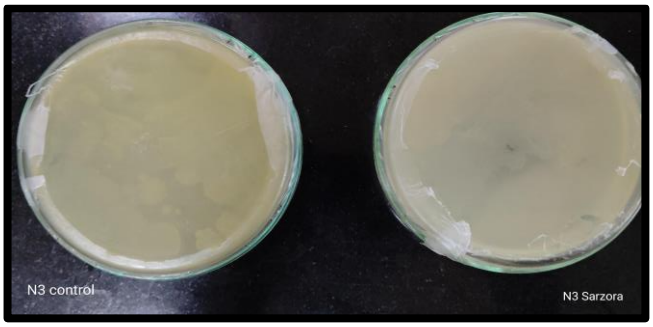


Fig 7.o

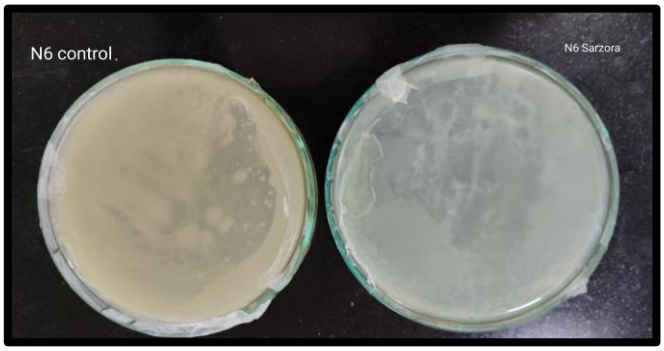


Fig 7.p

Fig7. n-pPlaque assay plates showing negative result.

The test flasks which showed reduction in growth were used to perform the plaque assay (Emb2 Sarzora, N3 Sarzora and N6 Sarzora). However, plaque formation was not observed on any of the host lawns.

Table 9: Absorbance of bacterial isolates inoculated in nutrient broth with water sample from Sarzora lake during dry season.

	1	2	3	4	5	6
SNS1 control	0.870	1.576	1.273	1.134	0.989	0.892
SNS1- Sarzora dry	0.776	1.149	1.234	0.987	0.892	0.851
SNS2 Control	0.518	0.652	0.869	0.960	1.234	1.509
SNS2- Sarzora dry	0.454	0.902	1.228	1.146	0.949	0.876
SNS3 Control	0.757	0.816	0.963	1.079	1.113	1.189
SNS3 -Sarzora dry	0.774	0.887	1.145	1.045	0.899	0.513
SNS4 Control	0.598	0.567	1.032	1.099	1.234	1.546
SNS4 -Sarzora dry	0.624	0.987	1.078	0.999	0.897	0.831

Fig 8.a

Fig 8.b

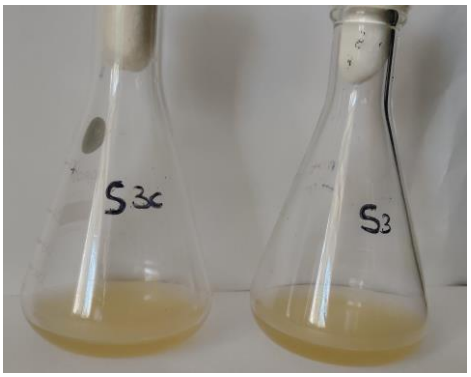


Fig 8.c

Fig 8.d

Fig 8.a –d The bacterial isolates inoculated in liquid broth for plaque assay.

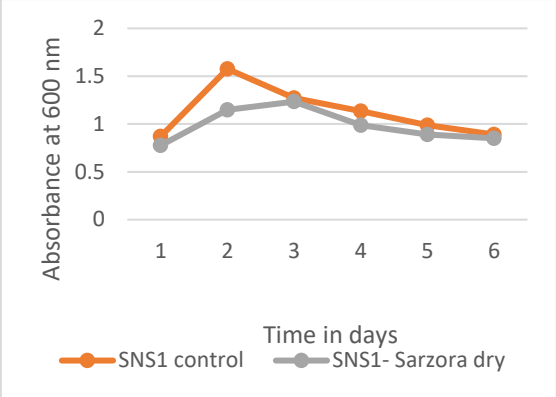


Fig 8.e

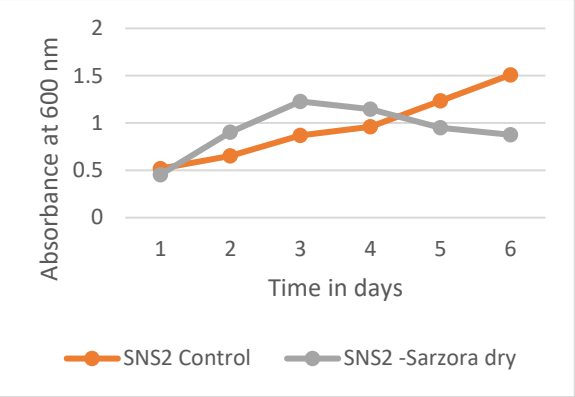
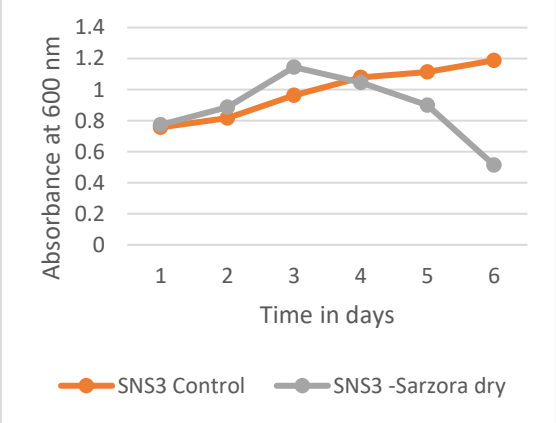


Fig 8



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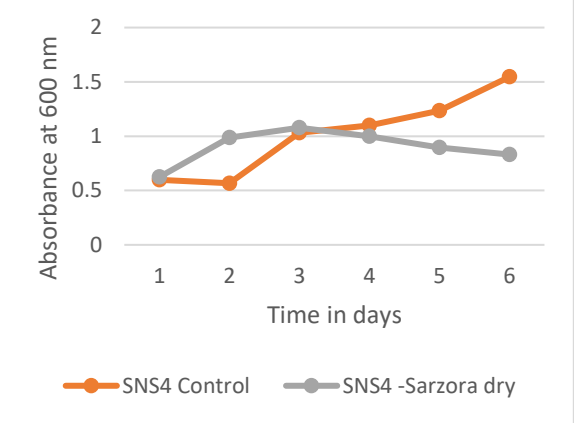


Fig 8.h

Fig8. e-h Growth of test and control cultures, measured in terms of absorbance at 600 nm, over a period of 6 days.

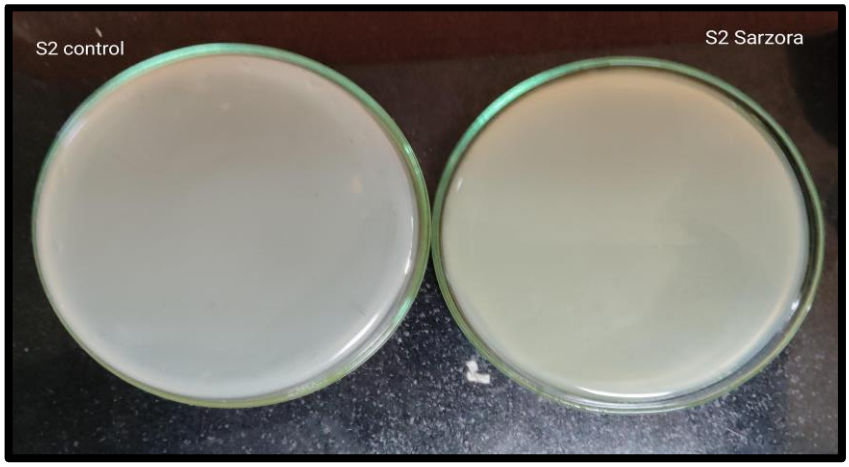


Fig 8.i

Fig 8.j

Fig 8.i –j Plaque assay plates of which S4 shows zone of clearance.

Here the two cultures SNS2 and SNS4 showed significant reduction in the absorbance over 6 days. Upon performing the plaque assay as per the protocol, the SNS4 test plate depicted a zone where the host lawn was cleared (Fig. 8 j). This indicated the presence of a lytic bacteriophage against host bacterium SNS4. Further attempts at purification of the bacteriophage, followed by repeated plaque assays were unsuccessful, indicating that propagation of the phage under laboratory conditions was unsuccessful.

4.9. Virus enrichment

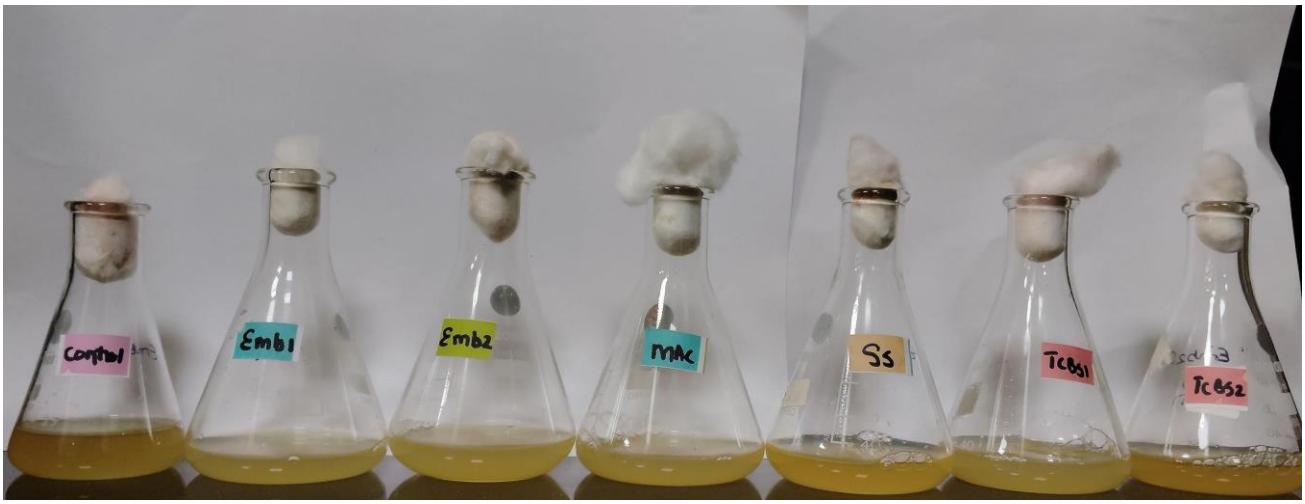


Fig 9.a – Virus enrichment method for isolation of bacteriophages in liquid medium

For virus enrichment, the cultures were inoculated in liquid broth, they were kept static for a week. They were checked visually for difference. There was no difference seen between the control and the cultures.

Identification of host cultures – molecular method

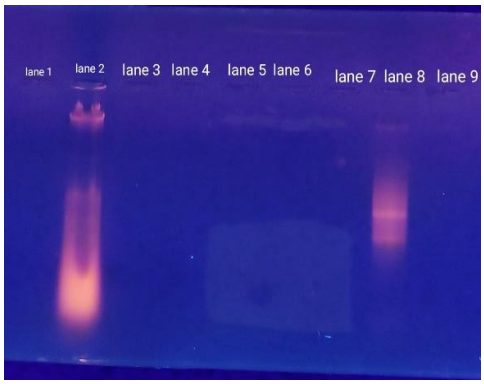


Fig 10 .a

Lane 1- SNN3, lane 2-SNN4, lane 3-SNN5, lane 4-SNN6, lane 5- SNN7, lane 6-SNN8, lane 7-SNS1, lane 8 - SNS2, lane 9-SNS3.

Lane 10- SNN7 and lane 11- SNS1

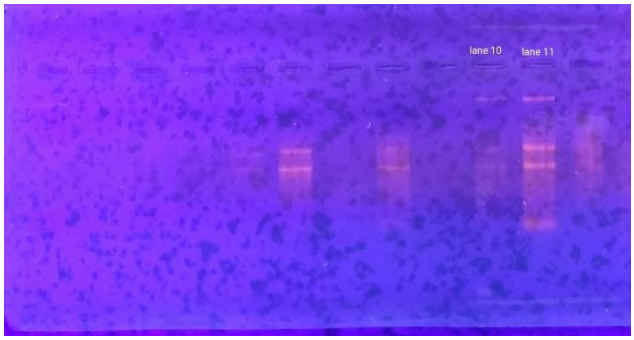


Fig 10 .b

Isolation of genomic DNA was successfully carried out for cultures SNN4, SNS2, SNN7 and SNS1, and was unsuccessful for SNN3, SNN5, SNN6, SNN7, SNN8, SNS1, SNS3.

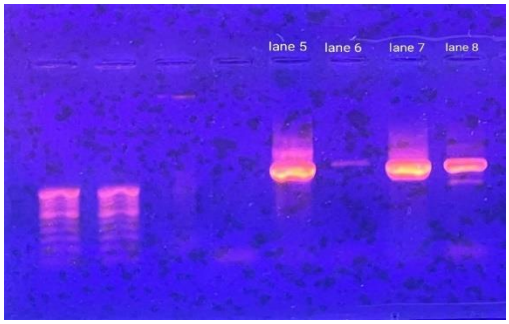


Fig 10c

Lane 5-SNS1, Lane 6-SNS2,

Lane 7-SNN7, Lane 8- SNN4

PCR amplification of the 16S rRNA gene was carried out, using the bacterial genomic DNA as template. The PCR products of expected size were further submitted for Sanger sequencing for the purpose of identification of host cultures. However, the sequencing could not be carried out, due to non-specific amplification products.

4.11. Identification of host cultures – biochemical method

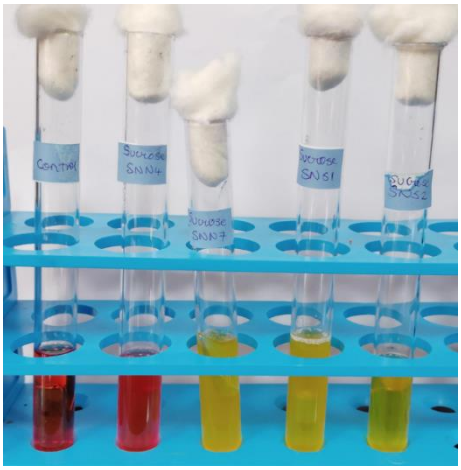


Fig 11.a

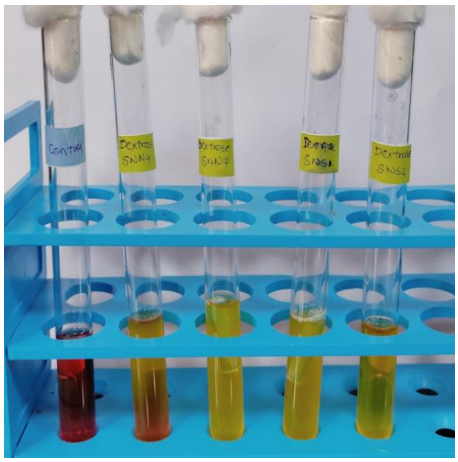


Fig 11.b

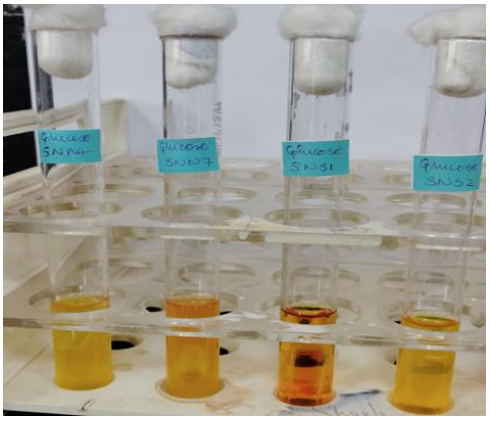


Fig 11.c

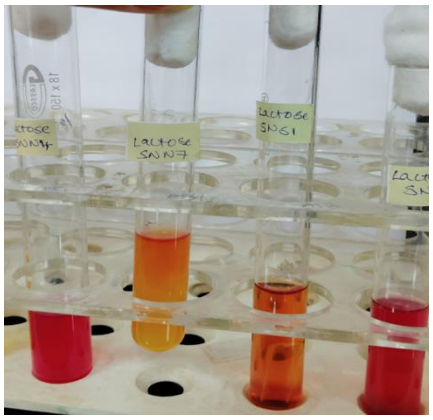
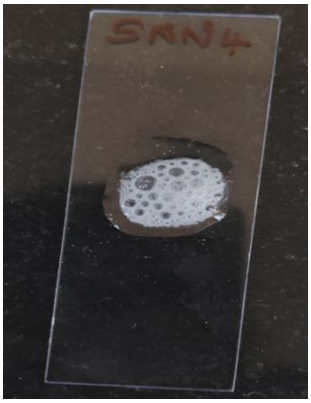


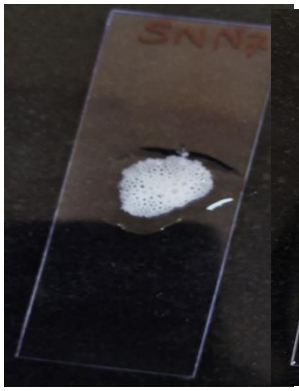
Fig 11.d

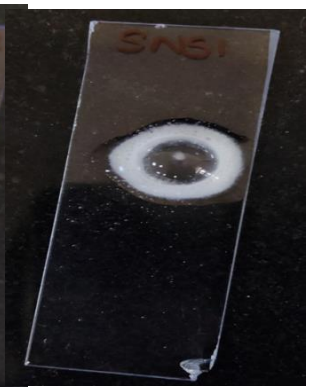
Fig 11 a-d : Sugar fermentation test results .

	SNN4	SNN7	SNS1	SNS2
Sucrose	+	+	+	+
Dextrose	+	+	+	+
Glucose	+	-	+	+
Lactose	-	+	-	-

Table 10: Result of sugar fermentation test.







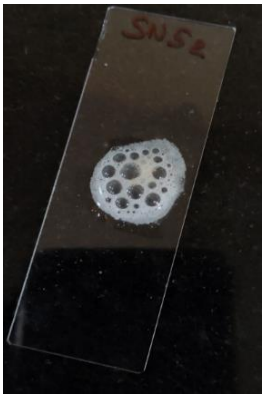
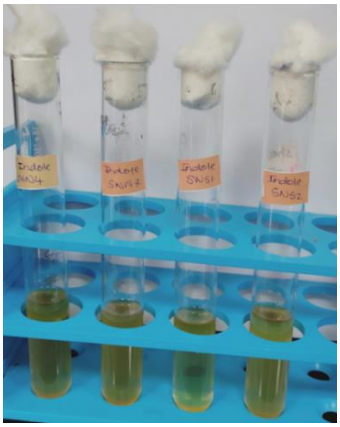
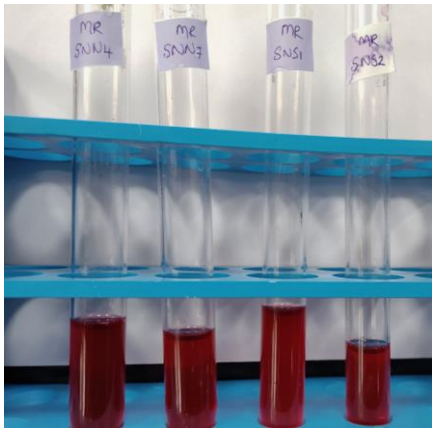


Fig 11.d Fig 11.d Fig 11.e Fig 11.f

Fig 11.d –h: Culture SNN4, SNN7, SNS1, SNS2 exhibit bubble formation indicting positive catalase activity





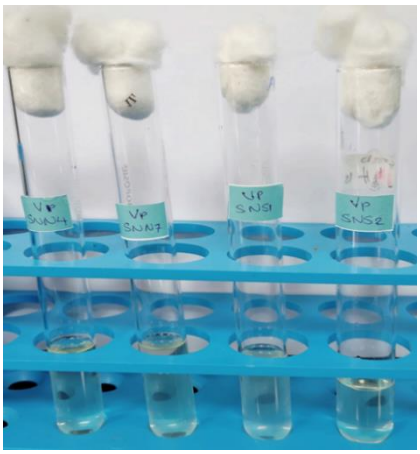
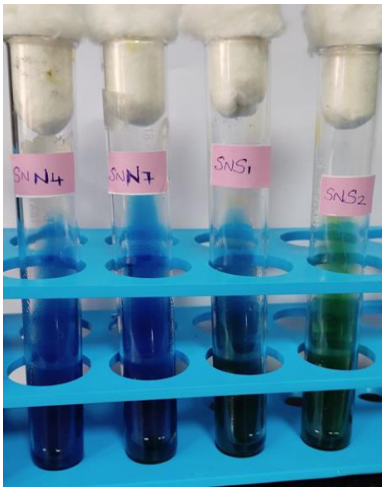


Fig 11.g Fig 11.h Fig 11.i

Fig 11.g Culture SNN4, SNN7, SNS1, SNS2 are Indole negative.

Fig 11.h Culture SNN4, SNN7, SNS1, SNS2 show positive result.

Fig 11.i Voges Proskauer test was negative for all 4 cultures SNN4, SNN7, SNS1, SNS2 .



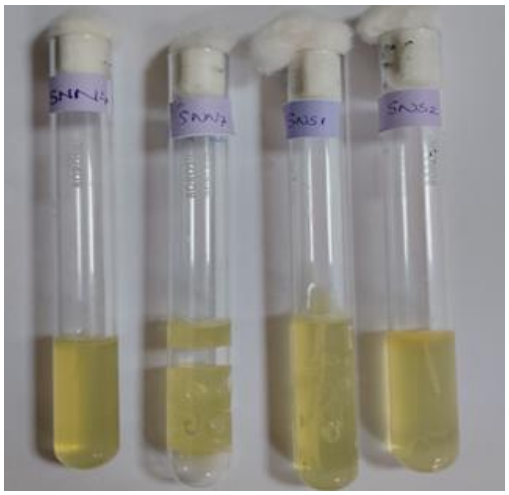


Fig 10.j Fig 10.k

Fig 11.j Citrate test was positive for culture SNN4, SNN7, SNS1 and was negative for SNS2

Fig 11.k Motility test was positive for culture SNN7, SNS1 and negative for SNN4, SNS2.

	SNN4	SNN7	SNS1	SNS2
Sucrose	+	+	+	+
Dextrose	+	+	+	+
Glucose	+	-	+	+
Lactose	-	+	-	-
Catalase	+	+	+	+
Indole	-	-	-	-
Methyl red	+	+	+	+
VP	-	-	-	-

Citrate	+	+	+	-
Motility	-	+	+	-

Table 11: Result of biochemical test.

In the present study, we have attempted to isolate bacteriophages against pathogenic bacteria, as well as bacterial hosts isolated from the same environments as the samples taken for phage isolation. Isolation of phages which could have potential use in phage therapy was unsuccessful, despite repeated screening. Several other researchers have faced similar situations. In one study, the researchers tried to isolate bacteriophages against *Staphylococcus* sp. from various sources but found it challenging (Mattila and Ruotsalainen., 2015). Limitations of the protocol used for screening could also be a factor. The processing of crude water samples by filtration through membranes of different pore sizes (0.45 or 0.22 µm) is carried out to remove bacteria; however, this step might also retain larger bacteriophages which might hinder isolation. (Hyman.,2019). Ghugare and Nimkande.,2017 used an enrichment method where bacteriophages that were retained along with their bacterial host on the membrane filter were enriched in liquid broth. Differences between the natural environment and the laboratory environment such as change in temperature, pH and surrounding medium, the influence of other members of the natural community which is absent in lab conditions, may also contribute to the difficulty of isolating bacteriophages (Ly-Chatain et l.,2014).

CONCLUSION

Though phages are abundant in nature, there could be various challenges that are faced while isolating bacteriophage due to which their isolation may be unsuccessful. Various factors like limitation in methods used, phage host interactions, contamination could hinder isolation. By understanding the reason behind unsuccessful isolation, optimizing conditions of the experiment and by refining the methods used one can increase the likelihood of isolating bacteriophage.

FUTURE PROSPECTS

- Identification of novel bacteriophage from wetland ecosystem.
- Molecular identification and characterization of the isolated bacteriophage.

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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

Lysis buffer

Ingredient	50ml
1M tris Hcl	50 µl
0.5 M EDTA	1000 µl
10% SDS	500 µl

Make up volume to 50 ml using distilled water.