

**STUDY ON BACTERIOPHAGE AGAINST MARINE
BIOFILM FORMING BACTERIA**

Submitted in partial fulfilment of the requirements for the award of Bachelor of Science in
Biotechnology

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Sundara vibu M** who carried out the project entitled **"STUDY ON BACTERIOPHAGE AGAINST MARINE BIOFILM BACTERIA"** under my supervision from August 2022 to April 2023.

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I **Sundara Vibu M (40770052)** hereby declare that the Project Report entitled “**STUDY ON BACTERIOPHAGE AGAINST MARINE BIOFILM FORMING BACTERIA**” done by me under the guidance of **Dr. D. Inbakandan(External guide)** at **CENTRE FOR OCEAN RESEARCH, COL. DR. JEPPIAAR RESEARCH PARK, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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ABSTRACT

Although antibiotics are used to treat biofilm-based illnesses, there is a significant risk to human health since pathogen-produced bacterial biofilms are known to be hundreds of times more resistant to antimicrobial drugs than planktonic cells. Bacteriophage (also known as phage) are thought of as potential treatments for bacterial infections because of their high specificity, safety, and non-toxicity as natural enemies of bacteria. The dynamic biofilm mode of life is enabled by the biofilm matrix, which is created during biofilm formation. Building biofilms and the lifestyle that result from them requires a series of well-defined steps that result in a spatially and temporally diverse bacterial community. A self-produced matrix of hydrated extracellular polymeric substances (EPS) serves as the immediate habitat for the microbes in biofilms. Polysaccharides, proteins, nucleic acids, and lipids make up the majority of EPS, which gives biofilms their mechanical resilience, facilitates their attachment to surfaces, and forms a cohesive, three-dimensional polymer network that links and momentarily immobilises biofilm cells. They also offer significant promise for defending against and eliminating harmful bacterial biofilms. The presence of bacteriophage was clearly correlated with the quantity of bacterial indicators. From this study, a Bacteriophage have been isolated. They were characterized based on the morphology of the plaque formation. This paper reviews on composition and formation process of bacterial biofilm and summarises the several phage-based applications for the control biofilm.

KEY WORDS Biofilm, Bacteriophage, Marine bacteria, Isolation of bacteriophage.

LIST OF CONTENT

CHAPTER	TITLE	PAGE NO
1	Introduction	
2	Review of literature 1. Bacteriophage 2. Marine biofilm 3. Biofilm formation 4. Marine sources 5. Bacteriophage in food pathogen 6. Bacteriophage in drinking water 7. Membrane bioreactor 8. Desalination 9. Materials 1. Zobel Marine Agar 2. Hard agar 3. Soft agar 4. Ca-Mg 5. 0.2µm syringe filter 6. Sterile syringe 7. Falcon tube (15ml) 8. Centrifuge 9. Bacterial isolation 10. 16s rRNA sequence	
3	Aim & Objectives 1. Aim 2. Objectives	
4	Materials and Method 1. Materials 2. Method 1. Phage isolation and purification 2. Passage & Phage lysate preparation 3. Bio-characterization 1. pH stability 2. Temperature 3. Salinity 4. Crystal Violet Assay 5. Fluorescence microscopy	
5	Results 1. pH stability 2. Temperature 3. Salinity 4. Crystal Violet Assay 5. Fluorescence microscopy	
6	Conclusion	
7	References	

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO
2.1	Composition of ZMB	
4.1	Bacterial isolation	
5.1	Result for pH stability	
5.2	Result for temperature	
5.3	Result for salinity	

LIST OF FIGURES

CHAPTER	TITLE	PAGE NO
2.1	0.2µm syringe filter	
2.2	Sterile syringe	
2.3	Falcon tube	
2.4	Centrifuged sample	
4.1	Phage trapping	
4.2	Phage titration	
4.3	Neat	
5.1	pH stability	
5.2	Temperature	
5.3	Salinity	
5.4	Crystal violet assay	
5.5	Control AK9- Day 1&2	
5.6	Phage BW1.1 – Day1&2	

CHAPTER 1

INTRODUCTION

Bacteriophage, or phage as they are more often known, are necessary parasites of bacteria. It is possible to isolate bacteria phage from a variety of habitats, including soil, water and the food industry. Bacteriophage are quite prevalent in the environment, with sewage sample values of 3.16×10^6 phage ml⁻¹. Phage are distributed throughout nature, as seen by the high concentrations ($>10^6$ PFU ml⁻¹) found in soil and ocean water. Nevertheless, only a small portion of phage are ever infectious to their host. Bacteriophage have been used in various fields, including medicine, agriculture, and food production. They have been used as an alternative to antibiotics to treat bacterial infections, particularly those that are resistant to multiple drugs. They have also been used to control bacterial populations in the food industry and as a biological control agent in agriculture. Research on bacteriophage continues to be an important area of study for understanding the diversity of viruses and their interactions with bacteria. This study's primary objective is to isolate Bacteriophage against marine Biofilm bacteria from marine effluent water. Also, to identify the host range of the Bacteriophage, to conduct salt tests, conduct different pH tests, and to conduct temperature tests. A multicellular population of bacteria known as a "bacterial Biofilm" forms a self-produced, highly hydrated extracellular matrix and adheres to both biological and non-biological surfaces. Antimicrobial drugs are prevented from penetrating the inside the Biofilm by the extracellular polymeric components of the Biofilm matrix. The term "extracellular polymeric material" refers to the sticky matrix that surrounds these communities and facilitates cellular organisation (EPS).

Bacterial biofilms are collections of bacteria that have formed a self-made matrix and are affixed to a surface or to one another. Proteins (such as fibrin), polysaccharides, and other materials make up the biofilm matrix. The three phases of biofilm development are adhesion, maturity, and dispersion. Desiccation and antibiotics cannot effectively treat biofilms. Antibiotics, acidic conditions, heat, desiccation, and biofilms are all very resistant to them. Phage may sterilise bacteria, making them an apparent replacement for antimicrobials and disinfectants in the fight against microorganisms. Phage have a significant role in determining microbial diversity and can have an impact on the structure and operation of microbial communities. Lysogenic and Lytic phage are two different forms of phage; Lytic phage may multiply, release lysine, and have strong lytic activity. The availability of nutrients and oxygen in the water, which affect cell metabolism, as well as the physical characteristics of the flowing system, such as flow velocity, temperature, and the makeup of the solid surface, all have a role in the development of Biofilm. The resistance mechanisms in biofilms appear to be multicellular in nature. It is necessary to explore the impacts of the system type, flow rates, density, and species composition in order to effectively regulate Biofilm growth in heat exchangers. Unwanted biofilm deposition and development is referred to as biofouling. The creation of ultra-pure drinking and

process water, the colonisation of medical equipment, and the fouling of ship hulls, pipelines, and reservoirs are all examples of settings in which this phenomenon might take place. Despite the fact that biofouling may arise in many different places, the biofilm is its common cause. A biofilm's bacteria may exhibit significant levels of resistance to biocides and antibiotics. The concentration of such compounds necessary to have an antibacterial impact can be over a thousand times higher than the concentration needed to kill free-living (planktonic) bacteria. Compared to conventional antibiotics or biocides, bacteriophage have a distinct effect on bacteria that are embedded in biofilms. In fact, it is possible to argue that phage and bacterial biofilms co-evolved, therefore it seems sense that they would infect adherent bacterial populations. At least four processes contribute to this discrepancy.

- Bacteriophage populations rise locally as a result of bacteriophage replication within host cells (amplification). As a result, the biofilm is exposed to a growing number of infectious bacteriophages. Bacteriophage can gradually eliminate the biofilm and lower the likelihood of renewal by spreading through the biofilm and killing the bacteria responsible for creating the EPS material.
- Bacteriophage are capable of expressing or carrying depolymerizing enzymes that break down EPS.
- Bacteriophage have the ability to activate depolymerizing enzymes from the host genome that break down EPS.
- Bacteriophage can infect persistent cells; although they can't reproduce inside of and kill off inactive cells, they can hang about these bacteria until they reawaken and then start a productive infection that kills the cells.

By significantly outnumbering their bacterial targets, bacteriophage may destroy their host cells without multiplying, a process known as "lysis from outside", although such quantities are seldom reached outside of the laboratory. By replicating inside the host cells and then lysing them during the discharge of offspring bacteriophage, which subsequently move on to eliminate further bacteria, the use of fewer bacteriophage destroys the host cells.

They most likely developed the methods to do so because they had to cope with bacterial capsular polysaccharides naturally occurring during infection. Known as "active penetration," this type of viral dissemination inside a biofilm relies on the lysis of bacteria as a crucial component. The ability of viruses to target specific hosts makes them an alluring tool for treating surroundings polluted with dangerous bacteria or battling diseases brought on by bacteria. Although there hasn't been much research on viral therapy's potential applications, studies have shown that it can successfully cure illnesses in humans, animals, plants. Phage therapy is a promising alternative to current treatments for bacterial infections because, unlike broad-spectrum antibiotics, it targets a specific host and is less likely to generate resistance in untargeted bacterial strains. Additionally, because phage may evolve

with their host, they are not vulnerable to the development of bacterial resistance, in contrast to chemical therapeutic agents.

Enzymes that break down bacterial polysaccharides may be carried by phage on their surface. These enzymes seldom interact with more than a few closely similar polysaccharide structures because of their extreme specificity. Several phage have been shown to elicit enzymes that can break down the EPS of different genera of Gram-negative bacteria. Phage for microorganisms that form biofilms is one of them. The multiple obstacles to creating antifouling (AF) solutions have been recognised in recent research of fouling management employing textured and biomimetic surfaces. The first surface deposition that leads to the colonisation of macrofouling is frequently formed by marine biofilms. Hence, choosing and managing AF coatings for maritime operations requires an understanding of how microfouling populations form. Cathodic and anodic depolarization, hydrogen creation, metal reduction, and formation of corrosive metabolites including organic acids and exopolymers are only a few of the ways by which biofilms cause metals to corrode.

Natural bacteriophage may and frequently do produce enzymes that can dislodge biofilms, but in this instance, these do not appear to be necessary for infectivity. It is obviously far more difficult to pinpoint the likelihood of such enzymes being induced from the host DNA. Bacteria that are metabolically active, are recently produced, and are found on biofilm or microcolony surfaces might logically serve as the prime targets for phage exploitation of biofilm bacteria.

The Biofilm Bacteria are Sensitive to Certain Phage

Planktonic bacteria and biofilm bacteria both have the ability to resist phage. It is always required to determine which phage the target bacteria are susceptible to for phage treatment to be effective. In contrast, bacteria that are connected with biofilms or planktonic bacteria but are not vulnerable to any of the phage present in that environment will not be preyed upon by phage. The resultant absence of phage predation pressure may enable bacteria to construct biofilms of their choosing.

Phage-Mediated Biocontrol of Bacterial Biofilms

Chronic bacterial illnesses frequently contain biofilms, and there is a wealth of research on the therapeutic use of phage to treat chronic bacterial infections. Although not all uses of phage as anti-biofilm agents, such as those related to industrial concerns or biofouling, occur in the context of treating chronic bacterial infections, this use of phage in treating chronic infections is the most obvious of these uses. Numerous marine creatures might live on any surface that is submerged in water. Marine biofouling describes the development of marine organisms on the surfaces of submerged or partially submerged items. Microorganisms including bacteria, fungus, and microalgae, as well as their secretions, are considered micro fouling.

Biofilm Control

There are several factors have to be taken to control Biofilm

- a. The microbial control in the bulk fluid.
- b.Changes in the water's composition and state.
- c. The biocide's potency.
- d. Cost-effectiveness and safety considerations.

This work mainly reviews the composition, structure, and formation process of bacterial biofilms, briefly discusses the interaction between phage and biofilms, and summarises several phage and derivative based anti-biofilm strategies, with the aim of developing novel, safe, and efficient treatment methods against Biofilm based damages in marine environment and promoting the application of phage in against biofouling. Phage are viruses that can live inside of cells as intracellular parasite deoxyribonucleic acid (DNA), or they can require bacteria as a result of their replication. Phage are particularly present in environment. This paper mainly reviews the composition, architecture, and formation process of bacterial biofilms, briefly introduces the two main life cycles of phage and bacterial biofilms and summarises several phage-based applications for the control of bacterial Biofilm.

CHAPTER 2

REVIEW OF LITERATURE

1. BACTERIOPHAGE

Bacteriophage is a kind of virus that attacks bacteria. Phage is another name for it. Bacteriophage exploit the machinery of the host bacteria to replicate and create new virus particles. They are made up of genetic material (DNA or RNA) encased in a protein coat. Bacteriophage have a complicated structure, and various phage kinds might differ in certain ways. Depending on how they live, they may be categorised into two main groups: lytic and lysogenic. Lytic bacteriophage enters the host cell, reproduce their genetic material there, and then lyse (rupture open) the host cell to release fresh phage particles. In contrast, lysogenic phage incorporates their genetic material into the host chromosome, where it repeats alongside the host DNA until the host DNA is propelled into the lytic cycle. Bacteriophage have been employed in a number of industries, including food production, agriculture, and medicine.

2. MARINE BIOFILM

Marine biofilm refers to a complex community of microorganisms, including bacteria, archaea, fungi, and microalgae, that adhere to a surface in the marine environment and form a slimy layer or film. Biofilms can develop on various substrates, such as rocks, shells, sand particles, and man-made structures like ship hulls. These biofilms are composed of a matrix of extracellular polymeric substances (EPS) produced by the microorganisms, which serve as a protective layer that helps the microorganisms to adhere to surfaces and resist environmental stressors. Marine biofilms play an important role in marine ecosystems, as they are involved in processes such as nutrient cycling, carbon fixation, and the breakdown of organic matter.

3. BIOFILM FORMATION

Microorganisms, such as bacteria, establish a population that clings to a surface and is encased in a protective extracellular matrix through the intricate process of biofilm development. This extracellular matrix serves to shield the bacteria from external pressures including antibiotics, host immunological reactions, and environmental influences. It is made up of a range of components, including proteins, polysaccharides, and nucleic acids. Biofilms may develop on a variety of surfaces, including pipelines, medical equipment, and uncultivated surfaces like rocks and soil. Additionally, they may develop in a number of habitats, including as soil, water systems, and the human body.

Single cell adhesion to a surface is often the first stage in the multistep process that results in biofilm development. The extracellular polymeric substances (EPS) that these cells later start to make assist them establish microcolonies and stick to the surface. The creation of diverse structures within the

biofilm may result from changes in gene expression and behaviour that the microcolonies experience as they develop and mature.

Human health is significantly impacted by biofilm development because biofilms may be a source of chronic infections that are challenging to cure with medicines. In industrial and environmental environments, where they can result in equipment failure, decreased efficiency, and higher maintenance costs, they are also a major concern.

4. MARINE SOURCES

Marine sources refer to various resources that are derived from the oceans, seas, and other bodies of saltwater. These resources can be both biological and non-biological in nature and have numerous uses in various industries, including food and nutrition, energy, pharmaceuticals, and more. Bacteriophage are also considered as a marine source with various applications, including;

1. Antibacterial agents

Bacteriophage can specifically target and kill bacteria, including antibiotic-resistant strains, making them potential alternatives to antibiotics in the treatment of bacterial infections.

2. Bio control agents

Bacteriophage can be used as biocontrol agents to regulate agents to regulate bacterial populations in marine environment.

3. Bioremediation

Bacteriophage can be harnessed for bioremediation, which is the use of biological agents to degrade or remove pollutants from marine environments.

4. Genetic resources

Bacteriophage are a rich source of genetic diversity, and their genomes can be studied and utilized for various purposes.

5. Research tools

Bacteriophage are important tools in marine microbiology research. They can be used to study bacterial populations, dynamics, and interactions in marine environments, providing insights into marine ecosystems and their functioning.

5. BACTERIOPHAGE IN FOOD PATHOGEN

A growing number of people are accepting the use of lytic bacteriophage for the biocontrol of food-borne viruses in food and in the food industry. In this study, four different food matrices (pig skin, chicken breasts, fresh eggs, and packaged lettuce) were experimentally contaminated with *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis. The efficacy of a bacteriophage cocktail made up of three different lytic bacteriophage was assessed. Pig skin that had been sprayed with the bacteriophage cocktail and then incubated at 33 °C for 6 hours saw a considerable bacterial decrease. *S. Typhimurium* and *S. Enteritidis* concentrations in chicken breasts that were dipped for five minutes in a bacteriophage cocktail solution and then kept chilled at four degrees for seven days also significantly decreased. Fresh eggs were sprayed with the bacteriophage cocktail, incubated at 25 °C for 2 hours, and then the bacterium content was reduced, although only little. These findings demonstrate the potential efficacy of this bacteriophage combination as a *Salmonella* biocontrol agent in several food matrices under production-like settings.

6. BACTERIOPHAGE IN DRINKING WATER

A direct bacteriophage presence testing method for 500 ml samples of drinking water has been devised that is quick, easy, and accurate. An immersion probe that is loaded with solidified soft agar that contains bacterial host cells is included in the method's glass apparatus, which also comprises a jar that holds the water sample. The experimental volume was filled with logarithmic phase host bacteria, and the probe was immersed. At 36°C, a water bath was used to incubate the whole apparatus. Within 3 hours, somatic bacteriophage plaques infecting *Escherichia coli* strain CN13 could be found. Within 6 hours, male-specific bacteriophage infecting *E. coli* F+ amp was found. After 8 hours, a bacteriophage that had infected the anaerobe *Bacteroides fragilis* subsp. *fragilis* HSP40 was discovered. In 500 ml of drinking water samples, the use of this instrument and the related approach allowed for the one-step identification of 1 pfu of *E. coli* or *Bact. fragilis* specific bacteriophage.

7. MEMBRANE BIOREACTOR

A membrane bioreactor is a wastewater treatment technology that combines a biological process, typically activated sludge or other biological treatment methods, with a membrane filtration process. This study used a variety of native and "spiked" bacteriophage (phage) of known size and shape to evaluate the possible removal effectiveness of viruses in a full-scale membrane bioreactor (MBR) wastewater reuse system. Nine locations at each treatment step of the water recycling plant (WRP) were sampled once a week for three months, and samples were then examined for a variety of microbiological characteristics (n = 135). The MBR product had mean levels of faecal coliforms that were 0.3 CFU/100 ml and undetectable in samples obtained following the chlorination step. Additionally, after MBR treatment, a sizable decrease (5.3 log) in somatic coliphage was seen.

However, GB124 and F-specific phage showed log decreases of 3.5 and 3.8 post-MBR, respectively, and were less prevalent at all steps. A post-MBR log decrease of 2.25 or 2.30 was seen for the suspended "spiked" phage MS2 and B-14 in the "spiking" studies. The removal of these suspended phage, which have a hole size (0.04 μ m) smaller than that of the membrane, thus emphasises the potential function of the membrane biofilm as a powerful additional barrier to viral transmission. The results of this investigation into a full-scale MBR system show that counting various phage groups may provide a useful and conservative method for determining if MBR is capable of eliminating enteric viruses that are important for human health. Additionally, they raise the possibility that phage removal in MBR systems may vary greatly and be directly connected to the size and morphology of the viruses as well as to whether or not they are attached to solids on the one hand.

8. DESALINATION

Desalination is the process of purifying saltwater, brackish water, or other forms of high-salinity water to remove salt and other contaminants and make it acceptable for human consumption or other uses, including agriculture or industry. A variety of methods and techniques are commonly used to remove salt and other impurities from water, leaving behind pure water that may be utilised for irrigation and drinking. Conventional disinfectants for water and treated wastewater effluents have drawbacks, including hazardous disinfection by-products, bacteria and related biofilms that are resistant to disinfectants, high costs, no residual disinfecting action, and significant risks associated with the production, transportation, and handling of large amounts of chlorine and ozone. Investigating fresh disinfecting options has therefore become necessary. Phage treatment, or the use of bacteria-eating phage, is used to treat bacterial infections. Recent research has made clear the importance of phage in the treatment of water and wastewater, particularly in disinfection. Numerous investigations have also revealed the prevalence of phage that are unique to enteric bacterial infections in natural water bodies. This review discusses the phage targeted at faecal coliform and other waterborne bacteria, the primary benefits of using phage to reduce pathogens, limitations of phage-based disinfection, and potential phage applications to enhance the layout and functionality of treatment plants.

9. MATERIALS

1. Zobel Marine Broth (ZMB)

The Zobel marine broth's composition closely resembles that of saltwater, which encourages the growth of marine microorganisms.

TABLE 2.1**Composition**

Ingredients	Gms/Litre
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160

2. Hard Agar

Hard agar is mainly used for grip. Hard agar is prepared by combination of Zobel marine broth with 1.5% of agar. Hard agar contains a higher concentration of agar than soft agar.

3. Soft Agar

This is the main layer of bacterial host. In soft agar the phage observation is faster. In soft agar the agar concentration is low with 0.5%. It is made by adding a small amount of agar.

4. Ca-Mg

It is used for bacterial growth. Calcium and magnesium are crucial for growth, skeleton formation and variety of biochemical processes. By adding CaMg, it gives quick result.

5. 0.2 µm syringe filter

0.2µm syringe filter is used to remove or capture bacteria. It is also known as wheel filter. It is attached to the end of syringe for use. Particles easily damage an HPLC due to the narrow bore and high pressures within.

**Fig 2.1 0.2µm syringe filter**

2.9.6 Sterile syringe

Sterile syringe is used for filtration. The sterile syringe is placed onto Lure Lock Inlet of 0.2 μ m syringe filter.



Fig 2.2 Sterile syringe

2.9.7 Falcon tubes (15 ml)

Falcon tubes are used to maintain sample integrity during spinning, freezing, and storage. Falcon tubes are made from high –clarity polypropylene.



Fig 2.3 Falcon tube

2.9.8 Centrifuge

The samples were centrifuged at 7000rpm at 4°C for 20 min. After completion of centrifuge, the supernatant was transferred into fresh sterile falcon tubes.



Fig 2.4 Centrifuged sample

10. MATERIALS

The materials used are Zobel Marine Agar (HIMEDIA), 0.2µm syringe filter (SARTORIUS), Sterile syringe (INDIAMART), Falcon tube (TARSONS).

2. METHODS

1.Bacterial isolation Table

2.10.1

S.NO	NAME	SCIENTIFIC NAME	GRAM STAINING	CATALASE
1	AK1	Pseudomonas	Negative	Positive
2	AK2	Pseudomonas	Negative	Positive
3	AK3	Pseudomonas	Negative	Positive
4	AK4	Pseudomonas	Negative	Positive
5	AK5	Pseudomonas	Negative	Positive
6	AK6	Pseudomonas	Negative	Positive
7	AK7	Pseudomonas	Negative	Positive
8	AK8	Pseudomonas	Negative	Positive
9	AK9	Pseudomonas	Negative	Positive

2.10.2.2 16s rRNA Sequence

A culture-free approach for identifying and comparing bacterial diversity from challenging-to-study complex microbiomes or settings is 16S rRNA sequencing. It is frequently employed to determine whether bacteria are present in a particular sample. The 16S rRNA in bacteria has 5–10 copies, which makes the detection incredibly sensitive. The 16S rRNA coding gene is around 1500 kb in length and has 50 functional domains. Polymerase chain reaction (PCR)-based 16S rRNA gene sequencing is based on DNA sequencing after PCR. The molecular biology technique of PCR is used to amplify certain DNA fragments through a number of cycles that include

- i) A double-stranded DNA template denatured
- ii) Annealing of complementing primers for the template
- iii) The DNA polymerase enzyme, which creates a new DNA strand, extends primers.

Chapter 3

AIM AND OBJECTIVES

1. AIM

To isolate bacteriophage against marine biofilm bacteria for the anti-biofilm application.

2. OBJECTIVES

- Isolation of bacteriophage against single genus of marine biofilm bacteria.
- Bio characterization of phage (Biochemical test)
 - Ph stability
 - salinity
 - temperature
 - chloroform stability
- One step growth and anti-biofilm assay.

CHAPTER 4

MATERIALS AND METHOD

4.2.3 Phage isolation and purification

For phage isolation 4 mL of AK9 culture was mixed with 10 mL of fresh LB broth (2X) and 10 mL of filtered sample and incubated over night at 37 °C. The mixture was then centrifuged for 20 min at 7000 rpm, and then the supernatant was filtered through 0.2 µm pore size filters. The lysate (supernatant) was examined for detection of the presence of lytic phage in the plaque assay by Double-layer Agar (DLA) method. For this purpose, 250 µL of lysate was mixed with 150 µL of a host bacterial culture. After 10 min of incubation at 37 °C, 3 mL of ZMA soft agar (10 mM MgSO₄, 0.7% agar, at 45 °C) was added, mixed, and overlaid onto fresh ZMA agar plates (1.5% agar) prepared before. After solidifying, plates were incubated overnight at 37 °C. Clear zones (plaques) over the bacterial lawn were considered as the presence of lytic phage. For phage purification, a single plaque was picked and placed in a tube containing 1 mL of LB broth and 250 µL of host bacterial culture and incubated over night at 37 °C. At the next day, the presence of phage was examined by DLA method. Three rounds of purification were conducted to assure about the purity of the phage. Finally, purified phage was named as BW1.1



Fig 4.1 Phage trapping



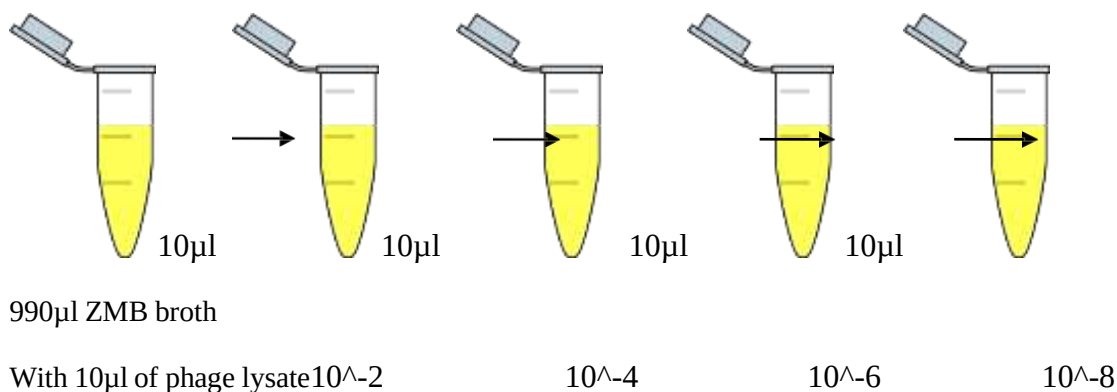


Fig 4.2 Phage titration

4.2.5 Passage and Phage lysate preparation

To increase the lytic ability is known as Passage. The act of continually moving a bacteriophage from one host bacteria to another in order to increase its population and create a pure culture of the virus is referred to as passage. The passage approach is frequently used to isolate bacteriophage with a restricted host range or low levels of presence in a sample. The purpose of passage is to choose bacteriophage that are most successful at replicating and infecting the host bacteria, increasing the probability that the bacteriophage will be successfully isolated and purified.

"Neat" or "Lysate" in the context of bacteriophage often refers to a bacteriophage suspension or lysate in its unaltered or undiluted state. A bacteriophage suspension is a concentrated solution of bacteriophage that have been purified from a lysate, whereas a bacteriophage lysate is a suspension of bacteriophage that have been created by infecting and lysing bacterial cells. Neat bacteriophage suspensions or lysates are often used in research, where the concentration and purity of the bacteriophage are important. The lysate or neat were collected and kept in shaker for 3-4 hr and centrifuged at 7000rpm for 20 min. After completion of centrifuge the sample were filtered using 0.2µm syringe filter and transferred to fresh falcon tube.



Fig 4.3 Neat

6. BIO-CHARACTERIZATION

1. pH STABILITY

Ten- fold serial dilutions of bacteriophage suspensions were prepared in ZMA media (10g of tryptone, 10g of NaCl, 5g of yeast extract). Different pH stability was checked (pH 2,pH5,pH9). Finally, phage titration (PFU/ml) was calculated by the number of plaques x 10/ dilutions. In pH the highest plaque was 1.82×10^{-4} .

2. TEMPERATURE

Similar to pH stability, bacteriophage temperature stability can change based on the kind of phage and the environment in which it is present. The phage was kept in different temperatures like 50°C, 70°C etc. The normal DLA were grown under room temperature. Finally, phage titration (PFU/ml) was calculated by the number of plaques x 10/ dilutions. In temperature the highest plaque was 3.26×10^{-6} .

3. SALINITY

Salt content is determined by a method called salinity testing. Plaque tests utilising agar plates with various salinities are a frequently used technique for phage salinity testing. Finally, phage titration (PFU/ml) was calculated by the number of plaques x 10/ dilutions. In salinity the highest plaque was 2.06×10^{-6} .

7. CRYSTAL VIOLET ASSAY

The crystal violet test is a technique for calculating the volume of biofilm that bacterial cells create. Communities of bacteria known as biofilms form on surfaces and create a protective extracellular matrix. The crystal violet test is a technique for calculating the volume of biofilm that bacterial cells create. Communities of bacteria known as biofilms form on surfaces and create a protective extracellular matrix. The crystal violet assay may be used to evaluate phage capacity to prevent or disrupt biofilms and to establish the ideal phage concentration and exposure period required to produce the desired result

8. FLUORESCENCE MICROSCOPY

Fluorescence microscopy can be an effective method for investigating how bacteriophage break down biofilms. In this technique, the biofilm is stained with fluorescent dyes before being examined under a fluorescence microscope. A bacterial strain that produces biofilms should be chosen, and a biofilm should be cultivated under the required circumstances, to undertake fluorescence microscopy-based phage biofilm degradation studies. After that, the biofilm is exposed to a known concentration of

phage particles for a predetermined amount of time. Following the procedure, the biofilm is stained with fluorescent dyes, such as SYTO 9, which binds to the DNA of bacterial cells and fluoresces green when excited.

The fluorescence microscope is then used to examine the dyed biofilm, and pictures are taken at various intervals to gauge the rate of biofilm deterioration. A visual and numerical evaluation of phage-mediated biofilm destruction is provided using fluorescence microscopy. It may be used to assess the effectiveness of various phage therapies and to compare the capacity of various phage to break down biofilms under diverse circumstances.

CHAPTER 5

RESULTS

5.1 pH stability

pH stability tolerance test showed that isolated phage BW 2.1 were 100.0% active in the pH range of pH2. At pH 9 approximately 50.0% (50 ± 5) of the phage formed clear plaques.

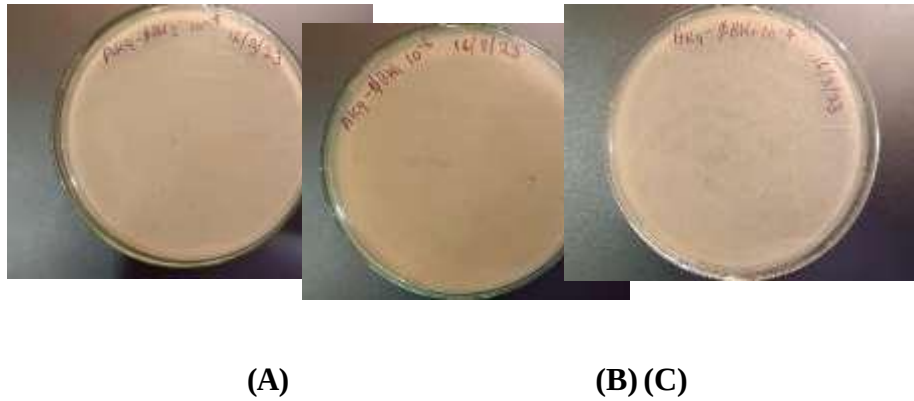


Fig 5.1 pH stability

(A) AK9-φBW2.1 pH2 at titration of 10^{-4} the plaques were formed.

(B) AK9-φBW2.1 pH2 at the titration of 10^{-6} the plaques were formed.

(C) AK9-φBW2.1 pH9 at the titration of 10^{-4} the plaques were formed.

RESULT FOR pH

TABLE 5.1.

TITRATION LEVEL	PFU/ML
10^{-4}	pH2 = 1.33×10^{-4}
10^{-6}	pH2 = 1.6×10^{-5}

10^{-4}	$\text{pH}9 = 3.6 \times 10^{-4}$
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5.2 Temperature

Temperature tolerance test showed that isolated phage BW2.1 were 100.0% active in the temperature range of 4-50°C, at 50°C approximately 50.0% (50 ± 5) of the phage formed clear plaques, and at 80°C no plaques formed.

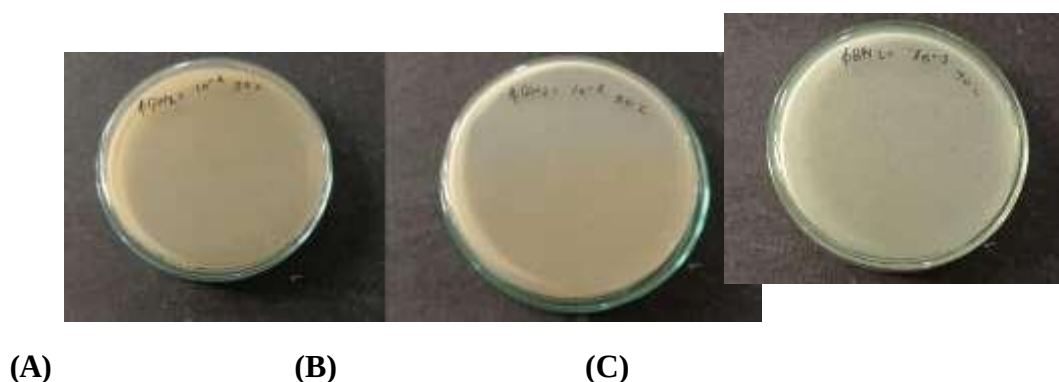


Fig 5.2 Temperature

- (A) AK9- ϕ BW2.1 at the temperature 50°C at the titration of 10^{-6} the plaques were formed.
 (B) AK9- ϕ BW2.1 at the temperature 50°C at the titration of 10^{-8} the plaques were formed.
 (C) AK9- ϕ BW2.1 at the temperature 70°C at the titration of 10^{-2} the plaques were formed.

RESULT FOR TEMPERATURE

Table 5.2.

TITRATION LEVEL	PFU/ML
10^{-6}	50°C = 1.16×10^{-8}
10^{-8}	50°C = 1.7×10^{-9}
10^{-2}	70°C = Completely chewed up.

BW2.1 was stable at 50°C, the highest plaque formed at 1.7×10^{-9} PFU. However, no plaques were observed at 80°C, which suggests there is no active phage at that temperature. Here the plaques are gradually decreasing according to its temperature.

5.3 Salinity

Salinity test showed that isolated phage BW2.1 were 100.0% active in salinity of % and %. At % approximately 50.0% (50 ± 0) of the phage formed clear plaques.

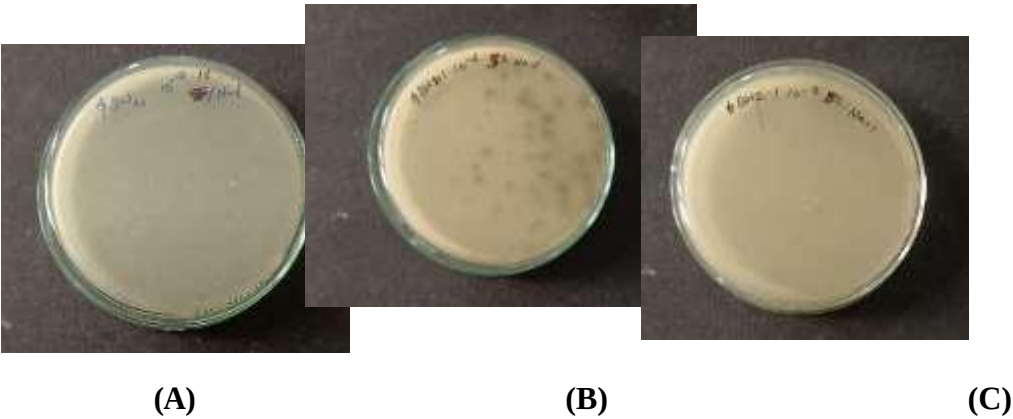


Fig 5.3 Salinity

- (A) AK9-φBW2.1 at 1% NaCl the titration of 10^{-8} the plaques were formed.
- (B) AK9-φBW2.1 at 5% NaCl the titration of 10^{-6} the plaques were formed.
- (C) AK9-φBW2.1 at 5% NaCl the titration of 10^{-8} the plaques were formed.

RESULT FOR SALINITY

Table 5.3

TITRATION LEVEL	PFU/ML
10^{-8}	1%NaCL= 7×10^{-8}
10^{-6}	5%NaCl= 1.8×10^{-7}
10^{-8}	5%NaCl= 1.09×10^{-10}

5.4 Crystal Violet Assay

In recent article, the results showed that when *S. Aureus* biofilms are grown in 96 well plate for more than 12hr, it is observed that biofilms grown in peripheral wells were thicker (with higher crystal violet absorbance at 570 nm). In this review, the original CV assay required the optical density of each well containing solubilised crystal violet-stained cells be read at 517nm. By comparing control in day 1, 2 and 3 the bacterial growth is increasing. The phage was analysed in crystal violet assay were assessed using 96 well plate. The absorbance readings for the crystal violet-stained biofilm assays after 24hr, 48hr, 72hr of treatment are displayed.

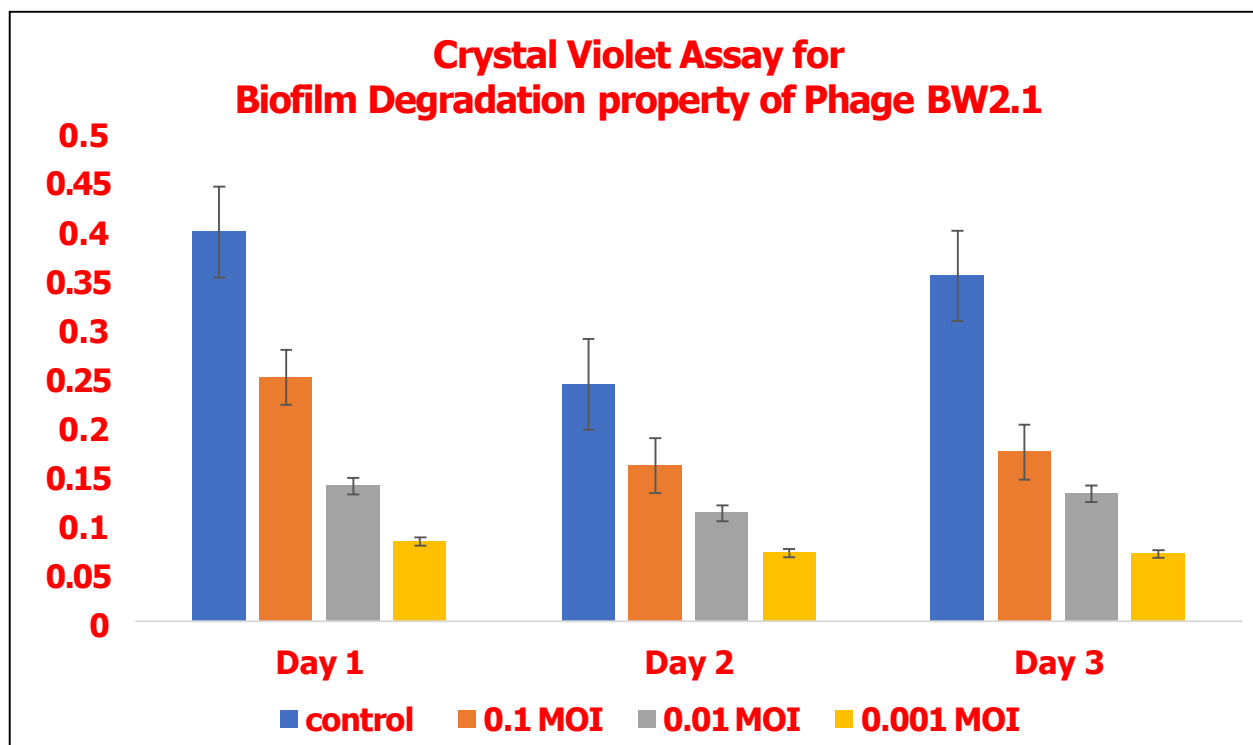
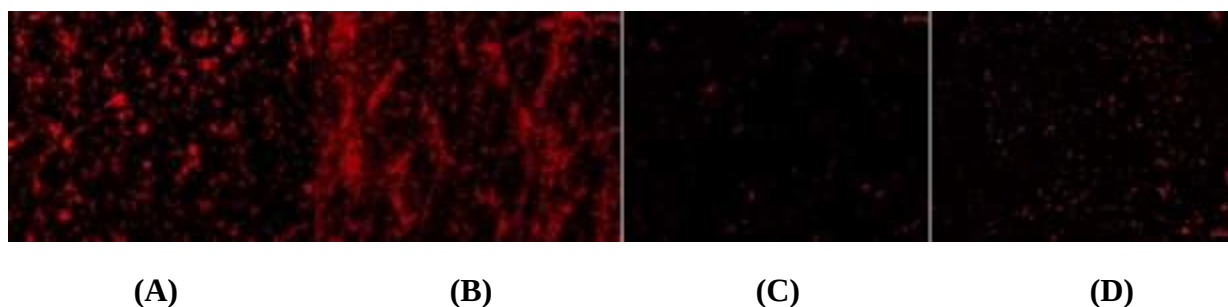


Fig 5.4 Crystal violet assay of phage BW1.1 are measured by absorbance at 517nm.

5.5 Fluorescence microscopy

The fluorescence microscopy result revealed the presence of bacteriophage attached to the bacterial host cells, indicating successful phage attachment and potential phage-mediated bacterial lysis. For fluorescence microscopy the acridine orange is used for detection and enumeration of microorganisms.

5.6 Fluorescence microscopy based phage biofilm degradation analysis



(A) Control AK9 – Day 1 (B) Control AK9 – Day 2 (C) Phage – Day 1 (D) Phage – Day 2

CHAPTER 6

CONCLUSION

The aim of the present study was to isolate bacteriophage against marine biofilm bacteria. The bacteriophage used in the study were found to be effective in reducing biofilm formation by marine bacteria. The study also demonstrated that bacteriophage was effective in inhibiting the growth of biofilm forming bacteria in marine environments. Bacteriophage specifically target and infect bacteria, leading to their lysis and death. This can significantly reduce the population of biofilm forming bacteria in marine environments, which can help to maintain the health and balance of marine ecosystems. Recent reviews have covered the topic of plaque formation. Plaque size appears to be inversely correlated with the latent period and directly correlated with burst size, phage adsorption constant and medium-level phage diffusion, each contributing in a unique way. In the presence of antibiotics, a shorter latent time and a larger burst size have been noted. According to reports, bacterial filamentation is what causes antibiotics to increase phage production. According to Krueger et al., penicillin-treated *S. aureus* formed filaments that were three times larger in diameter than typical bacterium and promoted the growth of phage. Hadas et al. discovered that the quantity of phage formation was increased by an equivalent amount, and that bacterial cells exposed to this antibiotic were 4-fold bigger.

The number of bacteria was decreased but not completely gone. Even when three distinct phage cocktails were used in earlier research that suggested using them to control *E. coli* O157 in an effort to assure that bacteria resistant to one phage would be vulnerable to the others. Phage have been isolated from the sewage of animal farms by a variety of employees. Rapid screening techniques including turbidity reduction and streak plate approaches, together with subsequent processing by the DLA method, were shown to be quite beneficial throughout the current inquiry. This emphasised the significance of early screening for a successful phage recovery. The use of either *B. subtilis* or *E. coli* is supported by a number of papers. On the basis of the recovery of thirty *B. subtilis* (BsHR) phage and three *E. coli* specific phage (EHR), it was determined that *B. subtilis* supported the growth of numerous morphotypes of phage as opposed to *E. coli*. The phage had the highest percentage of adsorption to the *Bacillus* host, according to Krasowska et al. The phage was isolated and named as BW1.1.

The phage were isolated and bio characterizations were done on different pH, temperature and salinity. In pH the plaques formed in pH2 were 87 pfu, in pH5 the plaques were 128 pfu and in pH9 the plaques were 8 pfu. In temperature the plaques formed in 50°C were 326 pfu, and in 70°C the plaques counted was 140 pfu. In salinity the plaques formed in 1% NaCl was 124 pfu, and in 3% NaCl the

plaques were 49 pfu, and in 5% NaCl the plaques are 35 pfu. The crystal violet assay and Fluorescence microscopy test were done.

CHAPTER 7

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