ANALYSIS OF LIPASE ACTIVITY OF JSF7 PHAGE

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Declaration

It is hereby declared that:

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

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Ethics Statement

We hereby declare that no animals, plants or humans were harmed throughout the process of the research.

Abstract

The lytic phage of Vibrio cholera, JSF7 has been found to have a GDSL-like lipase enzyme in its tail region. The sequencing of the organism showed that in the ORF30 of the organism there is a coding region of this protein. In this research process, we have tried to find out the effectiveness of the lipase enzyme of this phage. The experiment was conducted on trial and error, and a dye of target Rhodamine B was used throughout the process. Before the experiment, it was found that there were certain bacterial strains of vibrio (WT 324 and WT 346) that are the host of this phage. We have used these hosts to enrich our phage from time to time and used the phages of high titer around 10^7/ml. The research was conducted using heating techniques to extract fatty acids from the oil as our control process and then using rhodamine on the sample to find out the efficiency of Rhodamine B. Using phages on the sample we tried to find out the correlation between our obtained results. After getting acceptable results we compared our results with the negative control of the experiment which was JSF2 which had already been sequenced and no lipase enzyme coding region was found. In the last part of the research, we conducted the experiment using the process of dose dependency where we used different concentrations of phage on the sample and measured their optical density (OD) using spectrophotometer. We found that samples with the highest concentration of phage were getting the maximum result. We found that samples with the highest concentration of phage were getting the maximum OD result due to more complexing of molecules of fatty acids and rhodamine B. Thus, this was hypothesized that more phage in the sample was causing the formation of more fatty acids.

Dedication

We would like to dedicate this thesis to our beloved families for their love, prayers, support, and sacrifices.

Acknowledgment

First of all, we are dedicating our acknowledgment of this research work to the Almighty. Without the blessings and will of the Almighty, we would not have been able to get this far with our life and works. After that, we are expressing our heartfelt gratitude to the Chairperson of the Mathematics and Natural Sciences Department, Professor A F M Yusuf Haider sir. Without his approval this project would not have come this far today.

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List of Acronyms

LA	Luria-Bertani Agar
LB	Luria-Bertani Broth
ml	Milliliter
OD	Optical Density
ORF	Open Reading Frame
pfu	Plaque forming unit
RhB	Rhodamine B
rpm	Rotations per minute
µl	Microliter

Chapter 1: Introduction

In this diverse world comprising this huge ecosystem, there are microorganisms that we are unable to see. Among the huge and diverse family of microbes, there is a specific type called virus. These are the organisms that need a host to survive. The host will provide everything that the virus needs to show the traits of a living organism. A specific type of virus is called bacteriophage. These are the specific types of viruses that choose bacteria as their host [1]. Bacteriophages infect the bacteria and live on them. The bacteria on which the bacteriophages will thrive is very particular. Although the virus and bacteriophages seem to sound very harmful still, some of their traits can be utilized for the benefit of humans and the ecosystem [2].

Usually, phages have a common type of morphology. Like other types of viruses, the bacteriophages have a nucleic acid genome which is surrounded and enclosed by their self-encoded proteins. This protein protects the genetic material of the organism and mediates the delivery of the organism to the next host cell [1].

The target phage is JSF7 which had been isolated and sequenced already. It was seen that the phage was from a Vibrio family and also had a number of hosts in which it could replicate. These hosts were mostly seen to be different vibrio strains. Analysis was done on this phage and there was a gene that was found to have the potential of coding for lipase enzyme [3]. It is predicted that this bacteriophage can degrade lipid substances if proper environment and support is given. This lipase can be used in different needs varying from medicines to environmental cleanup.

The Phage JSF7 and its lipase enzyme has been focused on this study. It has been found from the genetic analysis of this phage that it has a lipase enzyme encoding gene in its genome. Here we have tried to find out about the activity of the lipase enzyme and how efficient it is. By predicting the presence of fatty acids using a dye Rhodamine B we were able to come to the conclusion that the enzyme can be active at certain stages. The study will also focus on the use of Spectrophotometric analysis of the assay findings [3].

Objective:

The main objective of this project was to find out the lipolytic activity of JSF7 phage following the reference of its genomic sequence.

Chapter 2: Literature Review

The bacteriophage JSF7 was isolated from the surface waters in Bangladesh along with two other types of phages which are JSF4 and JSF3. It was seen that JSF7 along with the other two types of phages was able to affect the active planktonic form biofilm-related form of the toxic-prone *V.cholerae*. Additionally, it was also found that JSF7 has been able to degrade the biofilm of *V.cholerae* strains 01 and 0139 [3]. It has also been found that JSF7 has similarities with JSF1. The JSF1 is a double-stranded DNA virus with 230 coding regions and has a genome size of around 126kbp. Similarly, JSF7 has been found to be active on the strain *V.cholerae* O1. It has a genome size of 46kbp with 48.42% GC content [3]. It was also seen that JSF7 was active and infectious from 65% to 98% at a pH range of 6 to 9. This JSF7 phage was also seen to be very active for 4 weeks when stored at room temperature using the SM buffer.

The genomic analysis of JSF7 showed the presence of 2 ORF that were predicted to encode GDSL-like lipase and polysaccharide degrading enzymes. It was estimated that the activity of these two enzymes may be the reason for the ability of JSF7 to degrade polysaccharides. It was seen in the study that JSF7 was able to degrade the biofilm of both susceptible and non-susceptible bacterial strains.

2.1 Mode of replication of JSF7

It has been seen that JSF7 follows the lytic mode of replication [4]. Using this information, it can be predicted that JSF7 has a specific type of bacteria on which it can thrive. Two such strains of bacteria have been found to be WT346 and WT324.

In the lytic phages, the host is infected and killed rapidly, as a result, they are able to control the population dynamics of the host and also are able to cause long-term evolution through a generalized transduction process [5]. Lytic phages release their progeny genetic materials in a burst amount as the cell is lysed. The interaction of the host is sometimes like a chronic infection. Here the viruses are released from cells without killing the host. This process follows parasitism. It can mostly be lytic infection where the infection of unicellular organisms can result in cell death. The replication of lytic phages like JSF7 has 6 stages which are adsorption, penetration, replication,

maturation, release and re-infection. The adsorption occurs when specific receptors are found on the host's bacterium. Specific enzymes penetrate the cell wall of bacteria and insert the nucleic acid inside the host. These genetic materials are synthesized using the host's enzymes and machinery to replicate itself. These materials are then assembled inside the host and then ejected outside by cell rupture of the host.

2.2 Lipid and lipase enzyme

Lipase are water-soluble enzymes. They have a tendency of having a preference to degrade apolar and water-insoluble substrates. This group of enzymes includes types referred as cholesterol esterases, lipases and cholesterol esterases. The substrate groups of lipases include in order of amphiatcity, long aliphatic chain acyl esters of cholesterol, triacyl esters of glycerol, acyl esters of long chain alcohols, diacyl esters of alcohol and monoacyl esters. Lipase converts the lipids to fatty acids and glycerol using the process of hydrolysis [6].

2.3 Lipids when heated

The action of heat on lipids, especially plant lipids, shows that the availability of free fatty acids increases with the increase of heat and heating time. Hydrolysis of fats to obtain free fatty acid can be done by heating the oil for a longer time at a lower temperature. Excess heating of the lipids can lead to the production of different other components as well [7]. In heated oil, the level of free fatty acids increases with time [8].

2.4 Introduction to coconut oil components

Coconut oil is made by crushing the copra giving low moisture content of around 6%-8% and which has an oil content of 60%-65% oil by the expellers. This form of oil has been consumed by people around the globe for a long time [9]. Coconut oil has a saturated fatty acid concentration of

92% in the form of triglycerides. Among them mostly are found to be medium-chain fatty acids. Most of these fatty acids are saturated and often cause it to be solid at room temperature [10].

Table 2.4 Fatty acid composition of coconut oil and some other vegetable oils

Vegetable oils	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	Others
Coconut	7.0	5.4	48.9	20.2	8.4	2.5	6.2	1.4	-	-	-	
Palm kernel	-	1.2	51.6	22.9	12.2	1.3	10.8	-	-	-	-	-
Sunflower	-	-	-	-	6.3	3.0	43.7	47.0	-	-	-	
Rice bran	-	-	-	0.4	22.9	1.8	42.5	30.5	1.4	0.5	-	
Safflower	-	-	-	0.3	11.9	2.3	29.2	55.9	0.4	-	-	
Sesame	-	-	-	-	10.3	5.8	42.9	41.0	-	-	-	
Groundnut	-	-	-	-	14.0	3.8	41.9	34.7	1.0	1.2	3.4	
Palm	-	-	0.2	1.1	42.6	3.8	41.9	10.4	-	-	-	
Olive	-	-	-	-	12.0	2.5	75.7	7.9	0.5	-	-	1.4
Soybean	-	-	-	-	11.6	4.0	18.8	56.1	8.5	-	-	1.0
Grape seed	-	-	-	-	7.2	4.8	19.4	68.1	0.1	-	-	0.4
Linseed	-	-	-	-	7.1	2.0	19.9	17.3	53.7	-	-	0.4

2.5 Rhodamine B

RhB is a dye that is used in the field of biology as a staining and a fluorescent dye. This type of dye is used widely in fluorescence related work in the field of biotechnology. The peak of the diluted RhB is around 565nm wavelength which is also known to be having the zwitterionic form of the chemical [11]. It is basically a green color powder having a molecular weight of 479.02. The excitation peak is around 546nm and have an emission peak around 568nm. Rhodamine B has previously been used to target lipids and has been used as a probe [12]. A quantitative assay of lipids have been done already using the dye RhB. The bonding was between the fatty acids and the RhB molecules [12]. The complex of fatty acids and RhB are capable of having fluorescence that can be recorded at particular wavelengths [13]. Previous studies had found that rhodamine shows good results at wavelength of 560nm in UV spectrophotometer [14].

2.6 Spectrophotometer

The principle of spectrophotometry is based on the principle of a solution absorbing light at specific intensities. In this process the light beam at a specific wavelength is passed through a

solution and the different absorption levels of the solution gives the prediction about the concentration of a substance in the solution. It is a quantitative analytical instrument. The amount of photons from the light being absorbed will give a predictive information about the concentration of that substance in the solution [15]. Beer Lamberts law of absorption is directly proportional to the concentration of the analyte is followed here.

2.7 Phage enrichment

The process of phage enrichment is needed to be done for increasing the phage count of different bacteriophages. In this process host bacteria are used to allow the phage to target the specific hosts and replicate using the machinery of the host. Process requires the identification of a host and adding it to the bacterial culture and grow a culture host followed by incubation. One of the main advantages of having phage enrichment is that to obtain phages in much larger volume of samples. This can be controlled as per the use of the experiment and get the best results [16].

Chapter 3: Methods and Materials

3.1 Place of study:

The whole research was conducted in the Biotechnology and Microbiology laboratory of the Department of Mathematics and Natural sciences, BRAC University, Dhaka, Bangladesh using two Vibrio cholerae strains (WT 324 and WT 346) and two Vibrio specific phage JSF7 and JSF2. The Vibrio species were previously isolated from the cholera patients and were stored for different experimental purposes at the Laboratory of the university. Also, the Vibriophage JSF7 was isolated from the environmental water samples beforehand and stored in the laboratory at 4°C.

3.2 Standard laboratory practices:

- A clean lab coat and hand gloves were worn while performing experiments. However, wearing lab coats outside the laboratory must be avoided.
- The experiments were performed inside a laminar flow cabinet which previously was cleaned with 70 % ethanol in order to avoid contamination. Also, after finishing the work, the laminar should be cleaned.
- All the glasswares (like: test-tube, conical flasks, and beakers) were at first washed under tap water then followed by a second time wash with distilled water.
- Before using, culture media, pipette tips, empty test-tubes, or conical flasks were autoclaved at 121°C at 15 psi for 15 minutes.
- After plating media into a petri dish, the remaining media should not be discarded. The extra media must be properly sealed with clean foil and masking tape and kept in the refrigerator in order to use in future.
- Proper labeling must be done on the media plates and containers with the initials of the group, media name, and date of preparation.
- Prepared culture plates and media must be kept on record.
- Culture plates should not be stored in the refrigerator for more than two weeks and culture plates should not be kept in the incubator for more than two days.
- After using a reagent, it should be kept on its assigned shelf.

- Care must be taken while using a spirit lamp or any kind of flammable component. After finishing the work, the spirit lamp should be turned off.
- While working if anything breaks down or is damaged, the lab attendants must be immediately informed about it.
- In case of any kind of burn or cut, proper first aid treatment must be taken.

3.3 Preparation of Culture Media and Solutions:

Reagents

- Distilled Water
- Commercially available Luria-Bertani Broth medium powder
- Commercially available Luria-Bertani Agar powder
- Rhodamine B
- Bile Salt
- Sodium Hydroxide
- Tryptone
- Sodium Chloride
- Yeast extract
- Bacteriological agar
- Saline Solution
- Coconut Oil

Apparatus

- Petri plates
- Micropipette
- Micropipette tips
- Test tubes
- Beakers
- Duran bottles
- Glass vials

- Eppendorf tubes
- Falcon tubes
- Conical flasks
- Needle, Loop
- Glass spreader
- Ethanol
- Spirit lamp
- Aluminum foil paper
- Water bath
- Cuvettes
- 0.22 Micron filter
- Syringe
- Autoclave
- Laminar
- Incubator
- Shaker incubator
- Analytical balance machine
- Centrifuge machine
- Vortex machine
- Spectrophotometer
- Refrigerator
- Paraffin oil
- Parafilm paper

3.3.1 Preparation of Luria-Bertani Broth (LB):

LB Broth or, Luria-Bertani medium is one of the most commonly used nutritionally rich mediums that is used for culturing bacteria. The main components of LB are: Tryptone, Sodium chloride (NaCl), and Yeast extract. While working, we used the commercially available LB that was present

in our laboratory. According to the instruction, in order to prepare 1000ml of liquid broth, 20gm of LB powder is required to be added with 1000ml distilled water.

Reagents and Apparatus:

- Commercially available Luria-Bertani Broth (LB) powder
- Distilled Water
- Conical flask
- Analytical balance machine
- Spatula
- Stirring/Glass rod
- Foil
- Bunsen burner

Procedure:

- Measuring in an analytical balance machine, 10gm of commercially available Luria-Bertani Broth (LB) powder was taken with a clean spatula on a piece of foil paper.
- In a clean conical flask, 500ml of distilled water was measured by using a measuring cylinder.
- The measured reagents were transferred to the conical flask.
- The reagent was mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
- The conical flask was then kept on a Bunsen burner and was given a good stir with the stirring/glass rod.
- When the media turned clear after a slight boil, the media-filled conical flask was covered with clean foil paper and kept in an autoclave machine at 121°C for approximately 2 hours so that the media is sterilized.
- After 2 hours in autoclave, the LB was taken out of the machine and kept outside so that the media cooled down for use.

3.3.2 Preparation of Luria-Bertani Agar (LA):

LA media or, Luria-Bertani Agar (LA) media is a non-selective powdered media that is required for the streaking of the inoculated stock bacteria as this medium is enriched in nutrition for the growth of pure culture strain. The main components of LA are: Tryptone, Sodium chloride (NaCl), Yeast extract, and 1.5% Agar. While working, we used the commercially available LA that was present in our laboratory. According to the instruction, in order to prepare 1000ml of nutrient agar media, 40gm of powder is required to be added with 1000ml distilled water.

- **Reagents and Apparatus:**
  - Commercially available Luria-Bertani Agar (LA) powder
  - Distilled Water
  - Conical flask
  - Analytical balance machine
  - Spatula
  - Stirring/Glass rod
  - Foil
  - Bunsen burner

- **Procedure:**
  - Measuring in an analytical balance machine, 16gm of commercially available Luria-Bertani Agar (LA) powder was taken with a clean spatula on a piece of foil paper.
  - In a clean conical flask, 400ml of distilled water was measured by using a measuring cylinder.
  - The measured reagent was transferred to the conical flask.
  - The reagent was mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
  - The conical flask was then kept on a Bunsen burner and was given a good stir with the stirring/glass rod.

- When the media turned clear after a slight boil, the media-filled conical flask was covered with clean foil paper and kept in an autoclave machine at 121°C for approximately 2 hours so that the media is sterilized.
- After keeping 2 hours in autoclave, the LA was taken out of the machine and kept outside so that the media gets cool down for use.
- After a bit of cooling down (~ 45-50°C), the media was poured into previously sterilized petri-dishes (in an oven at 160°C for 1 hour) inside a vertical laminar flow cabinet and left to solidify at room temperature. For small-sized petri-dishes approximately 10ml-15ml media; for medium-sized petri-dishes approximately 20ml media and for large-sized petri-dishes approximately 30ml of media was poured on each plate respectively.
- After solidification of the media, the plates were ready to use. In case the plates were not used, the plates were stored at 4°C.

3.3.3 Preparation of Bacterial Culture:

Reagents and Apparatus:

- LA plates
- Sample organism
- Loop
- Spirit lamp
- Masking tape and Marker

Procedure:

- The inoculating loop is sterilized in the spirit lamp by putting the loop into the flame until it is red hot.
- After the loop is cooled, a loopful of culture is streaked on a fresh nutrient agar plate.
- The plate is incubated for 24 hours at 37°C.
- After checking growth, the plate is wrapped with masking tape, properly labeled with the initials of the group, media name, and date of preparation, and stored at 4°C for further use.

- Before each experiment, bacterial samples are freshly subcultured and 24-hour culture is used this is to maintain the viability and purity of the organism.

3.3.4 Preparation of Bacterial Stock:

Soft nutrient agar is a non-selective powdered media that is enriched nutritionally for the growth of pure cultures of strains. It is prepared manually by mixing Tryptone, Sodium Chloride (NaCl), Yeast extract, and 0.8 % Bacteriological agar with Distilled Water for better results.

Reagents and Apparatus:

- Tryptone
- Sodium Chloride (NaCl)
- Yeast extract
- Bacteriological agar
- Distilled Water
- Conical flask
- Glass vials
- Inoculation needle
- Analytical balance machine
- Spatula
- Stirring/Glass rod
- Foil
- Bunsen burner
- Micropipette
- Parafilm
- Paraffin oil

Procedure:

3.3.4.1 Preparation of 0.8 % Soft Nutrient Agar:

- Measuring in an analytical balance machine, 0.5gm Tryptone, 0.5gm Sodium Chloride (NaCl), 0.25gm Yeast extract, and 0.4gm Bacteriological agar were taken with a clean spatula.
- In a clean conical flask, 50ml of distilled water was measured by using a measuring cylinder.
- The measured reagents were transferred to the conical flask.
- The reagents were mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
- The conical flask was then kept on a Bunsen burner and was given a good stir with the stirring/glass rod.
- When the media turned clear after a slight boil, the media-filled conical flask was covered with clean foil paper and kept in an autoclave machine at 121°C for approximately 2 hours so that the media is sterilized.
- After 2 hours in autoclave, the media was taken out of the machine and kept outside so that the media cooled down for use.
- After a bit of cooling down (~ 45-50°C), the media was poured into previously sterilized 3ml glass vials inside a vertical laminar flow cabinet and left to solidify at room temperature.
- In each vial, 2.5 ml of media was poured and after a few moments, the semisolid media gets prepared.

3.3.4.2 Inoculation of organism:

- The inoculation needle is sterilized in the spirit lamp by putting the needle into the flame until it is red hot.
- After cooling the needle, single bacterial colony is taken from a freshly subcultured bacterial sample and stabbed several times into the semisolid media inside the vial through a needle.

- After stabbing, the vial is wrapped with parafilm paper to keep it airtight and is put into an incubator for 24 hours for bacterial growth.
- After 24 hours, observing visible bacterial growth in the semisolid media, approximately 200µL paraffin oil is given in each vial using micropipette.
- The vial is sealed again with parafilm paper and stored in normal room temperature.

3.3.5 Enrichment of the pure phage (JSF7 and JSF2):

Phage enrichment means amplification of the number of phages that are capable to infect desired host. The protocol requires a host bacterium, nutritive medium and sample phage. For the protocol, the phage sample is enriched with the growth medium and incubated with host bacteria prior to centrifugation and filtration. Thus, if phage is capable of infecting the inoculated bacteria of the sample, then that phage will propagate and will increase in number to infect the host bacteria.

Reagents and Apparatus:

- Host bacteria WT346
- Vibriophages (JSF7 and JSF2)
- Inoculation loop
- LB Broth or, Luria-Bertani medium
- Falcon tube
- Vortex machine
- Shaker incubator
- Centrifuge machine
- Micropipette
- Syringe
- 0.22-micron syringe filter
- Parafilm

Procedure:

3.3.5.1 Preparation of Young Bacterial Culture:

- The host bacteria WT346 was streaked on LA medium plate and incubated at 37°C overnight.
- Using a micropipette, 3ml LB is taken into an autoclaved falcon tube.
- From the fresh 24-hour bacterial culture, few single colonies of WT346 bacteria were taken and inoculated in 3ml LB.
- With the help of a vortex machine, the solution is gently mixed.
- The falcon tube is then kept in the shaker incubator for 1.5 to 2 hours at 37°C.
- After 1.5 to 2 hours of incubation, the turbidity of the solution was checked, and logarithmic phase was ensured since the suspension was slightly turbid.
- Using a micropipette, 100µl of phage solution was added to the young bacterial culture of WT346.
- It was again incubated for 4 to 6 hours at 37°C in a shaker incubator.

3.3.5.2 Centrifuge:

- After 4 to 6 hours, the solution was taken out of the incubator and centrifuged in the centrifuge machine at 13000rpm for 5 minutes.

3.3.5.3 Filter:

- After centrifugation, the supernatant of the solution was collected by a fresh syringe.
- Using a 0.22-micron syringe filter, the supernatant was filtered to completely free the supernatant from any bacterial cells
- This filtered clear supernatant was then stored in an autoclaved falcon tube and wrapped with parafilm.
- For future use, the enriched phage solution is stored at 4°C.

3.3.6 Double Layer Agar method (DLA):

DLA method or Double Layer Agar method determines the concentration of functional bacteriophage particles (titer) and expresses as plaque-forming units (PFU/mL). It is necessary to determine the titer or the concentration of bacteriophage.

- **Reagents and Apparatus:**
  - Host bacteria WT346
  - Vibriophages (JSF7 and JSF2)
  - Inoculation loop
  - LB Broth or, Luria-Bertani medium
  - Falcon tube
  - Vortex machine
  - Shaker incubator
  - Micropipette
  - Tryptone
  - Sodium Chloride (NaCl)
  - Yeast extract
  - Bacteriological agar
  - Distilled Water
  - Bunsen burner
  - Eppendorf
  - Luria-Bertani agar plates (LA plates)

- **Procedure:**

3.3.6.1 Preparation of Young Bacterial Culture:

- The host bacteria WT346 was streaked on LA medium plate and incubated at 37°C overnight.
- Using a micropipette, 3ml LB is taken into an autoclaved falcon tube.

- From the fresh 24-hour bacterial culture, few single colonies of WT346 bacteria were taken and inoculated in 3ml LB.
- With the help of a vortex machine, the solution is gently mixed.
- The falcon tube is then kept in the shaker incubator for 1.5 to 2 hours at 37°C.
- After 1.5 to 2 hours of incubation, the turbidity of the solution was checked, and logarithmic phase was ensured since the suspension was slightly turbid.

3.3.6.2 Preparation of 0.8 % Soft Nutrient Agar:

- Measuring in an analytical balance machine, 1gm Tryptone, 1gm Sodium Chloride (NaCl), 0.5gm Yeast extract, and 0.8gm Bacteriological agar were taken with a clean spatula. The measured reagents were transferred to a clean conical flask.
- In the same conical flask, 100ml of distilled water was measured by using a measuring cylinder.
- The reagents were mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
- The conical flask was then kept on a Bunsen burner and was given a good stir with the stirring/glass rod.
- When the media turned clear after a slight boil, the media-filled conical flask was covered with clean foil paper and kept in an autoclave machine at 121°C for approximately 2 hours so that the media is sterilized.
- After keeping 2 hours in autoclave, the media was taken out of the machine and transferred into 10 test tubes.
- Until used, the test tubes were kept in the water bath (~52℃) so that the media is not solidified.

3.3.6.3 Dilution:

- Ten autoclaved clean Eppendorf tubes were taken and labeled from $10^{\wedge -1}$ to $10^{\wedge -10}$.
- For $10^{\wedge -1}$ dilution, in $10^{\wedge -1}$ Eppendorf tube, 900µL of LB was taken into which 100µL of enriched phage solution was mixed by re-pipetting.

- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-2} dilution, in 10^{-2} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-1} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-3} dilution, in 10^{-3} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-2} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-4} dilution, in 10^{-4} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-3} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-5} dilution, in 10^{-5} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-4} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-6} dilution, in 10^{-6} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-5} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-7} dilution, in 10^{-7} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-6} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-8} dilution, in 10^{-8} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-7} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-9} dilution, in 10^{-9} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-8} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.
- For 10^{-10} dilution, in 10^{-10} Eppendorf tube, 900µL of LB was taken into which 100µL solution from 10^{-9} was mixed by re-pipetting.
- With the help of a vortex machine, the solution is gently mixed.

3.3.6.4 Plating:

- Ten LA plates were taken and labeled from $10^{\wedge -1}$ to $10^{\wedge -10}$.
- From the water bath, one test tube containing 0.8 % soft agar was taken and bit cooled down. In the test tube, 300µL of young bacterial culture and 100µL of diluted phage solution from Eppendorf labeled $10^{\wedge -1}$ were taken.
- With the help of a vortex machine, the solution is gently mixed, and the mixture was poured onto LA plate labeled $10^{\wedge -1}$.
- From the water bath, another test tube containing soft agar was taken and bit cooled down. In the test tube, 300µL of young bacterial culture and 100µL of diluted phage solution from Eppendorf labeled $10^{\wedge -2}$ were taken.
- With the help of a vortex machine, the solution is gently mixed, and the mixture was poured carefully onto LA plate labeled $10^{\wedge -2}$.
- Similarly, all the other platings ($10^{\wedge -3}$ to $10^{\wedge -10}$) were done.
- All the plates were kept inside laminar for 15 minutes so that the media get solidified.
- Then all the plates were incubated at 37°C overnight.
- After the incubation period is over, single plaques were observed and then counted to determine the titer by using PFU (Plaque Forming Unit) formula. Each plaque is considered as on PFU and the titer (PFU/ml) is calculated by using the formula:

$$\frac{PFU \times Dilution\ factor}{Volume\ of\ phage\ lysate\ (ml)}$$

3.3.7 Spot titer:

Spot titer determines the titer or the concentration of bacteriophage. It is an alternative method to the DLA method or Double Layer Agar method. For this method, a lawn of bacteria is made and then droplets of different dilution phage solutions are given over the bacterial lawn. Following incubation if plaques are observed then the viability of the phage is ensured.

Reagents and Apparatus:

- Host bacteria WT346

- Vibriophages (JSF7 and JSF2)
- Inoculation loop
- LB Broth or, Luria-Bertani medium
- Falcon tube
- Vortex machine
- Shaker incubator
- Micropipette
- Tryptone
- Sodium Chloride (NaCl)
- Yeast extract
- Bacteriological agar
- Distilled Water
- Bunsen burner
- Eppendorf
- Luria-Bertani agar plates (LA plates)

Procedure:

- Young bacterial culture is prepared by inoculating few single colonies of WT346 bacteria in 3ml LB and incubated in a shaker incubator for 1.5 to 2 hours at 37°C.
- To make lawn of bacteria, in 3ml of soft agar, 200µL of young bacterial culture is mixed.
- The mixture is poured in an LA plate and kept for solidification.
- Phage dilution is done in LB.
- In the LA plate, 15µL of diluted phage solution are given in droplets over the bacterial lawn.

3.3.8 Preparation of Rhodamine B Solution:

Rhodamine B Solution is a mixture of Rhodamine B powder in a 0.2 M Sodium Hydroxide buffer. The concentration of the solution is mg/ml. That is, 1mg of Rhodamine B powder is mixed with 1ml of 0.2 M Sodium Hydroxide buffer. Rhodamine B is a xanthene dye that is used as a stain in

microbiology. It is a toxic and carcinogenic chemical compound. Thus, precautions should be taken while handling this toxic carcinogenic chemical compound

- **Reagents and Apparatus:**
  - Rhodamine B
  - Distilled Water
  - Sodium Hydroxide (NaOH)
  - Test tube
  - Analytical balance machine
  - Spatula
  - Foil
  - Vortex machine

- **Procedure:**
  - For making 0.2 M Sodium Hydroxide buffer, 40mg Sodium Hydroxide (NaOH) was measured in an analytical balance machine and transferred in a clean autoclaved test tube.
  - In the same test tube, measuring 5ml of distilled using a measuring cylinder was taken.
  - The reagent was mixed into the distilled water using a vortex machine until the powder gets dissolved.
  - Then 5mg Rhodamine B powder was measured in an analytical balance machine and mixed with the buffer using a vortex machine until the powder gets dissolved.
  - Thus, 5ml Rhodamine B Solution was made.

3.3.9 Preparation of Bile Salt Solution:

Bile Salt Solution is a mixture of 0.9% bile salt and distilled water.

- **Reagents and Apparatus:**
  - Bile Salt
  - Distilled Water
  - Conical flask
  - Analytical balance machine
  - Spatula
  - Foil
  - Vortex machine

- **Procedure:**
  - Measuring in an analytical balance machine, 0.9gm of Bile salt was taken with a clean spatula on a piece of foil paper.
  - In a clean conical flask, 100ml of distilled water was measured by using a measuring cylinder.
  - The measured reagent was transferred to the conical flask.
  - The reagent was mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
  - Then the solution was kept in an autoclave machine at 121°C for approximately 2 hours so that the solution is sterilized.

3.3.10 Preparation of Saline Solution:

Saline is a mixture of 0.9% Sodium Chloride (NaCl) and Water.

- **Reagents and Apparatus:**
  - Sodium Chloride (NaCl)
  - Distilled Water
  - Conical flask
  - Analytical balance machine

- Spatula
- Foil

Procedure:

- Measuring in an analytical balance machine, 0.45gm Sodium Chloride (NaCl) was taken with a clean spatula on a piece of foil paper.
- In a clean conical flask, 50ml of distilled water was measured by using a measuring cylinder.
- The measured reagent was transferred to the conical flask.
- The reagent was mixed into the distilled water by shaking the conical flask in a circular motion until the powder gets dissolved.
- Then the solution was kept in an autoclave machine at 121°C for approximately 2 hours so that the solution is sterilized.

3.4 Assay Protocols

3.4.1 Protocol 1 (Heating Time)

- **Reagents and Apparatus:**
  - Coconut Oil
  - Distilled Water
  - Test tube
  - Eppendorf
  - Cuvette
  - Micropipette
  - Centrifuge machine
  - Bunsen burner
  - Rhodamine B Solution
  - Spectrophotometer

- **Procedure:**
  - 4 test tubes were taken and labeled 1, 2, 3, and 4 respectively.
  - In 1, 4ml Coconut Oil and 4ml Distilled Water taken,
    In 2, 4ml Coconut Oil and 4ml Distilled Water taken,
    In 3, 4ml Coconut Oil and 4ml Distilled Water taken,
    In 4, 4ml Coconut Oil and 4ml Distilled Water taken.
  - Except 1, all the other test tubes were heated. 2 was heated for 10 minutes, 3 was heated for 20 minutes and 4 was heated for 30 minutes.
  - After taking all the test tubes out of heat, 200µL Rhodamine B Solution is given to each of the test tubes and mixed by inverting the tubes.
  - All the test tubes are kept at room temperature for 30 minutes to 1 hour.
  - After 30 minutes to 1 hour, using micropipette, the supernatant is extracted and transferred into 4 different eppendorfs.
  - All the eppendorfs are centrifuged in the centrifuge machine at 8000 rpm for 3 minutes.

- The supernatant is extracted and transferred into 4 different cuvettes.
- The OD of each sample is measured in spectrophotometer.

3.4.2 Protocol 2 (Oil Ratio)

Reagents and Apparatus:

- Coconut Oil
- Distilled Water
- Test tube
- Eppendorf
- Cuvette
- Micropipette
- Centrifuge machine
- Bunsen burner
- Rhodamine B Solution
- Spectrophotometer

Procedure:

- 5 test tubes were taken and labeled 1, 2, 3, Blank 1 and Blank 2 respectively.
- In 1, 2ml Coconut Oil and 4ml Distilled Water taken,
  In 2, 2.5ml Coconut Oil and 3.5ml Distilled Water taken,
  In 3, 3ml Coconut Oil and 3ml Distilled Water taken,
  In Blank 1, 3ml Coconut Oil and 3ml Distilled Water taken,
  In Blank 2, 6ml Distilled Water taken.
- Except Blanks, all the other test tubes are heated in boiled water for 30 minutes.
- After 30 minutes, the test tubes are taken out of heat and 200µL Rhodamine B Solution is given to each of the test tubes and mixed by inverting the tubes.
- All the test tubes are kept at room temperature for 30 minutes to 1 hour.

- After 30 minutes to 1 hour, using micropipette, the supernatant is extracted and transferred into 4 different eppendorfs.
- All the eppendorfs are centrifuged in the centrifuge machine at 8000 rpm for 3 minutes.
- The supernatant is extracted and transferred into 4 different cuvettes.
- The optical density (OD) of each sample is measured in spectrophotometer.

3.4.3 Protocol 3 (Plating Method 1)

Reagents and Apparatus:

- Host bacteria WT324
- Enriched Vibriophage JSF7 and JSF2 solution
- Coconut Oil
- Distilled water
- Saline solution
- Falcon tubes
- Micropipette
- Vortex machine
- Glass spreader
- Cotton swab
- Luria-Bertani agar plates (LA plates)

Procedure:

- Saline solution is made by mixing 0.45gm Sodium Chloride (NaCl) and 50ml of distilled water.
- From the fresh 24-hour bacterial culture, few single colonies of WT324 bacteria were taken and inoculated in 5ml saline solution and incubated in the shaker incubator for 2.5 to 3 hours at 37°C.

- After 3 hours, a sterile clean cotton swab is dipped into the saline with organism and streaked in the entire agar surface of an LA plate by rotating the plate approximately 60° each time to ensure an even distribution of the inoculum.
- Leaving the lid slightly ajar, the plate was allowed to rest at room temperature inside laminar at least 5-10 minutes, for the surface of the agar plate to dry before proceeding to the next step.
- Using a micropipette 2-3ml coconut oil is taken and using a sterile spreader the oil was spread evenly over the surface of agar, carefully rotating the Petri-dish.
- The plate is then incubated at 37°C overnight.
- After 24hrs, 20-25µL of JSF7 phage solution, 20-25µL of JSF2 phage solution, and 20-25µL of distilled water are given in droplets over the oil in the LA plate.
- The plate is then incubated at 37°C overnight.

3.4.4 Protocol 4 (Plating Method 2)

Reagents and Apparatus:

- Host bacteria WT346
- Enriched Vibriophage JSF7 and JSF2 solution
- Coconut Oil
- Distilled water
- Saline solution
- Falcon tubes
- Micropipette
- Vortex machine
- Cotton swab
- Luria-Bertani agar plates (LA plates)

Procedure:

- Saline solution is made by mixing 0.45gm Sodium Chloride (NaCl) and 50ml of distilled water.
- Using a sterile clean cotton swab, organism is taken from a fresh 24-hour bacterial culture of WT346 bacteria and directly dipped into the saline solution.
- Then the cotton swab is used to streak in the entire agar surface of an LA plate by rotating the plate approximately 60° each time to ensure an even distribution of the inoculum.
- Leaving the lid slightly ajar, the plate was allowed to rest at room temperature inside laminar at least 5-10 minutes, for the surface of the agar plate to dry before proceeding to the next step.
- Using a micropipette 300µL coconut oil is taken and given in three droplets over the oil in the LA plate.
- ~25µL of JSF7 phage solution is then given in droplet over one droplet of oil in the LA plate. In the other two oil droplets, ~25µL of JSF2 phage solution, and ~25µL of distilled water are given in droplets over the droplets of oil in the LA plate.
- The plate is then incubated at 37°C overnight.

3.4.5 Protocol 5 (JSF7 Activity)

Reagents and Apparatus:

- Enriched Vibriophage JSF7 solution
- Coconut Oil
- Distilled water
- Bile salt solution
- LB Broth or, Luria-Bertani medium
- Conical flasks
- Falcon tubes
- Test tubes
- Micropipette

- Centrifuge machine
- Cuvettes
- Rhodamine B Solution
- Spectrophotometer machine

Procedure:

- Three conical flasks are taken and labeled 1, 2, and 3 respectively. 5 ml of coconut oil and 5 ml of Bile Salt is taken in each of the three conical flasks and incubated in shaker incubator.
- After 24 hours from incubation, 500µL of phage solution is given in Conical 1.
- After 72 hours from incubation, 500µL of phage solution is given in Conical 2 and 500µL of LB is given in Conical 3.
- After 96 hours from incubation, 7ml of coconut oil and 3ml of distilled water are taken in a test tube and heated for 30 minutes in boiling water. After that supernatant is collected and transferred in a falcon tube and labeled 4.
- In the same day all the conical flasks are taken out from shaker incubator and the contents are transferred into three different falcon tubes and labeled 1, 2 and 3 respectively.
- 3ml of distilled water is added in each of the 4 falcon tubes and centrifuged in the centrifuge machine at 8000 rpm for 3 minutes.
- After centrifugation, from supernatant, extraction of 3-4ml oil from each of the four falcon tubes is done into autoclaved four different test tubes. The test tubes are also labeled as 1, 2, 3 and 4 respectively. In 1 remains, JSF7 24 hours, in 2 remains JSF7 72hours, in 3 remains heated 30 minutes and in 4 remains LB 24 hours sample.
- 5ml of distilled water is added in each of the four test tubes.
- 200µL Rhodamine B Solution is given to each of the test tubes and mixed by inverting the tubes.
- All the test tubes are kept at room temperature for 30 minutes to 1 hour.
- After 30 minutes to 1 hour, using micropipette, the supernatant from all the four test tubes is extracted and transferred into 4 different cuvettes.
- The OD at 560nm of each sample is measured in spectrophotometer.

3.4.6 Protocol 6 (Comparison of JSF2 and JSF7, Lipase Activity)

Reagents and Apparatus:

- Enriched Vibriophage JSF2 and JSF7 solution
- Coconut Oil
- Distilled water
- Bile salt solution
- LB Broth or, Luria-Bertani medium
- Conical flasks
- Falcon tubes
- Test tubes
- Micropipette
- Centrifuge machine
- Cuvettes
- Rhodamine B Solution
- Spectrophotometer machine

Procedure:

- Five conical flasks are taken and labeled 1, 2, 3, 4 and 5 respectively. 10 ml of coconut oil and 10 ml of Bile Salt is taken in each of the five conical flasks and incubated in shaker incubator.
- After 24 hours from incubation, 500µL of JSF2 phage solution is given in Conical 1 and 500µL of JSF7 phage solution is given in Conical 2.
- After 72 hours from incubation, 500µL of JSF2 phage solution is given in Conical 3 and 500µL of JSF7 phage solution is given in Conical 4 and 500µL of LB is given in Conical 5.
- After 96 hours from incubation, 7ml of coconut oil and 3ml of distilled water are taken in a test tube and heated for 30 minutes in boiling water. After that supernatant is collected and transferred in a falcon tube and labeled 6.

- In the same day all the conical flasks are taken out from shaker incubator and the contents are transferred into five different falcon tubes and labeled 1, 2, 3, 4 and 5 respectively.
- 5ml of distilled water is added in each of the six falcon tubes and centrifuged in the centrifuge machine at 8000 rpm for 3 minutes.
- After centrifugation, from supernatant, extraction of 4-5ml oil from each of the six falcon tubes is done into autoclaved six different test tubes. The test tubes are also labeled as 1, 2, 3, 4, 5 and 6 respectively. In 1 remains, JSF7 72 hours, in 2 remains JSF7 24 hours, in 3 remains JSF2 72 hours, in 4 remains JSF2 24 hours, in 5 remains heated 30 minutes and in 6 remains LB 24 hours sample.
- 5ml of distilled water is added in each of the six test tubes.
- 200µL Rhodamine B Solution is given to each of the test tubes and mixed by inverting the tubes.
- All the test tubes are kept at room temperature for 30 minutes to 1 hour.
- After 30 minutes to 1 hour, using micropipette, the supernatant from each of the six test tubes is extracted and transferred into six different cuvettes.
- The OD at 560nm of each sample is measured in spectrophotometer.

3.4.7 Protocol 7 (Dose Dependency of JSF7)

Reagents and Apparatus:

- Enriched Vibriophage JSF2 and JSF7 solution
- Coconut Oil
- Distilled water
- Bile salt solution
- LB Broth or, Luria-Bertani medium
- Conical flasks
- Falcon tubes
- Eppendorf tubes
- Test tubes

- Micropipette
- Centrifuge machine
- Vortex machine
- Cuvettes
- Rhodamine B Solution
- Spectrophotometer machine

Procedure:

- Five conical flasks are taken and labeled 1, 2, 3, 4, and 5 respectively. 10 ml of coconut oil and 10 ml of Bile Salt are taken in each of the five conical flasks and incubated in shaker incubator.
- After 24 hours from incubation, 500µL of JSF2 phage solution is given in Conical 5 and 500µL of JSF7 phage solution is given in Conical 1, and dilution of enriched JSF7 phage solution is done up to $10^{\wedge -3}$.
- For dilution three Eppendorf tubes are taken and labeled $10^{\wedge -1}$, $10^{\wedge -2}$, and $10^{\wedge -3}$ respectively.
- For $10^{\wedge -1}$ dilution, in $10^{\wedge -1}$ Eppendorf tube, 900µL of LB was taken into which 100µL of enriched JSF7 phage solution was mixed by re-pipetting and with the help of a vortex machine, the solution is gently mixed.
- For $10^{\wedge -2}$ dilution, in $10^{\wedge -2}$ Eppendorf tube, 900µL of LB was taken into which 100µL solution from $10^{\wedge -1}$ was mixed by re-pipetting and with the help of a vortex machine, the solution is gently mixed.
- For $10^{\wedge -3}$ dilution, in $10^{\wedge -3}$ Eppendorf tube, 900µL of LB was taken into which 100µL solution from $10^{\wedge -2}$ was mixed by re-pipetting and with the help of a vortex machine, the solution is gently mixed.
- 500µL of $10^{\wedge -1}$ JSF7 phage solution is given in Conical 2, 500µL $10^{\wedge -2}$ JSF7 phage solution is given in Conical 3 and 500µL of $10^{\wedge -3}$ JSF7 phage solution is given in Conical 4.
- After 96 hours from incubation, all the conical flasks are taken out from shaker incubator and the contents are transferred into five different falcon tubes and labeled 1, 2, 3, 4 and 5 respectively.

- 5ml of distilled water is added in each of the five falcon tubes and centrifuged in the centrifuge machine at 8000 rpm for 3 minutes.
- After centrifugation, from supernatant, extraction of 4-5ml oil from each of the five falcon tubes is done into autoclaved five different test tubes. The test tubes are also labeled as 1, 2, 3, 4, and 5 respectively. In 1 remains, JSF7 72 hours, in 2 remains JSF7 $10^{\wedge -1}$, in 3 remains JSF7 $10^{\wedge -2}$, in 4 remains JSF7 $10^{\wedge -3}$ and in 5 remains JSF2 72 hours sample.
- 5ml of distilled water is added in each of the five test tubes.
- 200µL Rhodamine B Solution is given to each of the five test tubes and mixed by inverting the tubes.
- All the test tubes are kept at room temperature for 30 minutes to 1 hour.
- After 30 minutes to 1 hour, using micropipette, the supernatant from each of the five test tubes is extracted and transferred into five different cuvettes.
- The OD at 560nm of each sample is measured in spectrophotometer.

Chapter 4: Results

4.1 Protocol 1 (Heating Time):

Table 4.1 Protocol 1 (Heating Time)

SAMPLE NO.	COMPONENTS	HEATING TIME (Minutes)	OD (560 nm)
1	4 ml Oil : 4 ml dH2O (r = 1:1)	0 (No heat)	0.153
2	4 ml Oil : 4 ml dH2O (r = 1:1)	10	0.289
3	4 ml Oil : 4 ml dH2O (r = 1:1)	20	0.455
4	4 ml Oil : 4 ml dH2O (r = 1:1)	30	0.569

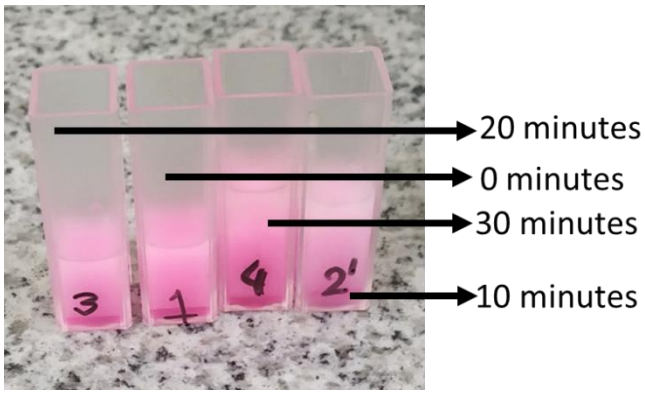
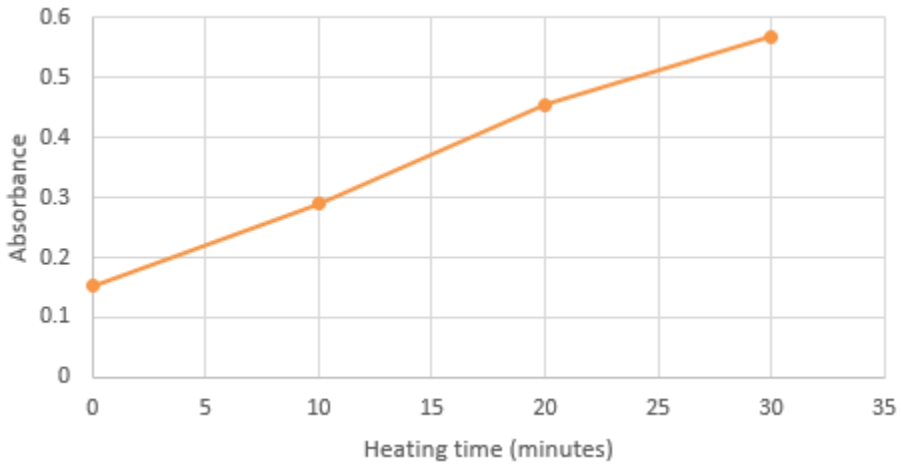


Figure 4.1 Samples of protocol 1 (Heating time)



Graph: Absorbance Vs. Heating Time.

4.2 (a) Protocol 2 (Oil Ratio):

Table 4.2(a) Protocol 2 (Oil Ratio)

SAMPLE NO.	COMPONENTS	HEAT (30 minutes)	RHODAMINE
1	3 ml Oil : 3 ml dH_2O (r = 1:1)	Yes	Yes
2	2 ml Oil : 4 ml dH_2O (r = 1:2)	Yes	Yes
3	2.5 ml Oil : 3.5 ml dH_2O (r = 1:1.5)	Yes	Yes
4 (Blank 1)	3 ml Oil : 3 ml dH_2O	No	Yes
5 (Blank 2)	6 ml dH_2O	No	Yes

OD 1 (560 nm)	OD 2 (560 nm)	OD 3 (560 nm)	AVERAGE
0.466	0.463	0.462	0.464
0.338	0.336	0.338	0.336
0.370	0.377	0.392	0.379
0.188	0.173	0.159	0.173
0.523	0.589	0.557	0.556

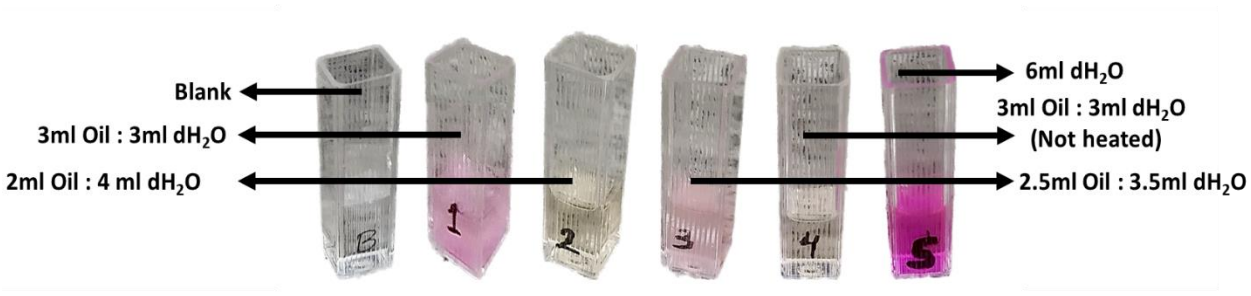
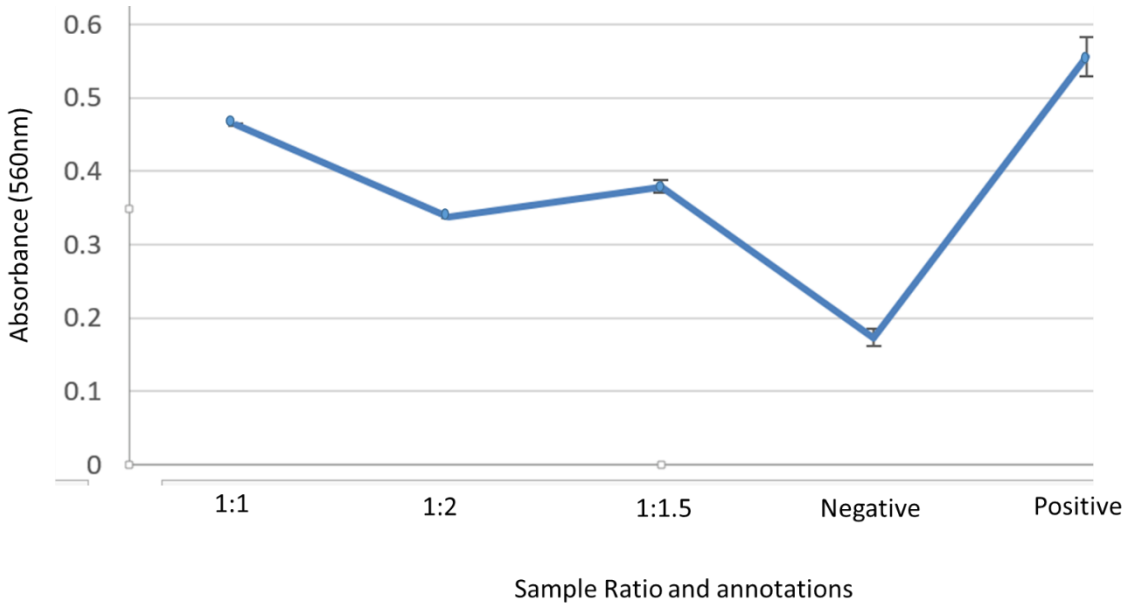


Figure 4.2 Samples of protocol 2 (Oil Ratio)



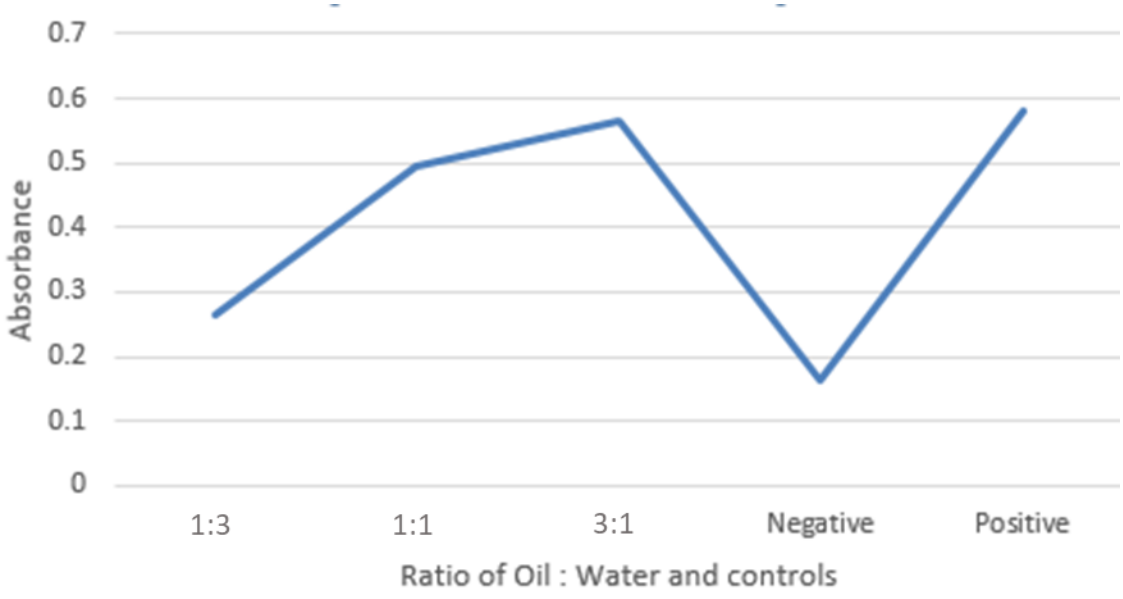
Graph: Oil Ratio Graph (First trial)

4.2 (b) Protocol 2 (Oil Ratio):

Table 4.2(b) Protocol 2 (Oil Ratio)

SAMPLE NO.	COMPONENTS	HEAT (30 minutes)	RHODAMINE
1	2 ml Oil : 6 ml dH_2O (r = 1:3)	Yes	Yes
2	4 ml Oil : 4 ml dH_2O (r = 1:1)	Yes	Yes
3	6 ml Oil : 2 ml dH_2O (r = 3:1)	Yes	Yes
4 (Blank 1)	4 ml Oil : 4 ml dH_2O	No	Yes
5 (Blank 2)	8 ml dH_2O	No	Yes

OD 1 (560 nm)	OD 2 (560 nm)	AVERAGE
0.267	0.263	0.265
0.490	0.497	0.493
0.530	0.599	0.564
0.160	0.169	0.164
0.572	0.588	0.580



Graph: Oil Ratio Graph (Second trial)

4.3 Protocol 3 (Plating Method 1):

Figure 4.1 Plating Method 1 of JSF7, JSF2 and dH_2O to check lipase activity

4.4 Protocol 4 (Plating Method 2):

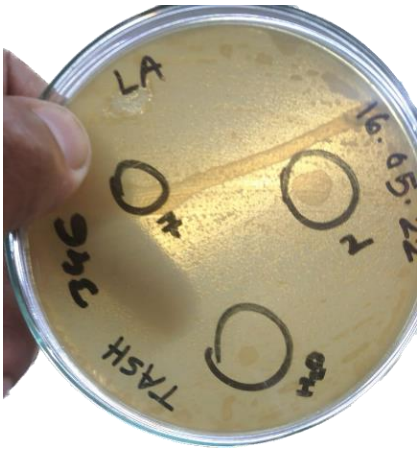


Figure 4.2 Plating Method 2 of JSF7, JSF2 and dH_2O to check lipase activity

4.5 Protocol 5 (JSF7 Activity):

Table 4.5 Protocol 5 (JSF7 Activity)

SAMPLE NO.	COMPONENTS	COMPONENTS	Time
1	10 ml Oil + 10 ml Bile Salt	JSF7	24 hours
2	10 ml Oil + 10 ml Bile Salt	JSF7	72 hours
3	10 ml Oil + 10 ml Bile Salt	Heat	30 minutes
4	10 ml Oil + 10 ml Bile Salt	LB	24 hours

OD 1 (560 nm)	OD 2 (560 nm)	OD 3 (560 nm)	AVERAGE
0.485	0.494	0.483	0.487
0.540	0.570	0.530	0.546
0.553	0.587	0.594	0.578
0.166	0.171	0.169	0.168

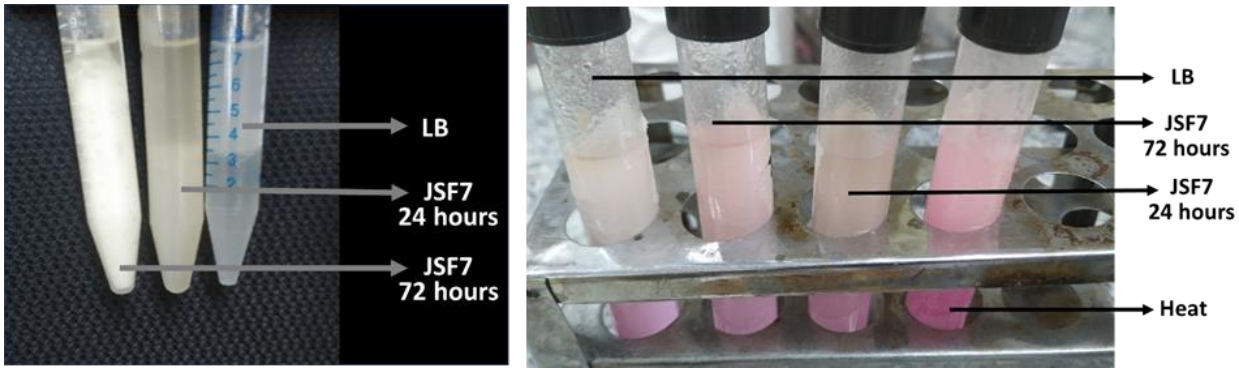
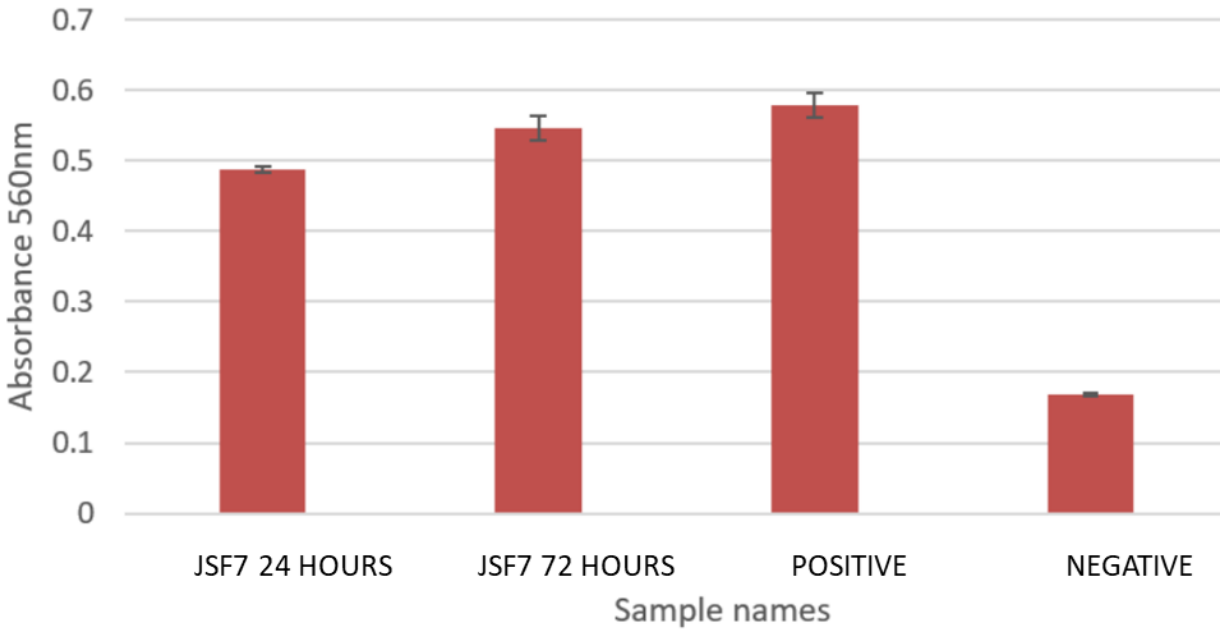


Figure 4.3 Samples of Protocol 5 (JSF7 Activity)



Graph: Protocol 5 graph showing lipase activity of JSF7

4.6 Protocol 6 (Comparison of JSF2 and JSF7, Lipase Activity):

Table 4.6 Protocol 6 (Comparison of JSF2 and JSF7, Lipase Activity)

SAMPLE NO.	COMPONENTS	COMPONENTS	Time
1	10 ml Oil + 10 ml Bile Salt	JSF7	72 hours
2	10 ml Oil + 10 ml Bile Salt	JSF7	24 hours
3	10 ml Oil + 10 ml Bile Salt	JSF2	72 hours
4	10 ml Oil + 10 ml Bile Salt	JSF2	24 hours
5	10 ml Oil + 10 ml Bile Salt	Heat	30 minutes
6	10 ml Oil + 10 ml Bile Salt	LB	24 hours

OD 1 (560 nm)	OD 2 (560 nm)	OD 3 (560 nm)	AVERAGE
0.573	0.584	0.594	0.583
0.568	0.557	0.541	0.553
0.210	0.184	0.185	0.193
0.206	0.190	0.179	0.191
0.593	0.575	0.555	0.574
0.127	0.200	0.170	0.165

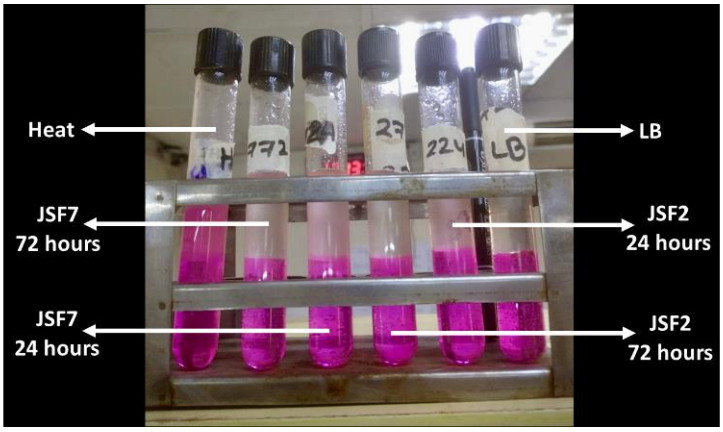
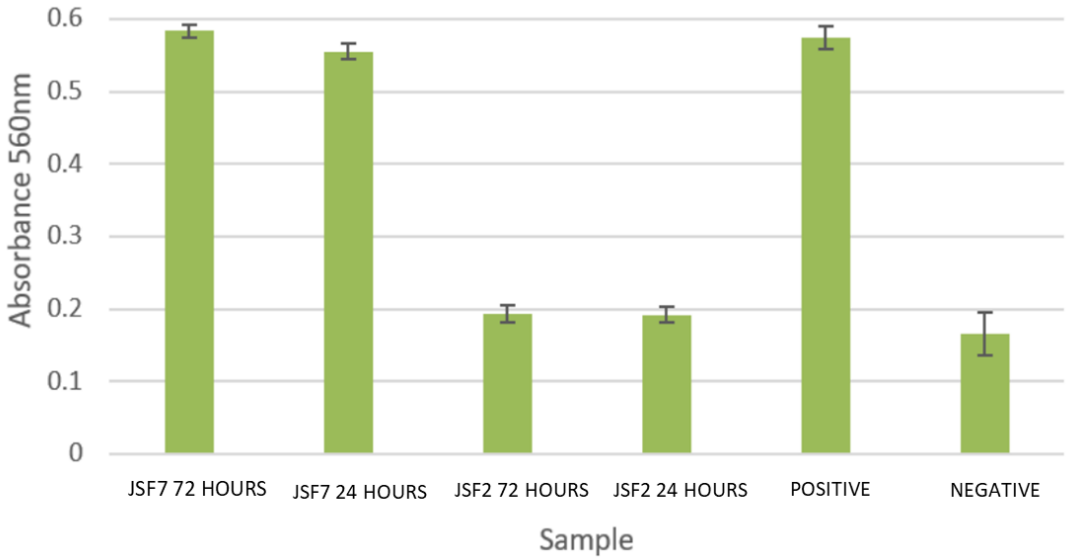


Figure 4.4 Samples of protocol 6 (Comparison of JSF2 and JSF7, Lipase Activity)



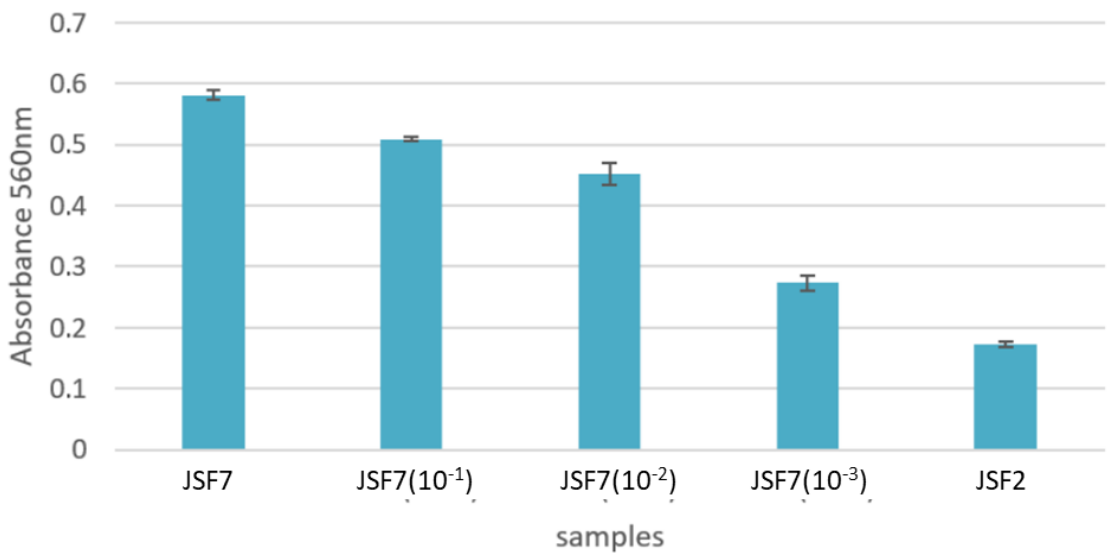
Graph: Protocol 6 showing the comparison of JSF7 and JSF2 lipase activity

4.7 Protocol 7 (Dose Dependency of JSF7):

Table 4.7 Protocol 7 (Dose Dependency of JSF7)

SAMPLE NO.	COMPONENTS	Phage	Time
1	10 ml Oil + 10 ml Bile Salt	JSF7	72 hours
2	10 ml Oil + 10 ml Bile Salt	JSF7 (10^{-1})	72 hours
3	10 ml Oil + 10 ml Bile Salt	JSF7 (10^{-2})	72 hours
4	10 ml Oil + 10 ml Bile Salt	JSF7 (10^{-3})	72 hours
5	10 ml Oil + 10 ml Bile Salt	JSF2	72 hours

OD 1 (560 nm)	OD 2 (560 nm)	OD 3 (560 nm)	AVERAGE
0.588	0.570	0.586	0.581
0.506	0.510	0.512	0.509
0.475	0.450	0.432	0.452
0.290	0.260	0.270	0.273
0.179	0.170	0.169	0.172



Graph: Dose dependency graph of JSF7

Chapter 5: Discussion

In the target experiment we tried to find out the effects of lipase enzyme of the phage JSF7. In this phage it was found that the lipase enzyme coding region of the phage genome was in the tail region of the phage. It was previously known that the proteins coded by the genome of the tail region will work even without the host. In our research we tried to find out the effectivity of this phage on a lipid source coconut oil. In the first part of our experiment, we tried to proof the use of rhodamine to detect the amount of fatty acids in a sample. Through multiple trial and error process in the experiment, we were able to prove the retention ability of rhodamine for fatty acids that were freed by JSF7 phages.

In our first protocol, we tried to see how much time of heating oil is optimum for using it as a positive control in our further experiments. In this experiment process we added equal ratio of water and oil at the ratio of 1:1 in all our samples. The heating time here was different for each of the samples. The time for heating was set at 0 minutes (no heat) for the first sample, 10 minutes for the second sample, 20 minutes for the third sample and 30 minutes for the fourth sample. We found that the OD obtained for sample 1 to 4 were 0.153, 0.289, 0.455 and 0.569 an average of multiple trials. From here we took the standard heating time as 30 minutes.

Next, we did two trials for our second protocol. In our first trial of second protocol of rhodamine assay, we used heat treatment on oil and water suspension. The ratio of the oil and water was varied from 1:1 to 1:2. The heat was equally given to the three samples. It was estimated that heating for a certain time will extract enough fatty acids from the solution. The negative control was a 1:1 ratio of oil and water with no heat treatment. In this case the rhodamine was fully transferred to the water leaving the oil with no color in it due to not having any fatty acids to bond to the rhodamine. In the experiment the multiple trials of the same process gave an average OD 0.336, 0.379 and 0.464 for the ratio of 1:2, 1:1.5 and 1:1 oil and water respectively. For positive control we got the highest OD value from the sample of 6ml distilled water which was 0.556. Here, the total rhodamine was fully mixed with water so gave the highest OD. So, comparing with the

positive control, from this assay we found that the more the portion of oil in the sample is present the more was the ability of retaining rhodamine. We found almost similar result in case of our first sample that had oil and water same ratio and the OD was of 0.464 that was near to positive control's OD (0.556). This meant that the ratio of oil and water used at the range of 1:1 is enough to extract some amount of fatty acid which was able to bind with rhodamine to give the result. Thus, this means the more there is fatty acid in the solution the more it can retain Rhodamine B.

In our next trial (of second protocol) we tried the similar process again but this time with a more variable ratio. The ratio of the oil and water were subsequently 3:1, 1:1 and 1:3 which was followed by heating for 30 minutes. Heating the oil and water sample meant that there is more chance of having more fatty acids. We did the same test again but with more ratio gap. The positive control was distilled water and the negative control was a 1:1 ratio of oil and water heated but not given any rhodamine. The obtained results were very promising for these trials we got better readings for our samples. The OD readings at 560nm were 0.564, 0.493 and 0.265 average of two trials at a ratio of 3:1, 1:1 and 1:3 oil and water. The reading of the positive control and the 3:1 were significantly higher than our other samples having less oil ratio. The other two samples were significantly higher than the negative control which was 0.164 in which no heat was given. So, it shows that solutions having more fatty acids are being able to hold more rhodamine B and give higher results.

In one of our next procedures, we tried two plating methods using LA Agar of 3% concentration. In this experiment we first made the host strain 346 fresh culture in LB broth and then spread the organism on the LA plate using cotton swab. Then we spread coconut oil on the organism layer using a spreader on the plate. This was followed by putting enriched phage samples on the plate at labelled positions and incubated overnight at 37°C. The idea was that the bacteria would grow at the layer above the LA agar and the bacteriophage would be able to degrade the oil layer and reach the bacteria layer underneath the oil and give clear zones. The phage JSF2 and distilled water were also placed on the labelled areas and was given to incubate overnight. However, the procedure did not show any significant achievements. We found no clear zones and the results were same like

those of negative controls. We estimated that the layer of oil was too much in comparison to the phage concentration.

In another plating method we tried doing the similar process but used swabbing method of oil instead of poring and then spreading. This made the oil layers very thin compared to our previous plating method. The JSF7, JSF2 and distilled water were given at the labelled spots on the plate. The JSF2 phage and distilled water was given as negative control. The results were not satisfactory as similar spots were seen to be made by phages JSF7, JSF2 and distilled water.

In our next step we took the samples of JSF7 and added them in similar samples of oil and bile salts. However, we gave them different incubation times to work with solution. We had hypothesized that phage samples having more time to incubate with the lipid solution will get more chance to use the lipase enzyme and extract more fatty acids from the solution. So according to our hypothesis, the quantity of fatty acids in the sample JSF7 for 72 hours will be the highest and JSF7 for 24 hours will have somewhat lower or similar quantity of fatty acids quantity. The complex will then form between the rhodamine B and fatty acids from the solution and give fluorescence following the Beer Lamberts law. In our experiment we found that the OD at 560nm for JSF7 for 72 hours and JSF7 for 24 hours were 0.546 and 0.487 after three trails average respectively. The negative control used here was LB broth that did not have any phage in the solution and the positive control was taken as heated solution of oil and bile salt solution. The values of the positive control were significantly higher than our samples and the reading at 560nm was recoded at an average of three trials to be 0.578. The negative control of the process was seen to have a value of OD around 0.168 after three trials average.

In our next step, we again focused on using bile salt as an intermediate to make smaller chains of lipids which could somewhat give the phages a better chance to separate the fatty acids from the chains. In the subsequent experiments, we tried to use the bile salts in equal volume of the oil given. The conical flask was used in this experiment for better rotational effect and better mixing of the oil with the bile salt. Next the phages JSF7 and JSF2 were added to the labeled flask. Sample

772 means that JSF7 is being added for 72 hours before the OD measurement and 272 means that JSF2 is being added to the oil and bile salt emulsion for 72 hours before OD is measured. There were two more samples that were prepared which were named 724 and 224. Among these 724 means that JSF7 has been added for 24 hours before the OD is being measured. The positive and negative controls being used here are Heat treatment and LB. The heat treatment was used because we found previous proof from our first assay technique that heating the oil for some time will release fatty acids that will be able to retain the rhodamine. The LB was used as a negative control because it had nothing to retain the rhodamine after the assay the obtained result was 0.165. In this process we found that most of the 772 annotated samples had an average OD of around 0.583, 724 had around 0.553, 272 annotated samples had OD of 0.193, 224 annotated samples had around 0.191. In our positive controls that were heated for 30 minutes approximately in all our experiments had an average OD of 0.574.

In this assay technique, we gave each pair of sample different time spans to work on the oil. It was estimated that the oil substrate and lipase enzyme of the JSF7 could work together without the host. The hypothesis was that there will be more free fatty acids when the JSF7 can interact with the oil. The negative controls used here were LB and JSF2 samples. It was hypothesized that these samples will give almost similar readings or less than those of JSF7. In the process, we extracted the oil from the bile salt by first pouring it into a falcon tube. Then the samples were given some water so that no phage bodies will remain in the oil. After giving it a centrifugation process it was estimated that there was no phage in the oil layer. The reason is that phages have more tendency to remain in the polar solvent than compared to a hydrophobic layer. Later the oil layer was extracted, and rhodamine was applied equally to all the samples and let to rest so that the dye can couple with the free fatty acids. In this part of our assay, we used 560nm wavelength which is the best wavelength for procedures with rhodamine. The OD values of the JSF7 samples incubated for 72 and 24 hours labeled 772 and 724 had significantly higher values compared to those of the other three negative controls, it was seen that the value of the positive control was somewhat close to that of our values of JSF7 supplied samples. The negative controls had a much lower value range compared to the positive control and JSF7-supplied phage. The range of the negative controls were also very close to each other.

The next experiment that we tried to do after the use of bile salt and phage for different time intervals was dose dependency. In this experiment, we tried to use different doses of the phage sample in the mixture of bile salt and oil. Here we used the same ratio of 1:1 oil and water as our last assay and then gave different doses of the same JSF7 phage in each sample. The hypothesis was different concentrations of phages will be able to achieve different quantity of fatty acids from the oil sample. In this protocol, we used JSF2 as our negative control and JSF7 as our positive control. By doing serial dilution to decrease the concentration of each consecutive sample by ten folds we were able to give three samples of phage each ten folds less than the previous sample in three different conical flasks. It was then allowed to be incubated for 72 hours and the OD was measured at 560nm wavelength using the UV spectrophotometer. The results showed the highest OD for 772 which means our positive control and gradual decrease in OD as the dilution increased by ten folds each time. It was seen that after 1000 times dilution the OD obtained was same as the negative control of our experiment. We could then conclude that the results by getting a confirmation that different concentrations of phages can cause the level of OD to be different also. Each time the OD decreases with the decrease in phage amount provided in the oil and bile salt solution. In our dose dependency test, we found that the sample annotated as 772 gave an average OD of 0.581, 7 (10^{-1}) gave an average OD of 0.509, 7 (10^{-2}) gave an average OD of 0.452 and 7 (10^{-3}) gave an average OD of 0.273. The last sample 7 (10^{-3}) had nearly the same OD as that of our negative control 272 that gave an OD average of 0.172.

We found that samples having multiple trials and readings had some standard deviation in them. It was found that there was slight standard deviation noticed in our second protocol of oil and water ratio. In one of our samples, we had found the deviation to be around 0.034. Which indicated the chances of error in this process is very less. In our protocol for testing the efficiency of JSF7, we found that there were two samples having standard deviation around 0.017 which means there is 17% chance of getting a value for our sample more than the mean. In our protocol six, we compared the functioning of JSF7 phage with JSF2 for lipolytic activity and there we had standard deviations around 0.029. Also, the negative control had deviation around 0.01 in multiple samples. Similarly, in our final protocol we had some deviations from the mean at the negligible level which were

mostly around 0.01 or less. Which indicates that very less chance is there that our values throughout the experiment have chance to get scattered from the mean.

Through this experiment, we proved that the availability of the free fatty acids from the lipase activity of JSF7 was proved by the dose dependency test. By following different protocols and having a trial-and-error method we were able to show that JSF7 phage has lipid degrading capacity in small levels even without the presence of any host. The presence of more fatty acids in 772 sample had a better chance to make complex with RhB and gave more fluorescence and OD values. The OD values were significantly decreasing as we increased the dilution for the phages. This is a proof that the presence of more phages in the solution gave more free fatty acids that were able to complex with Rhodamine B and give more OD values.

Chapter 6: Conclusion and Future perspectives

In our overall work, we tried to find out the efficiency of the lipase enzyme of JSF7 using different assay methods. Through these processes of the experiment, we were able to find out how much effective the phage can be against lipid substances. We could see that the experiment was more effective in the liquid phase of the experiment and showed less efficiency in the solid phase works. After the finding of the lipase enzyme in the genome of the organism we are now somewhat confirmed about it by this experimental process. In our future works, we are planning to get the specific lipase protein-encoding gene amplified. Using our self-designed primer, we are going to do a high-fidelity PCR using which we would amplify the DNA sequence first. Afterwards, future work will be more oriented toward getting the lipase protein extracted and testing its efficiency in different substrate samples.

References:

1. Kasman, L.M. and L.D. Porter, *Bacteriophages*, in *StatPearls*. 2022: Treasure Island (FL).
2. Sharma, S., et al., *Bacteriophages and its applications: an overview.* Folia Microbiol (Praha), 2017. **62**(1): p. 17-55.
3. Naser, I.B., et al., *Environmental bacteriophages active on biofilms and planktonic forms of toxigenic Vibrio cholerae: Potential relevance in cholera epidemiology.* PLoS One, 2017. **12**(7): p. e0180838.
4. Faruque, S.M., *Role of Phages in the Epidemiology of Cholera*, in *Cholera Outbreaks*, G.B. Nair and Y. Takeda, Editors. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 165-180.
5. Weinbauer, M.G. and F. Rassoulzadegan, *Are viruses driving microbial diversification and diversity?* Environmental Microbiology, 2004. **6**(1): p. 1-11.
6. Brockman, H.L., *Lipases*, in *Encyclopedia of Biological Chemistry (Second Edition)*, W.J. Lennarz and M.D. Lane, Editors. 2013, Academic Press: Waltham. p. 729-732.
7. N.A, S., K. Hassan, and S.N. Rahaman, *Preliminary Studies for Production of Fatty Acids from Hydrolysis of Cooking Palm Oil Using C. rugosa Lipase.* Journal of Physical Science, 2008. **19**.
8. Szabo, Z., et al. *Effects of Repeated Heating on Fatty Acid Composition of Plant-Based Cooking Oils*. Foods, 2022. **11**, DOI: 10.3390/foods11020192.
9. Nasution, I.P.M., et al., *Screening the Quality of Coconut Oil Heated on Several Temperatures by Liquid Chromatograph Mass Spectrometry.* IOP Conference Series: Earth and Environmental Science, 2020. **515**(1): p. 012025.
10. A.G, G.K., et al., *Coconut Oil: Chemistry, Production and Its Applications - A Review*. 2010.
11. Yamashita, H., et al., *Photochemical properties of Rhodamine-B dye molecules included within mesoporous molecular sieves.* Studies in Surface Science and Catalysis, 1998. **117**.
12. Jette, J.F. and E. Ziomek, *Determination of Lipase Activity by a Rhodamine-Triglyceride-Agarose Assay.* Analytical Biochemistry, 1994. **219**(2): p. 256-260.
13. Bartasun, P., et al., *A study on the interaction of rhodamine B with methylthioadenosine phosphorylase protein sourced from an Antarctic soil metagenomic library.* PLoS One, 2013. **8**(1): p. e55697.

14. Kizil, N., M. Soylak, and M. TÜZen, *Spectrophotometric detection of rhodamine B in tap water, lipstick, rouge, and nail polish samples after supramolecular solvent microextraction.* TURKISH JOURNAL OF CHEMISTRY, 2017. **41**: p. 987-994.
15. Marczenko, Z. and M. Balcerzak, *Principles of Spectrophotometry*. 2000. p. 26-38.
16. Middelboe, M., n.p. Amy M. Chan, and n.p. and Sif K. Bertelsen, *Bacteriophage isolation using enrichment cultures.*