INVESTIGATION OF THE ROLE OF FREE DNA IN THE FORMATION OF BACTERIAL BIOFILM

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A thesis submitted to the Department of Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
Brac University
September 2022

Declaration

It is hereby declared that

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

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The thesis titled "**Investigation of The Role of Free DNA in The Formation of Bacterial Biofilm**" submitted by

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is my own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

Abstract

Bacteriophage also known as bacterial virus are considered as natural enemy of bacteria since their discovery. In many medical settings, bacteriophages are used to eliminate bacteria from bacterial infected region. In our environment, the increase or decrease of bacteriophage depends on season. Naturally rise in bacteriophage will infect more bacteria resulting in the decrease of bacteria population and vice versa. Bacteriophage injects their genetic material into the bacterial cell and replicate inside it. The viral predators burst open the bacterial cell and come out of it. All the bacterial cellular components are released in the environment including bacterial DNA. Under such adverse condition bacteria will form biofilm and shield themselves from these bacterial viruses. In this study we will try to establish if the bacterial DNA provided by the bacteriophage helps in biofilm formation of bacteria.

This work is dedicated to

Our Dear Families

Acknowledgment

First and foremost, we would like to express our sincere gratitude to Almighty God for providing us with the perseverance, patience, and knowledge needed to finish this project within the desired time span. We convey our special thanks to Professor A. F. M. Yusuf Haider, Ph.D., Chairman, Department of Mathematics and Natural Sciences, BRAC University, who always has tremendous support for every student. We would like to showcase our deepest and most sincere gratitude to our project supervisor, Dr. Iftekhar Bin Naser, Associate Professor, Department of Mathematics and Natural Sciences, BRAC University, whose expertise, constant guidance, sincere monitoring, and motivational approaches in every sphere have inspired us to conduct this project efficiently. We sincerely express our appreciation and admiration for his patience and for providing a sense of direction whenever we encountered complications throughout this phase. We feel to acknowledge our indebtedness to him for his insightful corrections and directions which shaped the present work as it is today. We are indebted to all the extraordinary people of the Life Science Laboratories, BRAC University. Without their all-time support this would not be complete. Lastly, we would like to thank our parents for their constant support and encouragement that further pushed us to work harder and face the difficulties during this time frame.

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List of Acronyms

ARF6	ADP Ribosylation Factor 6
c-AMP	Cyclic Adinosine Monophosphate
CFTR	Cystic Fibrosis Trans membrane Receptor
CTX	Cholera Toxin
CVEC	Conditionally Viable Environmental Cell
DLA	Double Layer Assay
EAEC	Enteroaggregative *E. coli*
EPEC	Enteropathogenic *E. coli*
EPS	Extracellular Polymeric Substance
ER	Endoplasmic Reticulum
ETEC	Enterotoxigenic *E. coli*
FIB	Faecal Indicator Bacteria
GM1	Monosialotetrahexosylganglioside
LB	Luria Bertani
OD	Optical Density
PFU	Plaque-Forming Units
PP	Peyer's Patch
STEC	Shiga Toxin-producing *E. coli*
T1N1	Tryptone Salt Agar
UTIs	Urinary Tract Infections
UV	Ultra Violet
VBNC	Viable But Not Culturable

1. Introduction

1.1 Biofilm:

Bacterial biofilm is one of the main reasons behind antimicrobial resistance therapies which is an alarming issue. Bacteria form biofilm in adverse conditions and remain dormant until a favorable environment is created for multiplication and infection in a host body. Biofilm formation is also one of the survival mechanisms of bacteria. Some of the reasons for bacterial biofilm formation are adverse environments (excessive heat or cold), lack of nutrients, invasion of bacteriophages, etc.(Naser et al., 2017).

Bacterial biofilms are assemblages of bacteria that are embedded in a self-made extracellular polymeric substance (EPS) comprised of proteins, exopolysaccharides, and lipids and adhered to a surface or to each another. The composition and properties of the matrix and the interactions of molecules or Quorum sensing give biofilms their ability to adhere to different surfaces, maintain nutrient reservoirs, and shield themselves from the outside environment(Rabin et al., 2015).Five main steps can be characterized in the complex but well-regulated process of biofilm development: (i) Surface swarming, which is made possible by surface sensing carried out by planktonic bacteria's flagella; (ii) the stage of attachment, whereby the bacterial adhesions carry out the initial reversible attachment, which indicates loosely adhering to the surface and detaching, and the subsequent irreversible attachment, which is more precise and stable adhesion; (iii) the excretion of EPS matrix, which is a sign that a biofilm has been formed and was produced by recently attached bacteria; (iv) the maturation of the biofilm, which involves bacterial cell interactions that result in the development of micro-colonies; (v) the structure of the biofilm spreading after planktonic bacteria are released, causing the biofilm to form at new locations (Alhede et al., 2011), (Armbruster & Parsek, 2018).Bacteria in biofilms can use several survival techniques to invade the host defense mechanisms in addition to the protection provided by the matrix (Moser et al., 2017). They may damage local tissue by remaining dormant for a long time and hiding from the immune system and subsequently resulting in an acute infection. Alteration of metabolism, gene expression, and protein production within the biofilm, the bacteria adjust to environmental anoxia and nutritional limitation, which can result in a slower metabolic rate and a slower rate of cell division (Hall-Stoodley & Stoodley, 2009). The majority of biofilm-related diseases are slow progressive chronic infections, and often resistant to antimicrobial therapies.

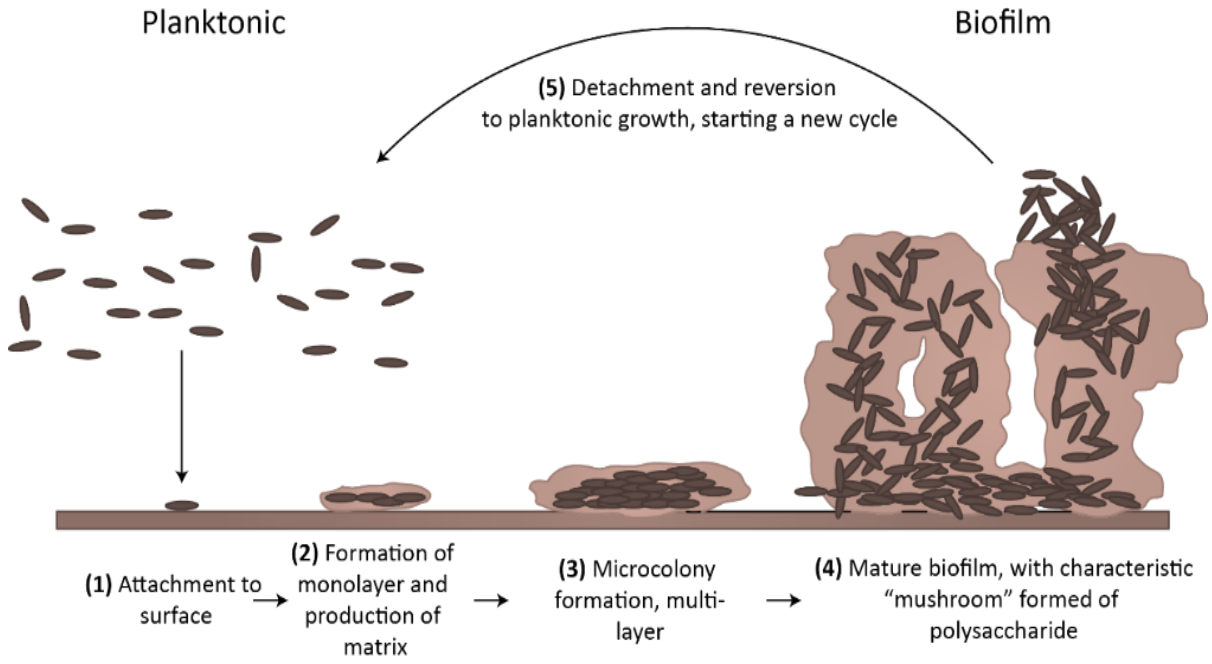

Figure 1: The process of biofilm formation

In figure 1, the basic process of biofilm has been depicted. Many extracellular materials contribute during matrix formation. Among these, the extracellular DNA of other lysed bacteria can be a contributor. In this study, the role of free bacterial DNA provided by bacteriophage in the formation of bacterial biofilm has been investigated.

1.2 Bacteriophage:

Bacteriophage is a kind of virus that invades bacteria, removes bacterial replication machineries, and injects its own nucleic acid to replicate inside the bacteria. After complete replication and multiplication, the new bacteriophages burst out of its host cell(Drulis-Kawa & Maciejewska, 2021). Specific bacteriophage will target a certain bacteria and attach itself to specific receptors on the bacterial cell wall (Edgar et al., 2008).

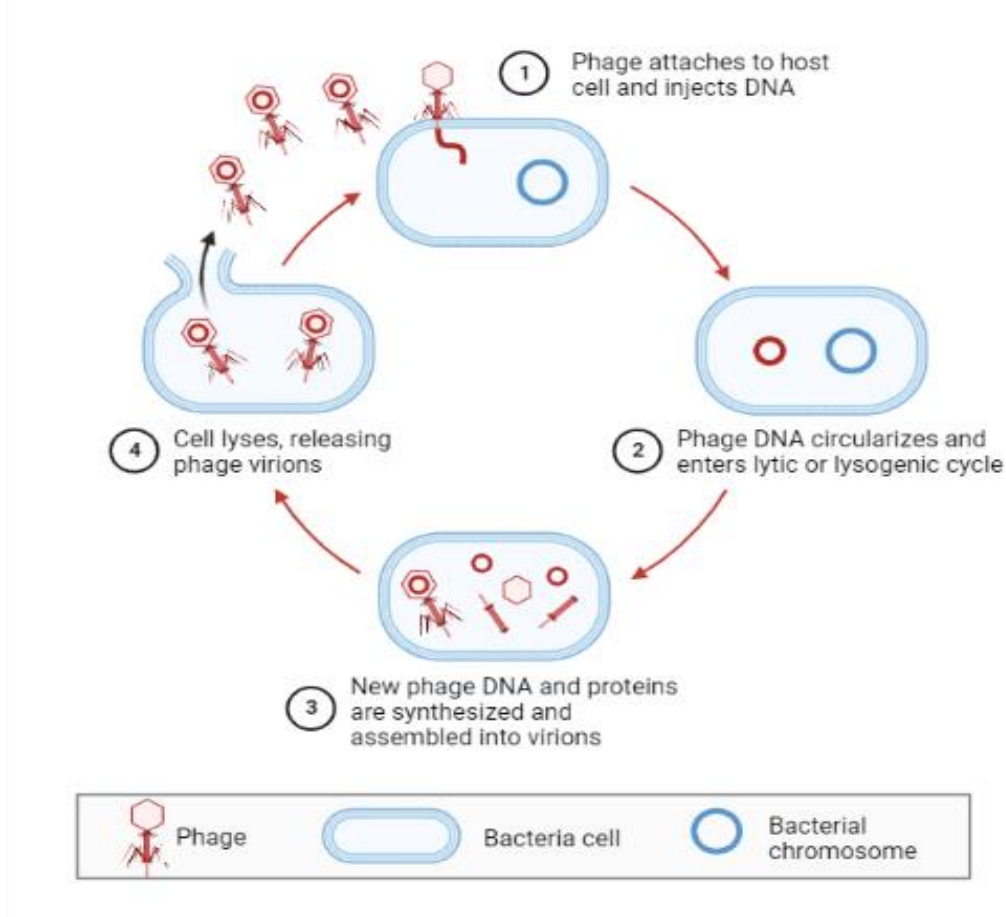

Figure 2: Common invasion process of bacteriophage in a bacterial cell

When the bacteriophage number increases the number of bacteria decreases and vice-versa. In Bangladesh between March to May, and September to December the bacteriophage number declines resulting in inclined number of bacteria and also rise of number of patients affected with bacterial infection. Although an opposite scenario is observed between June to August. As bacteriophage number rises, the number of bacteria decreases (Naser et al., 2017).

As the bacteriophages are multiplying inside the bacteria, they rupture the bacteria while coming out of bacteria. Therefore, bacterial DNA is found in abundance in the environment during this process. It is assumed that, these free bacterial DNA is responsible in biofilm formation of bacteria under this condition.

1.3 Aims of the study:

The data on the increase or reduction of phage numbers in different seasons in Bangladesh is available. However, it is still unknown if the free bacterial DNA provided by the phage by cutting the bacteria is responsible for the creation of biofilm. The aim of this study to investigate the fact that whether or not bacterial DNA provided by the bacteriophages is responsible for biofilm formation as bacteriophage numbers rise, free DNA levels rise as well, resulting in an increase in biofilm formation. The more we learn about the characteristics of this pathogenic bacteria, the better our chances of controlling the diseases and saving patients become.

2. Biofilm Forming Bacteria

2.1 *Vibrio cholerae*

The pathogen *V. cholerae* causes potentially fatal diarrheal maladies in people. There are numerous *V. cholerae* serogroups. Two of them, O1 and O139, are known to lead to serious illness (Faruque et al., 2005). The majority of the *V. cholerae* species is non-toxic strains. High genetic variability among environmental strains implies that *V. cholerae* is considerably resistant to environmental stress and engages with other aquatic organisms such as fish, algae, crustaceans, etc. (Baker-Austin et al., 2018).In the aquatic environment, *V. cholerae* exists as biofilms with the majority of the cells in the viable but non-culturable (VBNC) state or as clusters of dormant cells known as CVEC (Conditionally Viable Environmental Cells)(Faruque et al., 2006).

The pathogen *V. cholerae* has played a key role in human history. Beginning in the Bay of Bengal in the 1960s, *V. cholerae* spread in separate but merging waves. Significant outbreaks of various strains of this pathogen took place at various times in Spain, South America, France, the United States, Russia and Italy. It is still being studied in depth all over the world.

The symptoms of cholera, an extreme form of secretory diarrhea, can be deleterious within hours. Fluid loss can reach 1% of body weight or more per hour. Ingestion of a large inoculum is typically necessary for infection(Centers for Disease Control and Prevention (CDC), 2010). Almost all cholera infections have either no symptoms or only mild ones, like watery diarrhea. Oral rehydration solution is an effective treatment for the majority of infections. But if the illness is severe, antibiotics might be needed(Baker-Austin et al., 2018).

Cholera pathogenesis follows a certain pathway. The pathogen enters the human host by contaminated water. After reaching and harboring the target organ (small intestine), *Vibrio cholerae* begins expressing virulence factors, like cholera toxin.

The cholera toxin is made up of the two subunits CtxA and CtxB, and the CtxB pentameric subunit is what binds to the ganglioside GM1 on the cell's plasma membrane. The cell then takes up the GM1-bound cholera toxin and transports the complex inside of it to the endoplasmic reticulum (ER). The CtxA and CtxB subunits separate from one another there. Once ADP ribosylation factor 6 (ARF6) activates the enzyme's allosteric activation upon release from the ER into the cytoplasm (Baker-Austin et al., 2018).

The G protein-coupled receptor is catalyzed by the ARF6-CtxA complex, which further initiates adenylyl cyclase. This causes the cystic fibrosis trans-membrane receptor to be phosphorylated (P), which raises the levels of cAMP in the cell (CFTR). Watery diarrhea is the end result, which is caused by an ion and water efflux into the small intestinal lumen(Baker-Austin et al., 2018)

Bangladesh remains cholera-endemic, with biannual outbreaks and additional epidemics reported during floods, cyclones, or other natural disasters. In Bangladesh, approximately 66 million people are at danger of cholera, with an annual incidence rate of 1.64 per 1000 population, 100,000 cases, and 4500 deaths. More information is needed to answer questions concerning the cholera disease burden, as well as the effects, practicality, and cost-effectiveness of various cholera vaccination techniques in order to eradicate this disease from the country.

2.2 *Escherichia coli*

Escherichia coli is a gram-negative and rod-shaped bacterium that belongs to the Gammaproteobacteria class and is categorized as a member of the Enterobacteriaceae family. *Escherichia coli* can multiply in about 20 minutes when given the right conditions for growth(Jang et al., 2017). Although E. coli is widely employed as a faecal indicator bacteria (FIB) for evaluating water quality, its survival and proliferation in the environment raise issues about its suitability as a faecal indicator bacterium. Understanding the ecology of this bacteria is also crucial to preventing infection and the transmission of this pathogen to food, soil, and water since some E. coli strains and serotypes can lead to human disorders(Anderson, Whitlock, & Harwood, 2005).

Several extremely adapted *E. coli* clones that have developed certain virulence traits, which provide them a greater capacity for niche adaptation and enable them to cause a variety of diseases(Kaper, Nataro, & Mobley, 2004).Infection with one of the following pathotypes can cause enteric/diarrheal illness, urinary tract infections (UTIs), and sepsis/meningitis, three main clinical syndromes(Kaper et al., 2004).Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli*, and enteroinvasive *E. coli*, including *Shigella* strains, are among the six well-studied intestinal pathotypes of *E. coli*. These strains are categorized according to their virulence traits and pathogenicity mechanisms, which cause gastrointestinal disorders including diarrhea (Kaper et al., 2004).

Because they must endure the low-pH environment of the animal or human stomach to reach the intestinal system, many *E. coli* strains are acid-resistant. The population structure of *E. coli* can be impacted by host intestinal circumstances. Based on the host-animal and dietary, *E. coli* phylogenetic groupings are relatively abundant (Jang et al., 2017).*E. coli* is known to survive in natural habitats due to the formation of biofilms on surfaces in aquatic environments, such as sediments. The bacteria are guarded by biofilms from detrimental environmental factors including UV radiation, desiccation, protozoan predators, and chemicals like antibiotics and cleaners (McDougald, Rice, Barraud, Steinberg, & Kjelleberg, 2012).

2.3*Salmonella Typhi*

Gram-negative *Salmonella enterica* serotype *typhi* is the causative agent of typhoid disease and has affected developing countries for many years(Barnett, 2016). It is gram negative, flagellated and rod-shaped bacteria that resides only in human body (Crump, Sjölund-Karlsson, Gordon, & Parry, 2015). Generally, contaminated food or drink is the means of transmission. After being consumed or ingested, *Salmonella typhi* bacteria proliferate and spread throughout the body. Seasonal changes affect *Salmonella*. It has been shown that this sickness peaks between June and September(Britto, Wong, Dougan, & Pollard, 2018). An individual carrying the bacteria in their blood often displays serious symptoms like high fever, nausea, fatigue, abdominal pain, diarrhea etc. (Crump et al., 2015)

Typhoid has a specific mechanism for how it appears. *Salmonella* initially infects the host after passing via Peyer's patches (PP) and the small intestine's resident macrophages. Bacteria then travel from there into the mesenteric lymph nodes, where they proliferate. Once discharged into the circulation, germs quickly spread throughout the body. This stage is also known as transient primary bacteremia (Everest, Wain, Roberts, Rook, & Dougan, 2001). Secondary bacteremia is generally the stage where S. typhi can proliferate in the immune cells such as sinusoids of the liver, spleen and bone marrow from where they can again enter the blood stream and cause disease progression. From these sites, the re-entry of bacteria into the bloodstream can cause clinical disease. In microscopic histopathology, PPs, and lymphoid follicles are found to be swelled up due to increased number of macrophages and lymphocytes which indicates inflammation and onset of necrosis. This stage is followed by ulcer and generalized peritonitis and septicemia. Untreated typhoid fever can cause even death (Everest et al., 2001).

3. Materials and methods:

3.1 Organisms:

Four bacterial strains were chosen and tested under different settings for the investigation. Two of these were *Salmonella typhi* stains, while the other two were *Vibrio cholerae* and *Escherichia coli* (Table 1). In order to analyze the formation of biofilms, the *Salmonella typhi* strain S15c was employed as the positive control. In this experiment, no bacteria that do not produce biofilms were utilized. Because the goal of this study is to investigate how free DNA influences the development of biofilms. Therefore, organisms that do not produce biofilms are not included.

Table 1: List of bacteria tested

	Organism	Attributes
1.	*Vibrio cholerae* wt-346	Heavy Biofilm forming strain
2.	*Escherichia coli*Mac9A	Heavy Biofilm forming strain
3.	*Salmonella typhi* S15c	Heavy Biofilm forming strain
4.	*Salmonella typhi* S7f	Heavy Biofilm forming strain

Then, in this study, phages that can destroy the target bacteria were also employed. Several samples were obtained in order to assess the phages' effectiveness against the bacterial strains listed in the preceding table. Only the phages that produced the best results were chosen, and they are listed below:

Table 2: List of phages tested

	Organism	Attributes
1.	Phage JSF-7	Cuts the *Vibrio cholerae* wt-346
2.	Phage sample 3	Cuts *Escherichia coli* Mac9A
3.	Phage sample 14	Cuts *Salmonella typhi* S15c
4.	Phage sample 4b	Cuts *Salmonella typhi* S7f

3.2 Bacterial Culture Media:

In this experiment, LB broth and LB Agar media were utilized. All of the organisms here are gram negative bacteria, and LB is ideal for their growth. T1N1 was employed as preservation media in addition to that. Glycerol was used to coat the bacteria while they were stored there.

All of the cultures and media were obtained from the Life Science Laboratories at BRAC University. These cultures were revived, used and preserved by using standard protocols.

3.3 Overview of the Methods:

To conduct the investigation, a protocol was created and strictly followed. From reviving the bacterial stock to analyzing the results, every step is included in this.

The strains were initially turned into active culture after being revived from the preservation media. After that, young culture is created using a shaker incubator by adding fresh culture to fresh liquid media from plates. Then, after reaching the ideal cell density, the young culture is put in a vial to form static biofilms. Additionally, biofilm formation in phage soup containing free bacterial DNA was investigated. On the basis of using both regular LB broth and phage soup that contained free bacterial DNA, the optical density (OD) of these generated biofilms was assessed and compared.

3.4 Revival of Bacterial Culture:

Bacterial strains were revived from laboratory stocks that had been preserved in T1N1 Media. The culture was removed from there and revived using the streaking method of sub-culturing on LB agar plates. Single colonies were isolated from the plates after 24 hours of incubator incubation at 37°C.

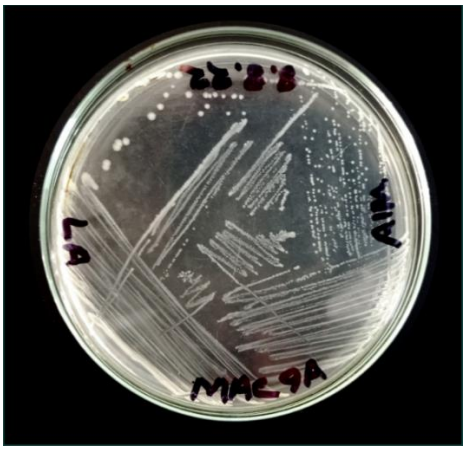

Figure 3: Sub-culture of Mac9A strain of E. coli bacteria using streaking method

Figure 4:Sub-culture of S15c strain of S. typhi bacteria using streaking method

3.5 Making Young Culture and Biofilm:

We had to first determine which stains of various bacteria available in the laboratory would form biofilm, as this is critical for the success of our research study.

We needed to make young culture for biofilm formation. For making a young culture, we put 3 ml of LB in a sterilized vial and added a single colony of a host bacteria. It was then placed in a shaker incubator for 3-4 hours in 37°C and 80 rpm for culture formation. Following that, we divided 500 µl of the inoculated LB among three vials. These vials were placed in a clean environment with no disturbance for 24, 48, and 72 hours respectively in order to form biofilm. We took the vials and rinsed them with distill water after 24 hours, 48 hours, and 72 hours of observation. After that, we applied crystal violet and waited 90 minutes. We washed those vials again with distill water. Then we noticed the ring around the vials, which indicates the formation of biofilm. If the young culture is left undisturbed for 48- 72 hours a good biofilm forms that can be seen well on the surface.

Since our key objective is to determine whether or not free bacterial DNA helps to develop a bacterial biofilm, we had to make a liquid LB broth that contained free DNA of a bacteria. In order to evaluate whether the free DNA contributed to the enhancement of bacterial biofilm development or not, we later inoculated this free bacterial DNA containing phage soup with a single colony of a different bacteria and left in a sterile environment as well as in a static state to create biofilm.

3.6 Double Layer Assay (Drop Plate Method):

The strains were individually inoculated in separate glass vials each containing 3mL LB broth. These vials were kept in a shaker incubator for 40 minutes to 1 hour to obtain young culture. 200µl of young culture were collected and poured in warm (not so hot and in melted condition) 3 mL soft agar aliquots. Then it was mixed using a vortex. The mixtures were poured into LA plates while still warm and liquid in order to avoid bubble formation and to achieve an even distribution. Then, the plates were solidified at room temperature. 15µl phage samples were deposited as a drop on the agar layer's top. The plates were kept for 30/40 minutes to drying. After that, these were incubated overnight at 37^{o}C. Then we observe if any lysis occurs or not. Lysis indicates the presence of phage in the tested sample, whereas no lysis indicates the absence of phage in the tested sample.

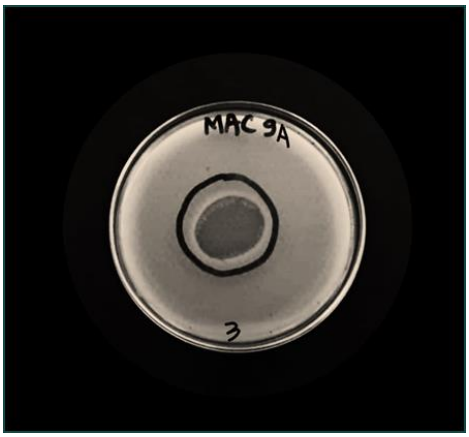

Figure 5: Double Layer Assay (Drop plate method) of Mac9A strain by using sample 3 phage drop

Figure 6: Double Layer Assay (Drop plate method) of S7f strain by using sample 4b phage drop

Using a 1ml micropipette, the DLA phage part was taken in an Eppendorf containing 200µl SM buffer. Then it was centrifuged for 5 minutes at 14000 rpm. The supernatant was collected and stored in another Eppendorf for phage enrichment.

3.7 Phage Enrichment:

At first, 10mL LB broth was inoculated with the host colony of a bacteria. It was then incubated in a shaker incubator for 2 hours at 37°C temperature and 80 rpm. Following that, 50µL phage was added and incubated again for 4 hours. Then it was centrifuged for 10 minutes at 13000 rpm. The soup was filtered through a 0.22 filter. Thus, single enrichment was obtained. This process was repeated for double and so on enrichment.

3.8 Serial Dilution and Phage Titer:

Serial dilation of phage was required to achieve various concentration of phage in order to check their activities. to make this, initially fresh 900 µl LB broth was taken and it was mixed with 100 µl phage by using vortex in an Eppendorf and thus 10-1 dilution was obtained.100µl was obtained from the vortexed mixture of phage and LB broth solution and added to another Eppendorf containing 900µl LB. So, we have also 10^{-2} dilution. Same process was repeated to obtain till the dilution concentration reached to 10^{-9}.

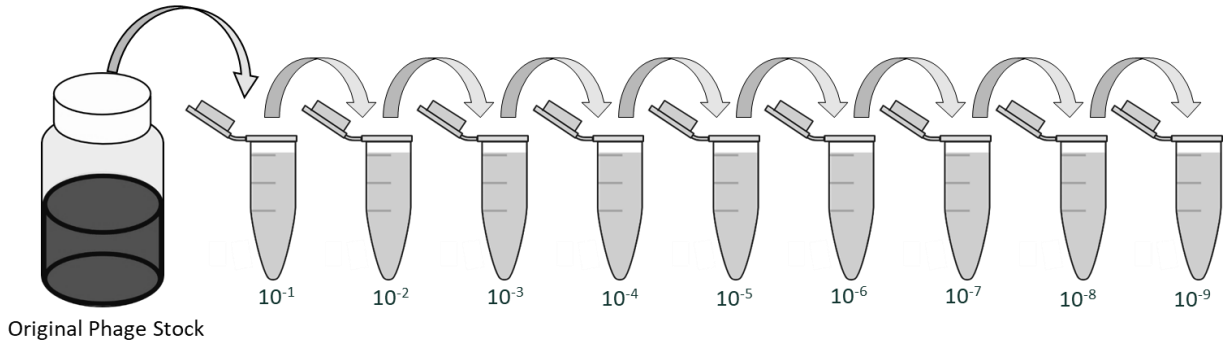

Figure 7: Serial dilution of Phage

Now, for phage titer, 3mL LB broth was inoculated with the host colony of the bacterial strain. It was then incubated in a shaker incubator for 2 hours. 300µL young host culture was added with 100µL phage in 3mL melted soft agar. The temperature of this melted agar should be neither too hot to kill the bacteria and phage, nor too cold that it gets solidified before mixing in the vortex and poring on the LA plate. After mixing, it was vortexed and poured in LA plate. After solidification of agar, the plate incubated at 37°C for 24 hours.Now, the plaque-forming units (PFU) was checked.

The PFU count for each plate is$\frac{Plaquecount}{DilutionFactorVolumePlated}$.

For instance, 125 plaques were counted when we obtainedthe result of tittering the phage sample 3, which kills the Mac9A strain of *Escherichia coli*. The dilution series value in this case is 10^{-5}. This is a result of setting up the initial dilutions with 0.1 ml of the phage stock. Therefore, 1.25×10^{8}PFU/ml in this lysis would be represented by all of these values.

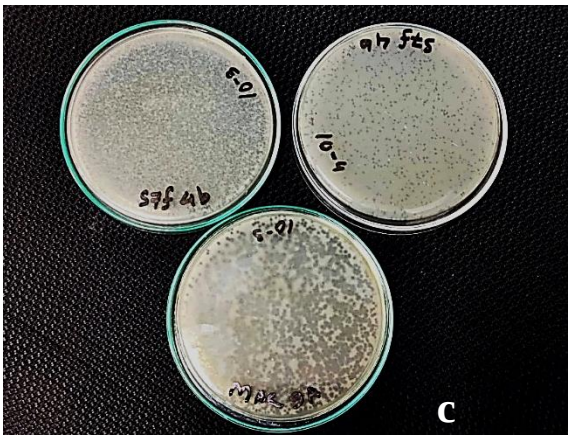

Figure 8: Phage titer assay. (a)10^{-3} dilution of Phage sample 4b for S7f stain of S. typhi gave almost clear pattern with just a small amount of the bacterial lawn still remaining. (b) 10^{-4} dilution of Phage sample 4b for S7f stain of S. typhi gave some clear plaques with comparatively vast area of the bacterial lawn still remaining. (c) 10^{-3} dilution of Phage sample 3 for Mac9A strain of E. coli gave almost clear pattern with very small amount of the bacterial lawn still remaining.

3.9 Making Phage Soup Which Contains Free Bacterial DNA:

The single colony of a specific bacteria was initially inoculated into a falcon tube with 3mL of LB broth. It was then incubated for the formation of a young bacterial culture for 2 hours at 37 °C and 80 rpm in a shaker incubator. Following that, 100L of phage, which is responsible for killing that specific bacteria was added to this and incubated for another 4 hours in a shaker incubator. We

wanted this phage to kill all the bacteria during this time frame so that their free DNA would be present in this soup. After completing four hours in a shaker incubator, it underwent a 10-minute centrifugation procedure at 25°C and 14000 rpm. In that falcon tube, all the debris formed a pellet, and the supernatant contained free bacterial DNA. Afterward, the pellet was removed and the supernatant was poured into another falcon tube. Thus, this phage soup was ready for cultivation with a single colony of a different host bacteria. After inoculation, the above-mentioned standard procedure for biofilm formation [3.5 Making Young Culture and Biofilm] was followed here as well.

3.10 Optical density (OD) Measurement of the Biofilm:

Following biofilm formation in both standard LB broth and phage soup containing free bacterial DNA, the optical density (OD) of the resulting biofilm was scaled. The measurement we have used here is in millimeters (mm). The stronger development of a biofilm is indicated by a biofilm with a higher OD. We evaluated the OD between the biofilms created in the regular LB broth and the phage soup containing free bacterial DNA. If the OD of a certain strain's biofilm is higher in phage soup containing free bacterial DNA than in regular LB broth, this suggests that free bacterial DNA has an effect on the development of biofilm.

3.11Preparing DNase and Protease:

In order to prepare DNase, we have applied 50 µl of DNase 1, was obtained from the NEB UK and the activity was 2000U/ml. So, we have applied 50 µl of DNase 1 in 100 µl of the normal LB broth. And then kept in shaker incubator for 4 hours. DNase was used for the degradation of the free bacterial DNA available in the phage soup.

We have applied 1 mg of Protease from *Streptomyces griseus* (activity- ≥3.5 U/mg solid) in 50 µl autoclaved distill water. The free protein present in the phage soup was broken down using protease.

3.12 Test of The Impact of Free Bacterial DNA on Increasing Biofilms Formation:

Here we have taken one of the best performing bacteria strains, S15c of *Salmonella typhi* and bacteria's phage soup WT346 strain of *Vibrio cholerae* to conduct this particular experiment.

1. Normal LB broth was inoculated with the single colony of S15c strain of S*almonella typhi* and followed the procedure for biofilm formation.

2. Host bacteria (S15c strain of S*almonella typhi*) was cultured in another bacteria's phage soup (WT346 strain of *Vibrio cholerae*). 300 µl of it was used for biofilm formation by following standard protocol.

3. DNase 1 was used for the degradation of the free DNA available in the phage soup in this bacteria culture. 100 µl of it was taken from it and mixed with 200 µl S15c culture in WT346 phage soup (which contains free bacterial DNA of S15c) and then biofilm was formed.

4. Protease was used for the degradation of the free protein available in the phage soup in this bacteria culture. 36 µl of it was taken from it and mixed with 264 µl S15c culture in WT346 phage soup (which contains free bacterial DNA of S15c) and then biofilm was formed.

OD for all of the biofilms formed here was checked and was compared with each other's.

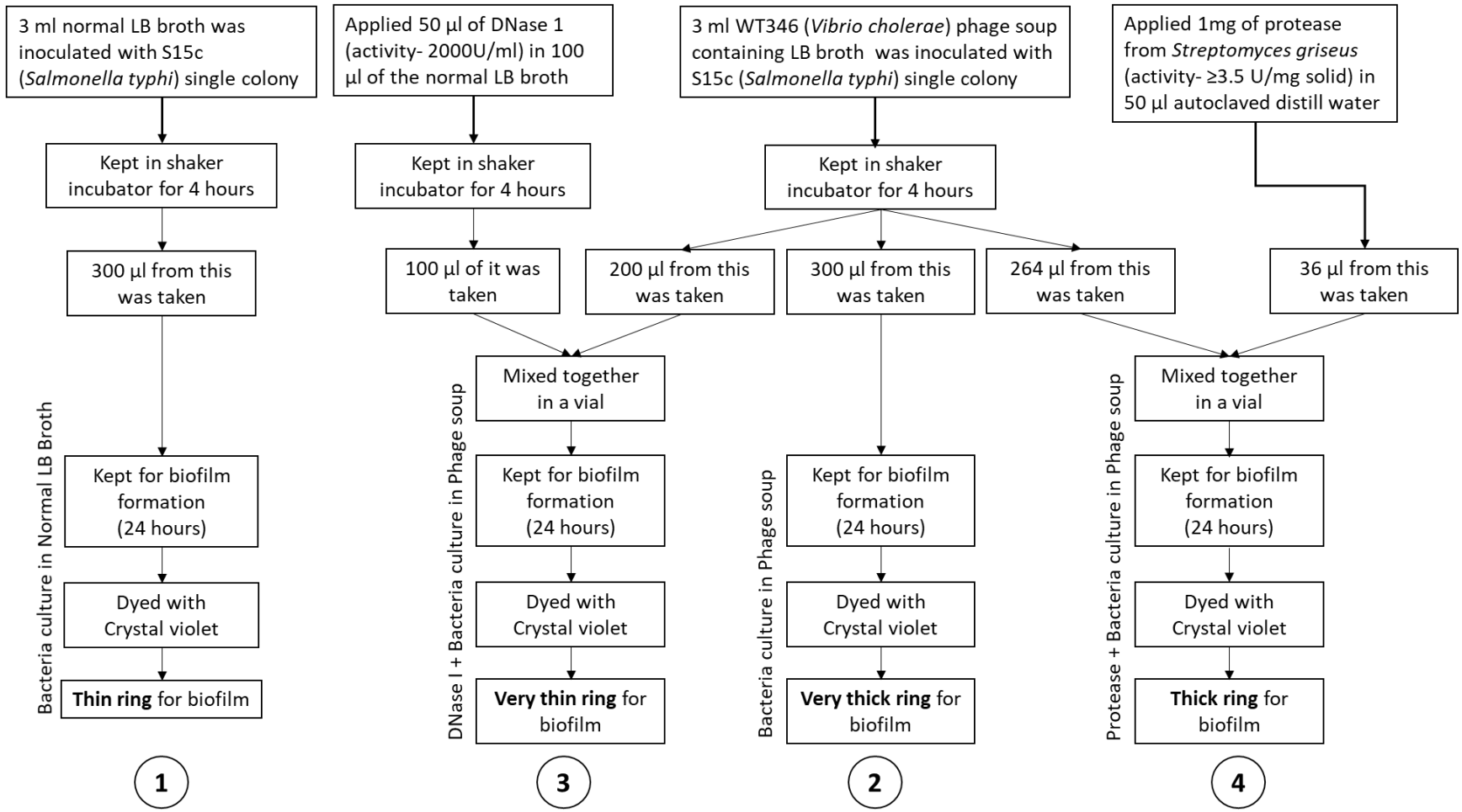

Figure 9: method of testing the impact of free bacterial DNA on increasing biofilms formation

4. Result and Discussion:

4.1 Biofilm Log and Comparison of the OD of Biofilm Rings:

All four strains (*Vibrio cholerae* wt-346, *Escherichia coli*Mac9A, *Salmonella typhi* S15c, and *Salmonella typhi* S7f) were cultured in two different types of LB broth conditions: 1. Standard LB broth and 2. Phage soup that contain free bacterial DNA (S7f phage soup, Mac9A phage soup, S15c phage soup, and WT346 phage soup) to determine how free bacterial DNA affected the boost in biofilm development. The OD of the produced biofilm rings were measured and compared according to their visibility in the naked eyes and the thickness (in millimeter).

4.1.1 S7f strain (*Salmonella typhi*)

Biofilm was developed for 24, 48, and 72 hours after culturing the S7f strain in the standard LB broth, Mac9A phage soup, WT346 phage soup, and S15c phage soup. The results obtained after 24 hours revealed that the S7f strain's biofilm ring was absent in the normal LB broth, the WT346 phage soup, and the S15c phage soup (Table 3). Only a thick biofilm ring with an OD of 1.2 mm was detected for Mac9A phage soup. For 48 and 72 hours, biofilm rings were seen in all LB conditions, also the results show lower OD values and thin biofilm rings in the normal LB broth. However, compared to the OD of the biofilm ring at 48 hours (1.3 mm), the OD of the biofilm ring in the Mac9A phage soup was higher for a period of 72 hours (1.4 mm). Again, the OD of the biofilm rings in both WT346 phage soup and S15c phage soup were higher and thicker for 48 hours (2.0 mm and 0.9 mm respectively) than 72 hours (1.0 mm and 0.7 mm respectively).

Table 3: The results of the OD of S7f strain biofilm rings under various LB conditions throughout three different time periods (24 hours, 48 hours, and 72 hours)

Host Strain Name	**LB condition**	**Time of observation**					
		24 Hours		**48 Hours**		**72 Hours**	
		Appearance	**OD (mm)**	**Appearance**	**OD (mm)**	**Appearance**	**OD (mm)**
S7f (*Salmonella typhi*)	Normal LB broth	-	-	Thin ring	0.2	Very thin ring	0.1
	Mac9A phage soup	Thick ring	1.2	Thick ring	1.3	Very thick ring	1.4
	WT346 phage soup	-	-	Very thick ring	2.0	Thick ring	1.0
	S15c phage soup	-	-	Thick ring	0.9	Slight thick ring	0.7

Here is the graphical representation of biofilm rings' OD of S7f strain (*Salmonella typhi*) over time. As we can see, the OD of the biofilm rings that have developed in the phage soups has larger values for all of the three time periods, which suggests that it occurred as a result of the presence of free bacterial DNA. However, the absence of free bacterial DNA in the normal LB broth caused the OD of the biofilm rings to have the lowest value, which indicates that the production of the biofilms was not as boosted in this instance as it was in the prior one.

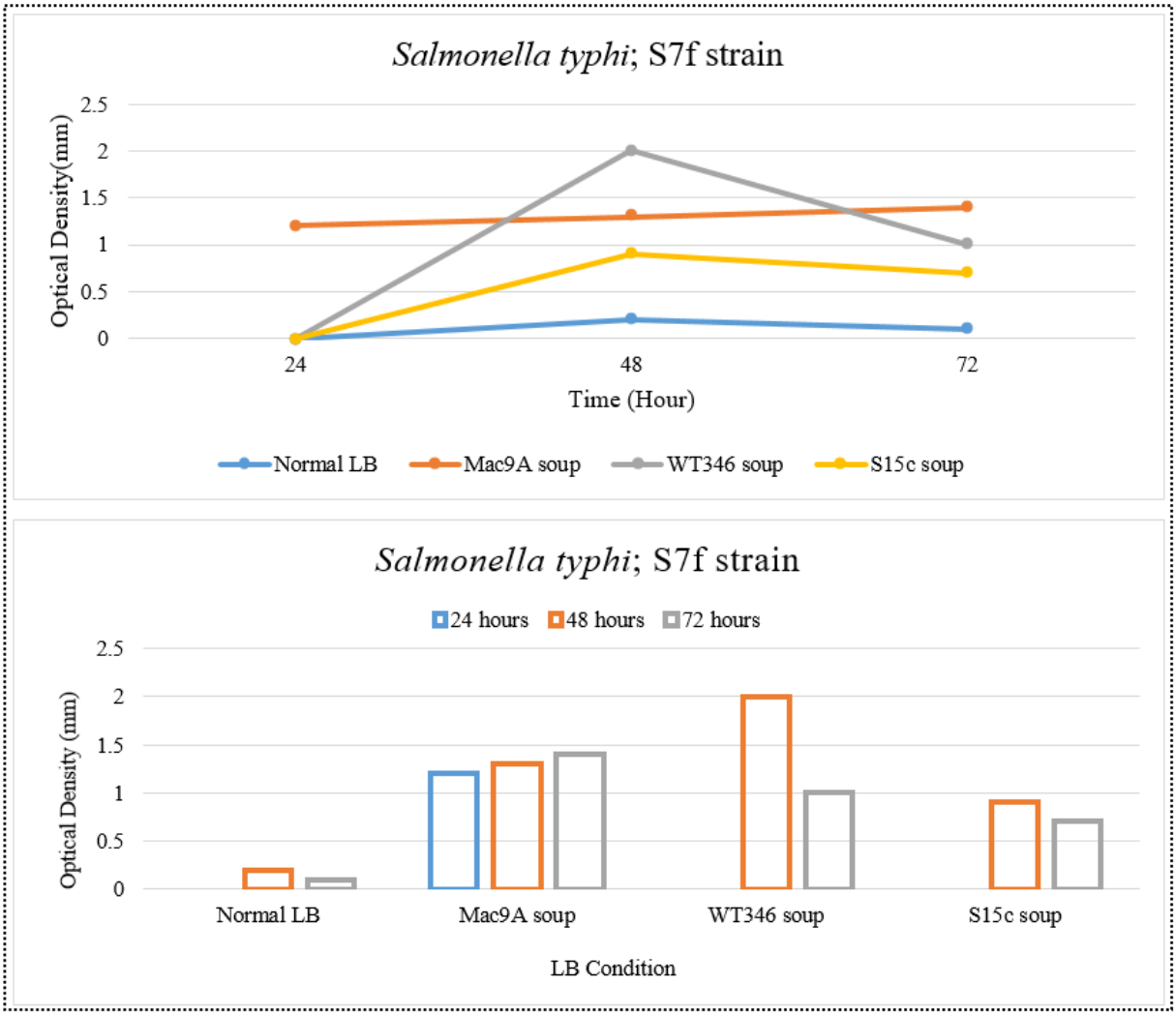

Figure 10: OD of the biofilm rings of S7f strains in different LB conditions at 24, 48 and 72 hours

4.1.2 WT346 (*Vibrio cholerae*)

The WT346 strain was cultured in the normal LB broth, Mac9Aphage soup, S7f phage soup, and S15c phage soup and then biofilms were formed for 24, 48, and 72 hours. This is the only strain that showed biofilm rings in all LB conditions at three different time frames of 24, 48 and 72 hours. In comparison to 48 and 72 hours, all of the biofilms developed at 72 hours had the highest OD values and thickness. The results, however, indicate lower OD values and thin biofilm rings in the normal LB broth over the entire time span than biofilm rings that have been formed in phage soups.

Table 4:The results of the OD of WT346 strain biofilm rings under various LB conditions throughout three different time periods (24 hours, 48 hours, and 72 hours)

Host Strain Name	**LB condition**	**Time of observation**					
		24 Hours		**48 Hours**		**72 Hours**	
		Appearance	**OD (mm)**	**Appearance**	**OD (mm)**	**Appearance**	**OD (mm)**
WT346 (*Vibrio cholerae*)	Normal LB broth	Thin ring	0.5	Thick ring	1.0	Thick ring	1.1
	Mac9A phage soup	Thick ring	1.1	Very thick ring	1.5	Very thick ring	2.0
	S7f phage soup	Thick ring	0.7	Thick ring	1.3	Very thick ring	1.6
	S15c phage soup	Very thick ring	1.7	Very thick ring	1.8	Very thick ring	1.9

Here is a graph showing the OD of biofilm rings of the *Vibrio cholerae* strain WT346 over time. As we can see, the OD of the biofilm rings that have formed in the phage soups has greater values for each of the three time periods, which shows that it did so because free bacterial DNA was prevalent. However, the absence of free bacterial DNA in the normal LB broth resulted in the lowest OD of the biofilm rings, demonstrating that biofilm formation was not as enhanced in this case as it was in the preceding case.

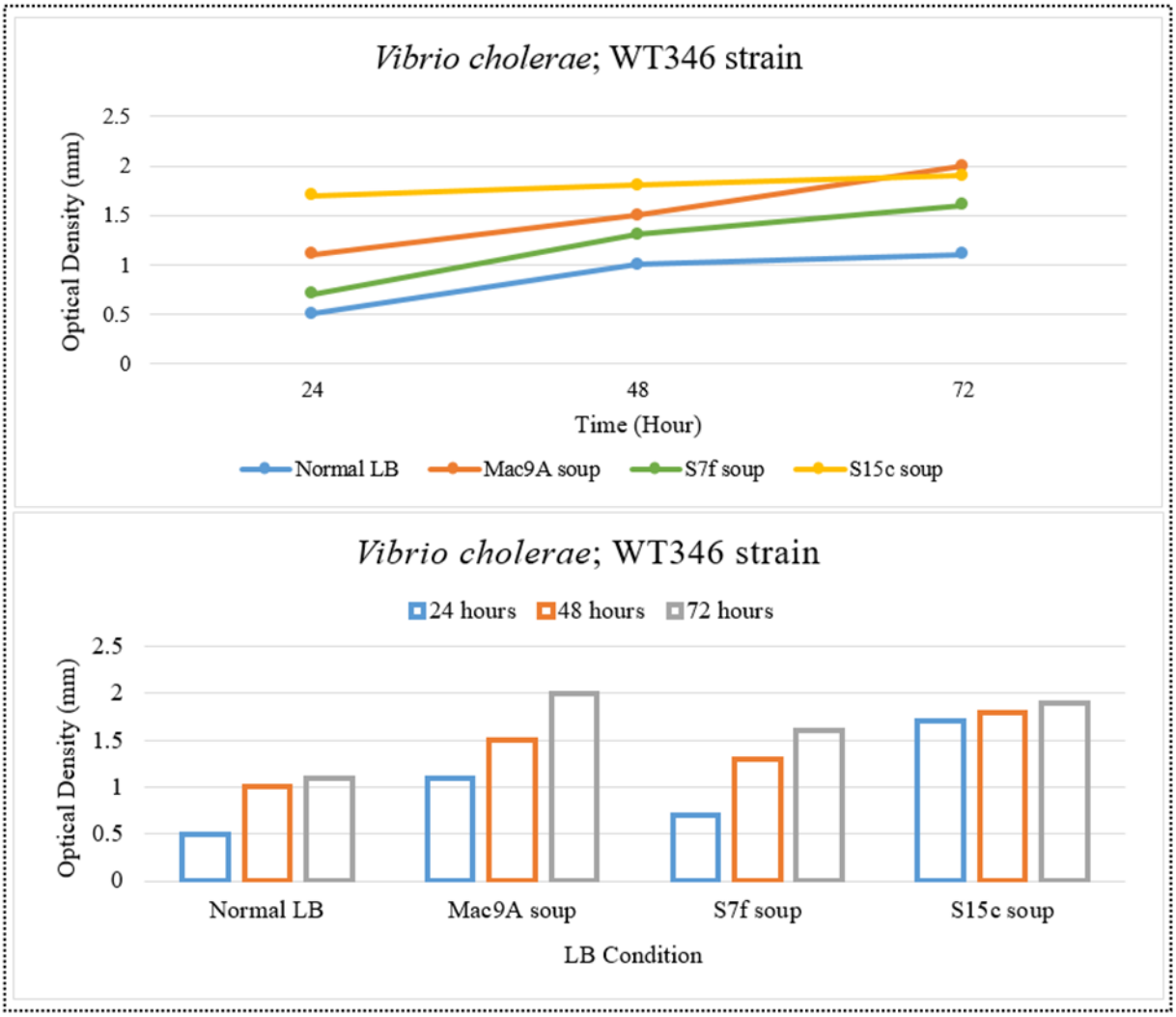

Figure 11: OD of the biofilm rings of WT346 strains in different LB conditions at 24, 48 and 72 hours

4.1.3 Mac9A (*Escherichia coli*)

Following the culturing of the Mac9A strain in the normal LB broth, S7f phage soup, WT346 phage soup, and S15c phage soup, biofilm was produced for 24, 48, and 72 hours. The Mac9A strain did not form a biofilm ring in the normal LB broth, the WT346 phage soup, or the S15c phage soup after 24 hours. For S7f phage soup, only a thick biofilm ring with an OD of 0.8 mm was found. For 48 and 72 hours, biofilm rings were seen in all LB settings and the outcomes there indicate decreased OD values (0.5 mm and 0.1 mm respectively) and thin biofilm rings in the normal LB broth. However, the OD of the biofilm ring in the S7f phage soup, WT346 phage soup and S15c phage soup was higher for a period of 48 hours (2.0 mm, 2.0 mm and 0.9 mm

respectively) compared to the OD of the biofilm ring at 72 hours (1.4 mm, 1.0 mm and 0.7 mm respectively).

Table 5: The results of the OD of Mac9Astrain biofilm rings under various LB conditions throughout three different time periods (24 hours, 48 hours, and 72 hours)

Host Strain Name	**LB condition**	**Time of observation**					
		24 Hours		**48 Hours**		**72 Hours**	
		Appearance	**OD (mm)**	**Appearance**	**OD (mm)**	**Appearance**	**OD (mm)**
Mac9A (*Escherichia coli*)	Normal LB broth	-	-	Thin ring	0.5	Very thin ring	0.1
	S7f phage soup	Thick ring	0.8	Very thick ring	2.0	Very thick ring	1.4
	WT346 phage soup	-	-	Very thick ring	2.0	Thick ring	1.0
	S15c phage soup	-	-	Thick ring	0.9	Slight thick ring	0.7

The OD of biofilm rings produced by the E. coli Mac9A strain over time is shown in the following graph. We can see that the OD of the biofilm rings that have formed in the phage soups has higher values for each of the three-time windows, which implies that it happened as a result of the existence of free bacterial DNA. The OD of the biofilm rings was lowest in the normal LB broth without free bacterial DNA, reflecting that biofilm formation was not as accelerated in this event as it had been in the prior one.

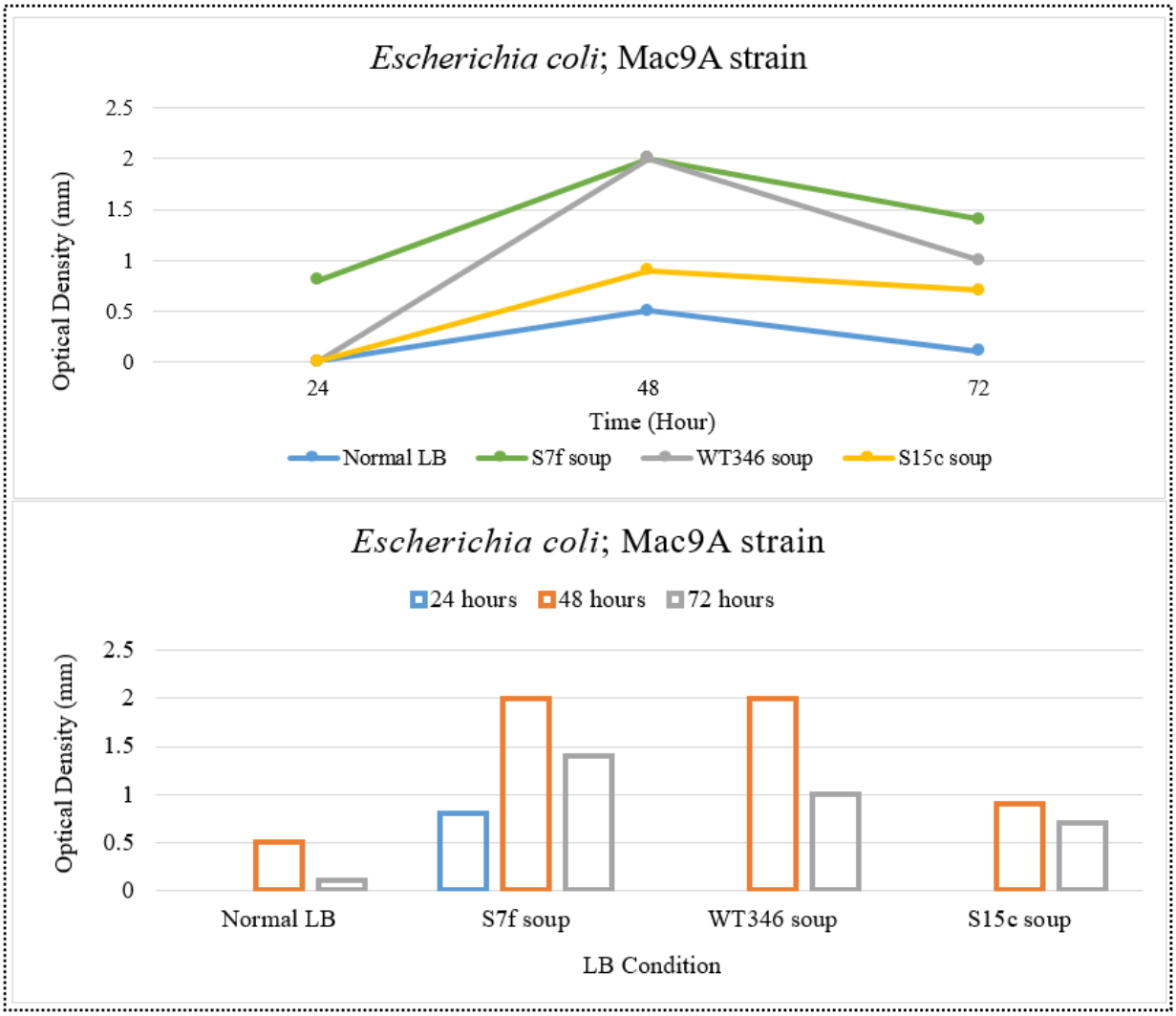

Figure 12:OD of the biofilm rings of Mac9A strains in different LB conditions at 24, 48 and 72 hours

4.1.4 S15c (*Salmonella typhi*)

The S15c strain was cultured in the normal LB broth, Mac9Aphage soup, WT346 phage soup, and S15c phage soup and then biofilms were formed for 24, 48, and 72 hours. Biofilms were formed in all type of LB conditions for all time sets, except the 24 hours' one in the normal LB broth. Also, the results for 48 and 72 hours indicate lower OD values (1.0 mm and 1.3 mm respectively) and thin biofilm rings in the normal LB broth than biofilm rings that have been formed in phage soups. The OD of the biofilm ring in Mac9Athe phage soup, WT346 phage soup and S7f phage soup was higher for a period of 72 hours (1.6 mm, 2.0 mm and 1.6 mm respectively) compared to the OD of the biofilm ring at 48 hours (1.5 mm, 2.0 mm and 1.3 mm respectively).

Table 6: The results of the OD of S15c strain biofilm rings under various LB conditions throughout three different time periods (24 hours, 48 hours, and 72 hours)

Host Strain Name	LB condition	Time of observation					
		24 Hours		48 Hours		72 Hours	
		Appearance	OD (mm)	Appearance	OD (mm)	Appearance	OD (mm)
S15c (*Salmonella typhi*)	Normal LB broth	-	-	Thick ring	1.0	Very thick ring	1.3
	Mac9A phage soup	Thin ring	0.5	Very thick ring	1.5	Very thick ring	1.6
	WT346 phage soup	Very thick ring	1.5	Very thick ring	2.0	Very thick ring	2.0
	S7f phage soup	Thick ring	0.9	Thick ring	1.3	Very thick ring	1.6

The graphical depiction of biofilm rings' OD of S15cstrain (*Salmonella typhi*) over time is shown here. From this illustration, it can be observed that the OD of the biofilm rings that have developed in the phage soups has larger values for all of the three time periods, which suggests that it occurred as a result of the presence of free bacterial DNA. But the deprivation of free bacterial DNA in the normal LB broth affected the OD of the biofilm rings to have the lowest value, which designates that the generation of the biofilms was not as enhanced in this case as it was in the phage soup.

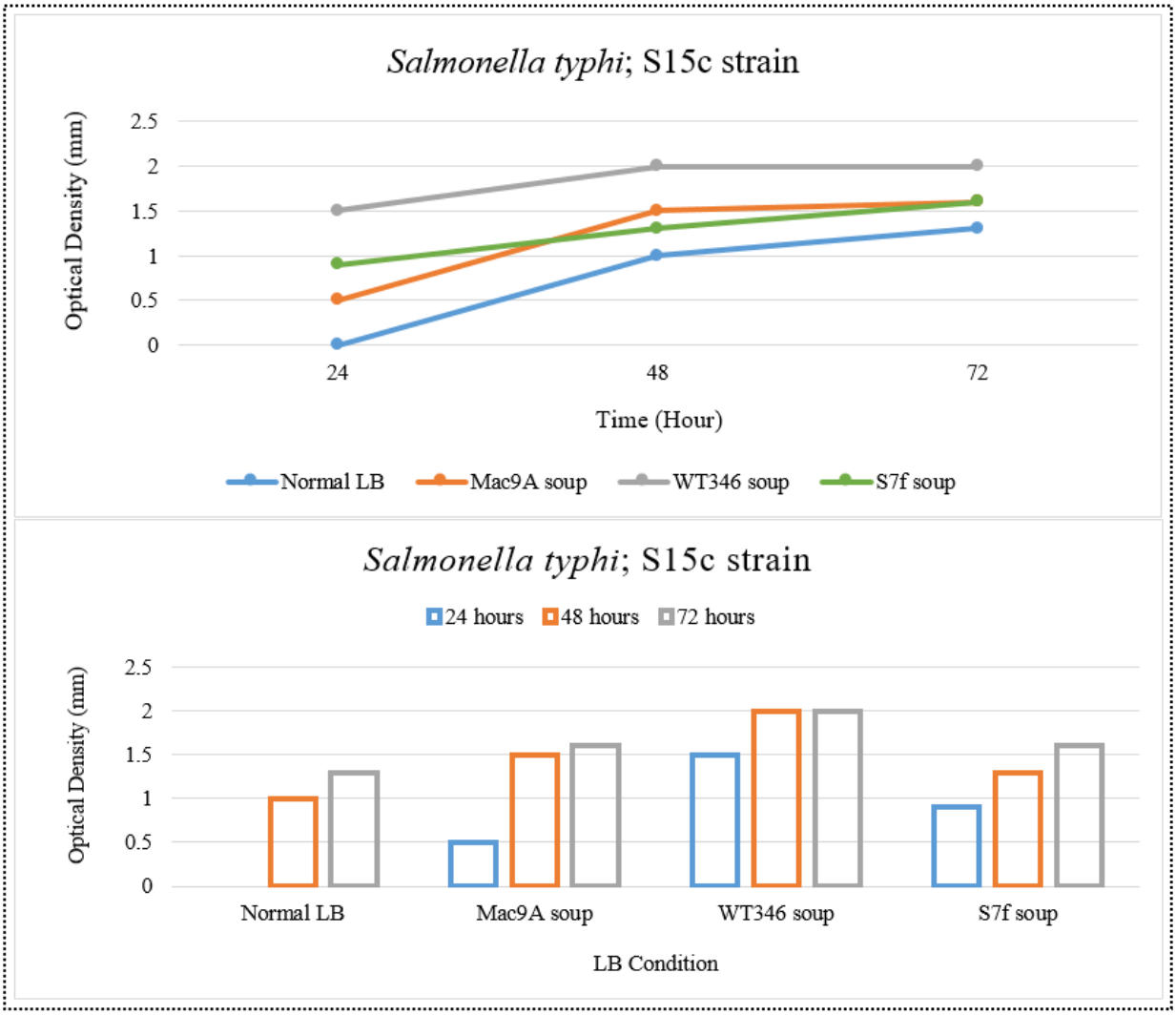

Figure 13: OD of the biofilm rings of S15c strains in different LB conditions at 24, 48 and 72 hours

4.1.5 Comparison of OD of Biofilms on Normal LB vs Phage soup

Here is the graphical representation of normal vs phage soup containing biofilm od of all stains over time. Mean Optical Density (OD) of the biofilms in each type of LB conditions (Normal LB broth, Mac9A phage soup, WT346 phage soup, S15c phage soup and S7f phage soup) are exhibited in these pictorials here. It can be observed that biofilms produced in the normal LB broth contain lowest OD values compared to the ones formed in the 4 different phage soups. this graph is implying that due the lack of free bacterial DNA in the Normal LB broth, the formation of the biofilm was not enhanced. on the other hand, due to the availability of free bacterial DNA in the

phage soups, all of the biofilms that have produced in them have showed higher OD values which indicates the positive impact on the biofilm formation. Thus, the result we have obtained in this study kind of proves that free bacterial DNA enhances the biofilm formation.

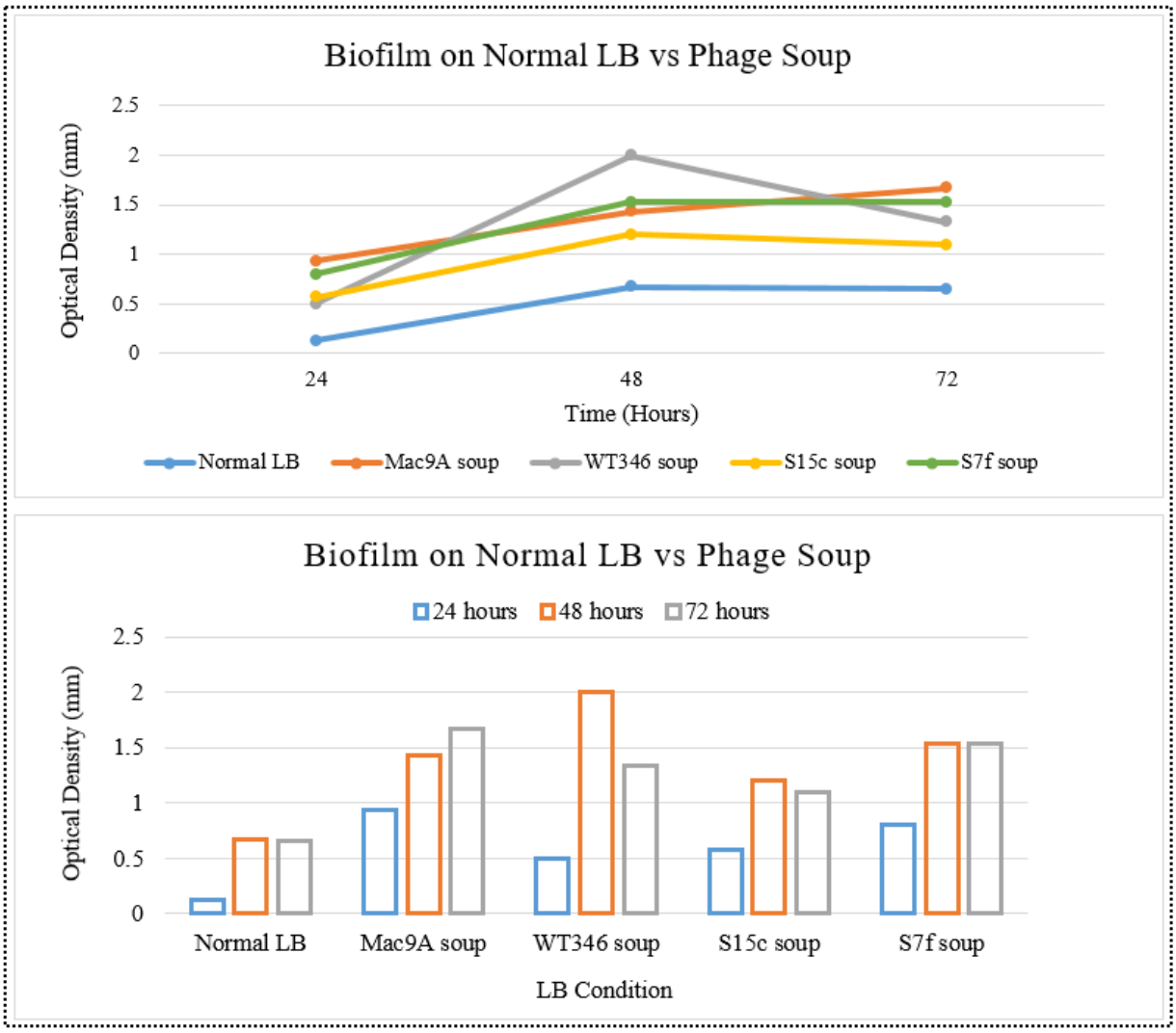

Figure 14: Comparison of Mean OD of biofilms on normal LB broth vs phage soups

4.2 Treatment with DNase and Protease

We have used DNase enzyme in order to degrade the free bacterial DNA available in the WT346 phage soup to prove that it is actually DNA but not any other component is helping on the enhancement of biofilm formation. Additionally, protease enzyme was employed to break down proteins to see if the production of biofilms would be hindered by the lack of proteins. Biofilm of S15c strain was obtained in both normal LB broth and WT346 phage soup so that we can compare all of these biofilms together.

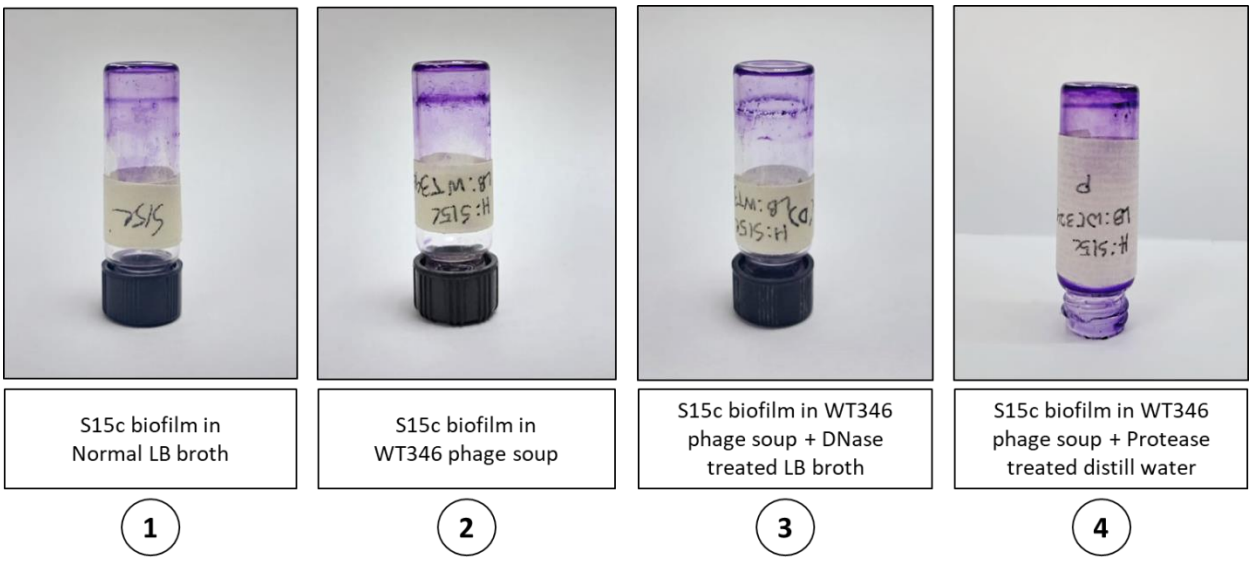

Figure 15: Biofilm rings of S15c strain in different conditions

Here,

1. Biofilm of S15c strain of S*almonella typhi* in normal LB broth was obtained. It was a thin biofilm ring and OD of it was 0.6 mm. As LB broth contained only basic nutrition but no free bacterial DNA, this outcome was expected.

2. Biofilm of S15c straining WT346 phage soup showed the thickest ring among all of them and its OD was 1.3 mm. It contained free bacterial DNA which was responsible for the enhancement of the biofilm formation here.

3. The S15c strain was cultured in WT346 phage soup, and DNase 1 was introduced to this for the purpose of degrading the free DNA present in the WT346 phage soup. This mixture's biofilm didn't

produce a better outcome; instead, it produced a thin ring with an OD value of 0.5 mm. This occurred because there was no free DNA present in the solution.

4. S15c strain was cultured in WT346 phage soup and Protease was added here to break down free protein molecule in the WT346 phage soup. The biofilm ring was thicker in comparison to number **(1)** and **(3)** biofilms as this mixture only lacked protein but not the free DNA. The free DNA helped in the advancement of biofilm formation. The OD of the biofilm was 1.1 mm.

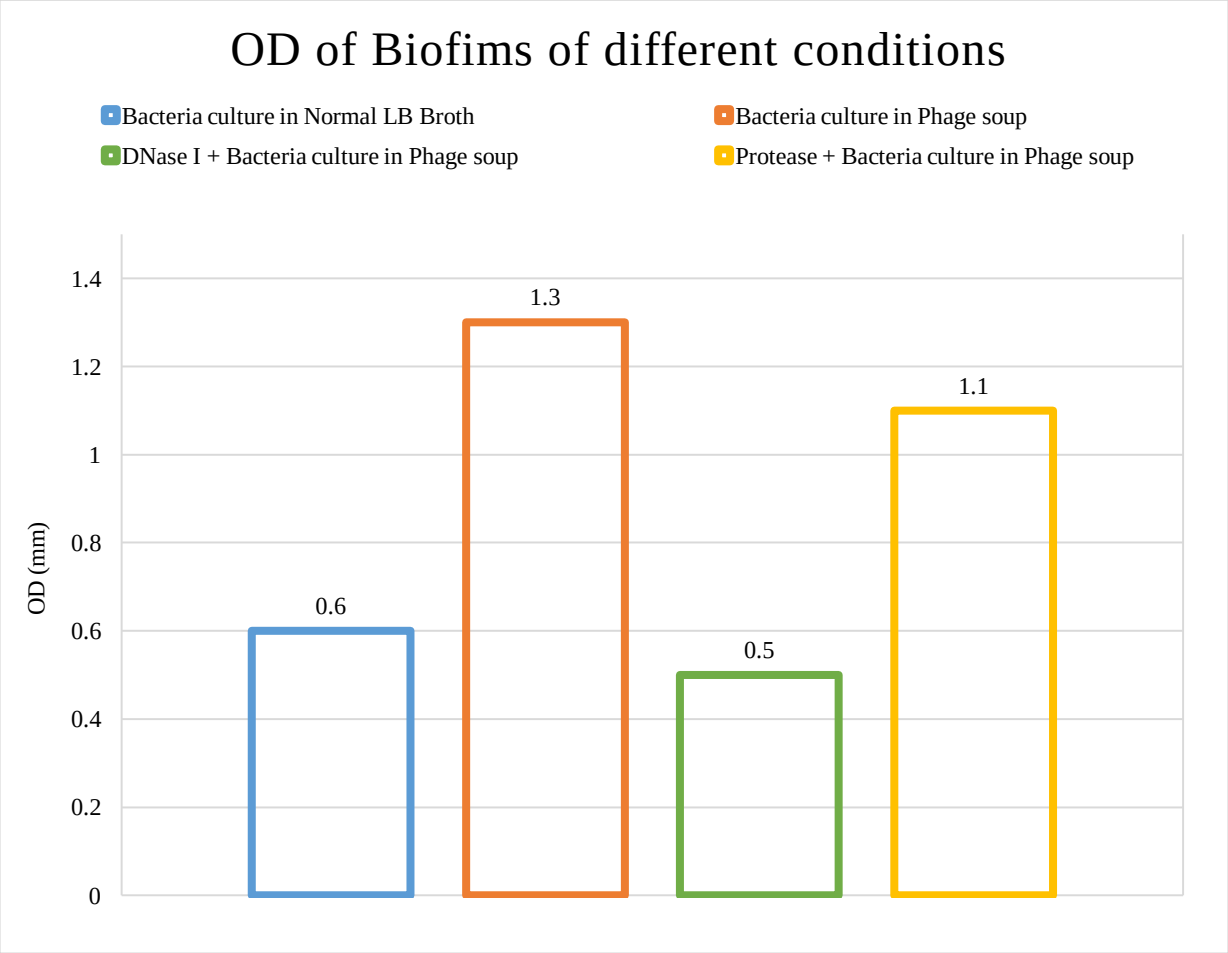

Figure 16: Graphical representation of OD (mm) of Biofilms of different conditions

Thus, this experiment proves that, it is the free DNA which enhances the formation of biofilm.

4.3 Conditions Maintained During the Study:

To prevent contamination or interference from other organisms, sterility was maintained throughout. All of the lab work was completed in a Class 2 Biosafety Cabinet, and all of the equipment had been thoroughly sterilized. Our biofilms were formed in a sterile environment and the place was remote so that no movement occurs there.

The same amount of time was given for the creation of the biofilm in each subsequent repeat of the experiment. Based on the traits of the species, the biofilm developed thicker or thinner. However, they didn't get a different timing. DNase and Protease was diluted to adjust their concentration in according to the requirement for our study to conduct. All the organisms were grown on the same culture medium. Additionally, the same amount of media was used in each instance to ensure that each subject received the same amount of nourishment. To maintain healthy growth, always new media was employed while working.

4.4 Importance of Biofilms and bacteriophages in Understanding Pathogenesis

Although, free planktonic bacteria cell responsible for the increased infectious diseases, biofilm itself can cause various diseases as well. In several instances, it has been observed that while the same bacteria do not normally cause disease when they are free-living, they do so when embedded in a biofilm. Many nosocomial infections from indwelling medical devices, including enteropathogenic infections, biliary tract infections, mouth infections, ocular infections, and others, have been linked to biofilm (Kolpen et al., 2022; Vestby, Grønseth, Simm, & Nesse, 2020). Numerous research point to a close relationship between biofilm dynamics and disease etiology. Due to the clinical significance of biofilm dynamics in such disorders, further research is required for gathering knowledge regarding it.

Bacteriophages, because of the co-evolution mechanism, are actively involved in biofilm development in two opposing ways, as dispersing or promoting agents. Phages may contain matrix-degrading enzymes that enable the efficient infection of cells trapped in biofilms. In this situation, phages are a natural and practical weapon against microbial biofilms. Prophages, on the other hand, control phage-mediated cell lysis and bacterial DNA release, which is a crucial mechanism for preserving the biofilm matrix (Drulis-Kawa & Maciejewska, 2021).By causing cell

lysis (at least in a portion of the bacterial population), phage is crucial to the development of the biofilm lifecycle because this causes eDNA (Environmental DNA) to accumulate in the biofilm matrix. The formation of a liquid crystalline structure in the biofilm matrix would be helped by the concurrent rise in DNA and phage concentrations (Secor et al., 2015).

Now, we assumed that the phages, through destroying the bacteria, could be the cause of this free environmental DNA. More biofilms are formed at the same time as phage numbers rise, as more phages attack more bacteria, releasing DNA in the process which is helping in biofilm development. Because of this, we have employed bacteriophages to eliminate their designated bacteria in our lab in order to produce a free bacterial DNA-containing solution. We cultured bacterial strain there, and we observed that the bacterial biofilm growth was improved. By doing so, we can attest to the fact that free DNA always has a significant influence for constructing the structure of bacterial biofilms.

5. Future Prospects of the research

As the quantity of bacteriophages is lower in March to May and September to December, there will be less free DNA and there might be less biofilm formation of bacteria. The number of phages that kill bacteria and generate free DNA in the environment increases in May to August. Bacteria that survive during this period are induced to create biofilms by these free DNAs. That is why we have worked here with the sample that has been obtained during the May of 2022 and stored in the lab. Also, the source of our sample was Gulshan Lake, Dhaka, Bangladesh. So, sample from different sources and different times should be used to conduct this research in future.

Again, the study's sample size was limited. Due to the various biofilm forming capacities of the bacteria, a total of four distinct strains were collected from the lab. They could be seasonal disease-causing substances. They were taken to determine whether all microorganisms would react the same way to the experiment. It was discovered that they all do respond similarly.

It would be preferable if more seasonal pathogenic agent strains could be incorporated into the study. Both clinical and natural sources for the strains should be used. Potential sample sources include the natural reservoirs of these diseases. To further assess the data, clinical samples should also be used. Thus, it will be understood what effect free bacterial DNA obtained by cutting down

the bacteria by using phage has upon them. In this experiment, only LB media was employed. It's crucial to observe the outcomes of using different growth media. Other basic or enriched media, or even sterile water from natural sources, can be used in place of this. The goal of our research can be more strongly supported if similar results are observed across all potential configurations. If the hypothesis is accepted, more detail study can bring to light more information about biofilm. This study will open the doors in designing many anti-biofilm therapies.

6. Conclusion:

Even though bacterial viruses are considered as enemy of bacteria they often work together to prevent total elimination of bacterial strains from the environment. The bacterial DNA of predated bacteria aids in biofilm formation even in slightest concentration. The major target of our project was to establish this hypothesis in laboratory settings. The biofilm rings in our studies depicts the difference in the result. As many contributing factors come together to build up the biofilm hence why, to know these factors are important to target anti-biofilm therapies. Bio-film enables the bacteria to communicate among themselves even in adverse condition. The co-existence of phage and bacteria in the environment and their diversified activities needs to be unraveled to understand the prognosis of many diseases. Researchers around the world have already made progress studying the bio-film. The findings of this study will add to our understanding of bacteriophage's impact on the environment, including humans. The information can be used by scientists to develop new and improve pre-existing preventative measures.

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