PARTIAL GENOMIC CHARACTERIZATION AND ISOLATION OF ENDOLYSIN FROM BACTERIOPHAGE SFN6B AGAINST *Shigella flexneri*

By

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ABSTRACT

PARTIAL GENOMIC CHARACTERIZATION AND ISOLATION OF ENDOLYSIN FROM BACTERIOPHAGE SFN6B AGAINST *Shigella flexneri*

Muhilarasi Sungevie

Bacteriophages are obligate intracellular parasites that target specific bacteria and cause the subsequent lysis of the bacterial cells. Bacteriophages release a lytic enzyme called endolysin at the end stage of the lytic replication cycle. This enzyme aids in the breakdown of bacterial cell wall, which allows the release of new progeny. With the emergence of antibiotic resistant bacteria, different therapeutic approaches need to be implemented to overcome the problem. Phage therapy plays an important role as antimicrobial agent due to their nature in causing lysis of the targeted bacterial cells without harming other cells. However, bacteria are also capable of developing resistance towards bacteriophages. Therefore, endolysin has been studied as an alternative antimicrobial agent to replace antibiotics and phage therapies. Hence, this study focused on the partial genomic characterizations and also the isolation of endolysin from the bacteriophage SFN6B against *Shigella flexneri.* The SFN6B phage sample was first grown in large scale and precipitated using the polyethylene glycol precipitation. The PEG-precipitated phage sample was then subjected to density gradient caesium chloride ultracentrifugation to

determine the buoyant density of SFN6B phage in caesium chloride gradients and the effectiveness of the method in purifying phage particles. Apart from that, the nucleic acid of the PEG-precipitated phage was extracted and subjected to enzymatic digestions using DNase, RNase and six different restriction enzymes such as *Bam*HI, *BgI*II, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I. Subsequently, agarose gel electrophoresis was carried out to determine the size of the phage genome and the outcomes of enzymatic digestions of the phage genome. On the other hand, ammonium sulphate precipitation was carried out on crude phage lysate to precipitate proteins at different percentage of ammonium sulphate saturations. Following SDS-PAGE, lytic activity assay was performed on the precipitated proteins to determine the protein precipitate that contains the endolysin. Results obtained showed that the buoyant density of the SFN6B phage was approximately 1.5 g/mL. The average phage titre after density gradient caesium chloride ultracentrifugation was approximately 200 times higher than the average phage titre obtained after PEG precipitation. Apart from that, agarose gel electrophoresis revealed that the size of the phage genome was above 10, 000 base pairs and the phage genome was made up of DNA. Besides that, the vulnerability of the phage genome to the different restriction endonucleases used was also observed based on the different number of bands produced. SDS-PAGE revealed that high concentrations of proteins were precipitated at 40-60% of ammonium sulphate saturation. The expected result of the lytic activity assay was to obtain a single quadrant containing area of bacterial lysis which indicate the presence of endolysin. However, in this study, all the protein precipitates spotted on lawns of *S. flexneri* showed area of lysis. Plaque assays were performed to determine the

presence of phage particles in the protein precipitates and it was found that phage particles were co-precipitated with other proteins during ammonium sulphate precipitation. As a result, the protein precipitate containing endolysin was unable to be determined. Therefore, it is suggested that removal of phage particles prior to ammonium sulphate precipitation via high speed centrifugation should be carried out to prevent co-precipitation of the phage particles with the proteins. Besides, alternative methods such as running the precipitated protein samples through gel filtration column may also aid in phage removal before determining the precipitated protein containing endolysin via lytic activity assay.

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Lastly, I would like to thank my family and all my friends for their constant helps, encouragements and support throughout this project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

MUHILARASI SUNGEVIE

APPROVAL SHEET

This project report entitled "**PARTIAL GENOMIC CHARACTERIZATION AND ISOLATION OF ENDOLYSIN FROM BACTERIOPHAGE SFN6B AGAINST *Shigella flexneri***" was prepared by MUHILARASI SUNGEVIE and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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(Dr Michelle Ng Yeen Tan) Date:…….

Supervisor

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FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **MUHILARASI SUNGEVIE** (ID No: **12ADB07004**) has completed this final year project entitled "**PARTIAL GENOMIC CHARACTERIZATION AND ISOLATION OF ENDOLYSIN FROM BACTERIOPHAGE SFN6B AGAINST *Shigella flexneri***" under the supervision of Dr Michelle Ng Yeen Tan (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(MUHILARASI SUNGEVIE)

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LIST OF ABBREVIATIONS

×*g*	Times gravity (acceleration due to gravity)
ºC	Degrees Celcius
µL	Microlitre
µg	Microgram
%	Percentage
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bp	Base pair
CsCl	Caesium chloride
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g/mL	Gram per millilitre
HCl	Hydrochloric acid

h	Hour
Kb	Kilo base pair
kDa	Kilodaltons
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide
mA	Milliampere
min	Minutes
mM	Millimolar
mL	Millilitres
MWCO	Molecular weight cut off
NaCl	Sodium chloride
nm	Nanometer
OD_{600}	Optical density measure at wavelength 600 nm
PAGE	Polyacrylamide gel electrophoresis
pfu/mL	Plaque forming units per millilitre
PEG	Polyethylene glycol
pH	Power of hydrogen

RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
S. flexneri	*Shigella flexneri*
S. dysenteriae	*Shigella dysenteriae*
S. sonnei	*Shigella sonnei*
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
V	Volts
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

The absolute basic structural form of bacteria comprise of a protein-lipid envelope that surrounds the cell cytoplasm. Apart from the more unusual bacteria such as *Rickettsia* and *Mycobacteria*, most bacterial species can be classified into two different types based on the presence or absence of an outer membrane (Chart, 1994). Bacteria can be classified into Gram-positive and Gram-negative depending on the ability of the bacterial cell wall in retaining the Gram stain (Liu, 2011). Due to the presence of a distinctively thicker peptidoglycan layer, the peptidoglycan itself can value a higher amount of cell wall mass in Gram-positive bacteria compared to Gram-negative bacteria (Seltmann and Holst, 2002). Generally, the absence of the outer membrane structure results in Gram-positive bacteria having a less complicated cell envelope structure compared to Gram-negative bacteria. Gram-negative bacteria also express lipopolysaccharides (LPS) that are surface-exposed carbohydrate structures, showing a prominent difference from Gram-positive bacteria that do not contain LPS (Chart, 1994).

It is true that Gram-negative bacteria are generally more resistant to antibiotics than Gram-positive bacteria (Silhavy, Kahne and Walker, 2010). This is due to the fact that apart from the mechanisms involved in the general transport of substrates, the uptake of antibiotic into bacterial cells also depends on the

structure of the cells (Fischer and Braun, 1981). The outer membrane of Gram negative bacteria contains porins, which are proteins of a special class that produce non-specific aqueous diffusion channels across the membrane (Nikaido, 1994). Porins limit the diffusion of hydrophilic molecules larger than approximately 700 Daltons and this, coupled with the non-fluid continuum formed by the LPS molecules that act as effective barrier for hydrophobic molecules, make the outer membrane a selective, yet very effective permeability barrier (Silhavy, Kahne and Walker, 2010). Hence, the Gram positive bacterial cell wall is more permeable to a series of antibiotics due to its simple structure as compared to the Gram negative bacterial cell wall (Fischer and Braun, 1981). The protein and lipid compositions of the outer membrane have a very strong impact on the bacterial sensitivity to various types of antibiotics, and development of drug resistance that involves the modifications of these macromolecules is common (Delcour, 2008). For instance, strains of *Shigella flexneri* that are resistant to multiple antibiotics are often found in patients in both community hospitals and urban areas in north-eastern Brazil (Lima, et al., 1997).

Serious infections caused by Gram-negative pathogens continue to be highly associated with considerable mortality (Paterson, 2008). Multidrug-resistant bacteria are becoming very common due to their multiplicity of mechanisms and this daunting spectre has become the target of many research efforts into alternative approaches (Tillotson and Theriault, 2013). The use of bacteriophages as bactericidal agents have been employed for over 90 years as

a way of treating bacterial infections in humans as well as other species in a process called phage therapy (Abedon, et al., 2011).

Over billions of years, phages have co-evolved with their hosts and acquired mechanisms to counter bacterial defences such as extracellular biofilm production that has severely reduced the effectiveness of conventional antibiotics (Burrowes, et al., 2011). Bacteriophages are also commonly known as bacterial viruses and they exist in all habitats where bacteria proliferate. Since the past 30 years, researches conducted on phages have revealed the diversity of phage genomes, the abundance of phages in nature and their impact on the microbial diversity evolution, their influence in the regulation of microbial balance and their control of infectious diseases (Mc Grath and Sinderen, 2007). Bacteriophages are capable of inserting their nucleic acid into bacteria and multiply in them (Passarge, 2007). Phage-infected bacteria produce endolysins that are double-stranded DNA bacteriophage-encoded peptidoglycan hydrolases toward the end of the lytic cycle (Borysowski, Weber-Dabrowska and Gorski, 2006).

In Gram-negative bacteria, "lysis from within" that occur during the lytic cycle is via holins and endolysins (Hyman and Abedon, 2012). Endolysins do not have the self-replication characteristics like phages but they are specific for the bacteria from which they are generated from, hence they also have the same specificity as the bacteriophages (Denyer, Hugo and Russell, 2011). In Gram positive bacteria, the absence of an outer membrane enable endolysins to

access the peptidoglycan layer and cause the destruction of these organisms when applied externally (Schmelcher, Donovan, Loessner, 2012). Interestingly, some endolyins such as the SPN9CC endolysin could cause the lysis of intact Gram negative bacteria in the absence of an outer membrane permeabilizer (Lim, et al., 2014). This allows endolysins to be used as effective therapeutic antimicrobials against specific pathogens (Nelson, et al., 2012).

Hence, the objectives of this project were to extract the nucleic acid of the bacteriophage SFN6B against *Shigella flexneri*, partially characterize the genomic properties of the bacteriophage and to carry out preliminary extraction of endolysins from the bacteriophage SFN6B against *Shigella flexneri*.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteriophages

2.1.1 Biology of Bacteriophages

Bacteriophages or phages are bacterial viruses that only infect and multiply within their bacterial hosts with high host specificity of species or strain level (Tan, Chan and Lee, 2014). They are rightly known as obligate intracellular parasites due to their fundamental characteristic of absolute dependence on specific host organisms (Sundar, et al., 2009). Phages are ubiquitous in nature and can be readily isolated from environmental samples such as water, sewage and soil (Keary, et al., 2013).

According to Deresinski (2009), currently there are 13 families and 30 genera of bacteriophages. They are believed to be the most abundant life form on Earth, suffusing the biosphere with a predicted 1×10^{8} species that comprises of an estimated total of 1×10^{30} to 1×10^{32} phage particles. If these phage particles were gathered, they would weigh approximately 1×10^{9} metric tons (Deresinski, 2009). Structurally, bacteriophages contain a core nucleic acid, usually double-stranded DNA (dsDNA), encapsulated with a lipoprotein or protein capsid which is connected to a tail that interacts with various bacterial surface receptors via fibres extending from the tail (Tan, Chan and Lee, 2014).

Ninety-six percent of all bacteriophages present on earth are tailed bacteriophages belonging to the order *Caudovirales* (Almeida, et al., 2004). According to the International Committee on Taxonomy of Viruses, they can be classified into three different families: the *Podoviridae* (short non-contractile tail), the *Siphoviridae* (long non-contractile tail) and the *Myoviridae* (long contractile tail) (Tan, Chan and Lee, 2014). Figure 2.1 shows the electron micrographs and the schematic representations of phages of the three different families.

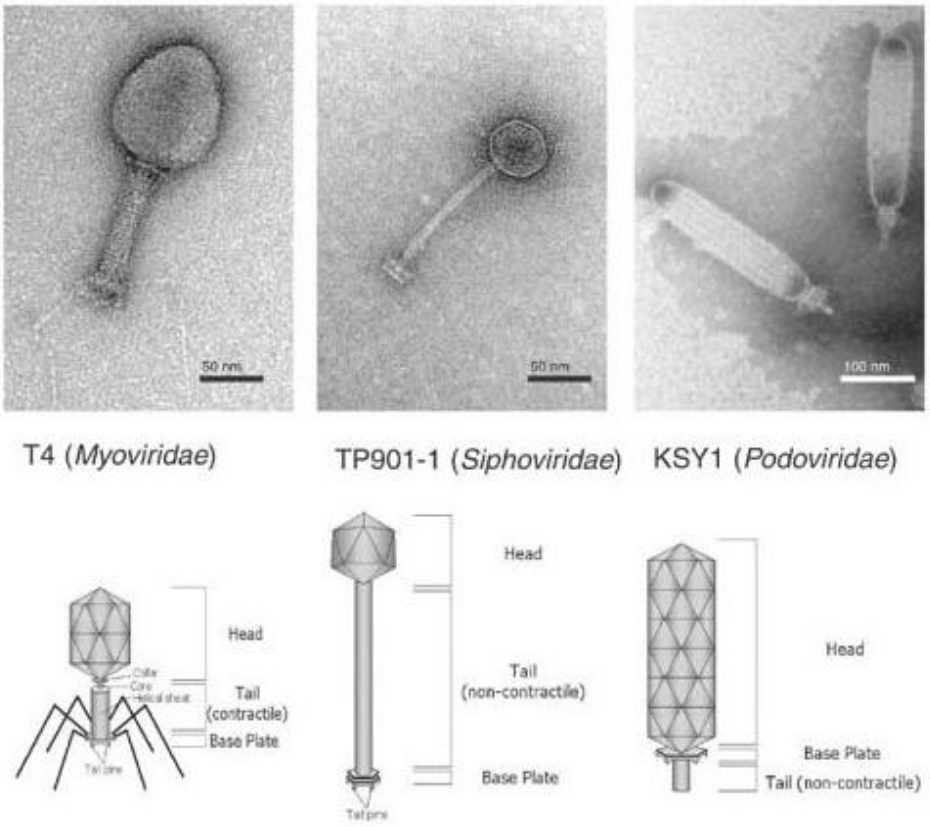

Figure 2.1: Electron micrograph (upper panel) and schematic representation (lower panel) showing T4 phage with a long contractile tail (*Myoviridae*) and a prolate head; TP901-1 (P335 species) temperate phage of *Lactococcus lactis* (*L. lactis*) with a long non-contractile tail (*Siphoviridae*) and an isometric head; and KSY1 virulent phage of *L. lactis* with a short non-contractile tail (*Podoviridae*) and a rare morphology characterized by an elongated head (Mc Grath and Sinderen, 2007).

2.1.2 Discovery of Bacteriophages

In 1896, Ernest Hankin reported the presence of marked antibacterial activity against the causative agent of cholera, *Vibrio cholera* (Sandeep, 2006). In 1915, Frederick Twort hypothesized that the antibacterial activity could be due to the presence of phages. However, he did not pursue his discovery further (Haq, et al., 2012). A French-Canadian microbiologist, Felix d'Herelle "officially" and independently discovered bacteriophages at the Institut Pasteur in Paris in 1917. This discovery or rediscovery was associated with an outbreak of severe haemorrhagic dysentery among French troops stationed at Maisons-Laffitte in July-August 1915 (Sulakvelidze, Alavidze and Morris, 2001).

In 1917, D'Herelle isolated a so-called "anti-Shiga microbe" from stools of patients with recovering shigellosis by filtering the stools that were incubated for 18 hours. This active filtrate was able to cause arrest of culture, death and finally the lysis of the bacilli when added either to an emulsion or culture of the Shiga bacilli (Wittebole, Roock and Opal, 2014). According to Wittebole, Roock and Opal (2014), D'Herelle went on to describe his discovery as an obligate bacteriophage. Furthermore, by inoculating laboratory animals with this phage as a treatment for shigellosis, he demonstrated the activity of this anti-Shiga microbe. According to Sulakvelidze, Alavidze and Morris (2001), D'Herelle then used phages to treat dysentery not long after his discovery whereby, the phage preparation was administered to a 12-year old boy with severe dysentery. It was noted that the patient's symptoms ceased after a single

administration of the anti-dysentery phage, leading to the boy's full recovery within a few days. In addition, when three patients with bacterial dysentery treated with one dose of the preparation showed signs of recovery within 24 hours of treatment, the efficacy of the phage preparation was confirmed. (Sulakvelidze, Alavidze and Morris, 2001). Phage therapy was a modest success during the 1920s and 1930s. However, phage therapy was largely abandoned in Western countries due to the commercialization of penicillin in the 1940s (Keary, et al., 2013).

2.1.3 Classification of Bacteriophages

Based on their replication cycles, phages can be commonly classified into either temperate (lysogenic) or virulent (lytic) (Keary, et al., 2013). According to Birge (2000), a temperate phage establishes a stable relationship with the host cell whereby, some phage functions are expressed. In this state, the phage nucleic acids are usually replicated together with the host's genetic material (Elbreki, et al., 2014). According to Todar (2012), temperate phages integrate their chromosome into a specific section of the host cell chromosome but do not result in the death of the host bacteria. In this state, the host cells are said to be lysogenized and the phage DNA is called a prophage, whereby all the phage genes are repressed except one. The one expressed phage gene codes for the synthesis of a repressor molecule that prevents the synthesis of proteins and phage enzymes that are required for the lytic cycle (Todar, 2012). The lysogens usually undergo the occasional metabolic shift that causes the reactivation of the viral DNA which results in lytic response (Birge, 2000).

The inactivation of the repressor molecule synthesis will result in the synthesis of enzymes that excises the viral DNA from the host cell chromosome (Todar, 2012).

On the other hand, virulent phages lack independent replication systems. Hence, they take over the protein synthesis and DNA replication machinery of their host cells for the production of new progeny (Oliveira, et al., 2012). Within several minutes to hours following the initial phage infection, the new progeny will be released following the host cell death (Elbreki, et al., 2014). During lytic growth, the phages utilize macromolecular pool of the host cells to carry out virally-encoded functions while suppressing the host-directed synthetic activity. The host cells need to be metabolically active in order to support phage replication (Fry and Day, 1992).

According to Parija (2009), the lytic cycle of a virulent phage generally consists of four different stages; adsorption, penetration, synthesis of phage components and release of new progeny phages. Adsorption is a specific process whereby it is dependent on the susceptibility of the host bacterium to the specific phage and the specificity is determined by the presence of chemical receptors on the bacterial surface (Parija, 2009). According to Hyde (1987), the attachment of the phage tail fibres and pins to the surface of the host cells in the penetration step triggers a contraction of the tail sheath, resulting in the subsequent discharge of the phage nucleic acid into the host cell. Next, intracellular development begins with the transcription of the phage

nucleic acid by host RNA polymerase followed by the translation of the phage mRNA by the host protein synthesizing machinery resulting in the formation of "early" proteins (Hyde, 1987). Subsequently, the synthesis of "late" proteins occurs followed by the assembly and maturation of the progeny phages. Lysozyme causes the weakening of the host cell wall during the replication of the phages hence, the cell wall eventually bursts as a results of osmotic pressure and this is also known as "lysis from within" (Parija, 2009). Figure 2.2 shows the two different replication cycles of phage particles.

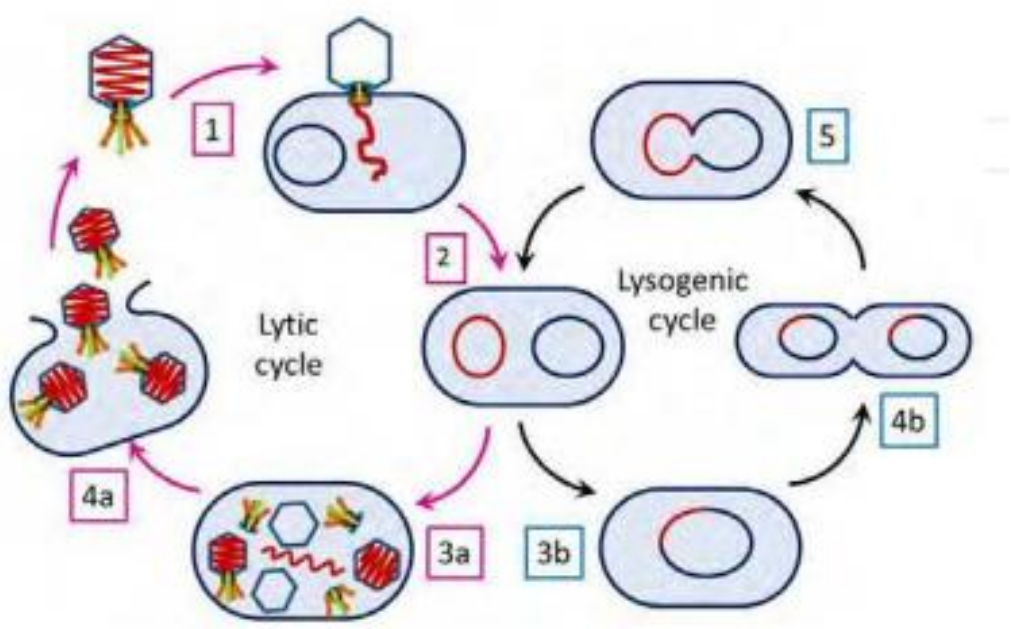

Figure 2.2: The replication cycles of the bacteriophage. 1- Attachment of phage to the host cell followed by the injection of the viral DNA into the host cell; 2- Phage DNA undergoes either the lytic or lysogenic cycle; 3a- New phage nucleic acid and proteins are synthesized and new virions are assembled; 4a- Lysis of the cell causing the release of virions; 3b and 4b- Steps involved in the lysogenic cycle whereby phage genome is integrated within the bacterial chromosome forming prophage; 5- Phage excises from the bacterial chromosome and initiates the lytic cycle (Orlova, 2012).

2.1.4 Applications of Bacteriophages

Phage therapy is one of the important applications of bacteriophages. Phage therapy involves the use of phage particles or their products as bioagents in treating bacterial infections (Matsuzaki, et al., 2005). According to Inal (2003), the increasing incidence of antibiotic resistance in bacteria has led to the increasing interest in phage therapy for combating bacterial infections. Over the last decade, phages have emerged as the major alternative to antibiotics in treating antibiotic-resistant infections (Borysowski and Gorski, 2008). Phages have been administered systemically, orally and topically to treat a wide variety of infections caused by antibiotic-resistant bacteria which include *Streptococcus, Escherichia, Staphylococcus, Pseudomonas, Proteus, Klebsiella, Salmonella* and *Shigella spp*. (Alisky, et al., 1998). Besides that, a study in 1989 on the effect of phage therapy on 131 cancer patients who were suffering from post-operative wound infections showed that 81.5% of the patients produced positive clinical results. Antipseudomonal phages appeared to be the most effective followed by antistaphylococcal phages (Mathur, Vidhani and Mehndiratta, 2003).

According to Loc-Carrillo and Abedon (2011), the advantages of phage therapy include their bactericidal property whereby, phage-infected bacteria will not be able to regain their viability. Besides that, phages are highly specific to the bacteria that they infect hence, will not harm the normal microflora of the body (Sandeep, 2006). Apart from that, phages are inherently non-toxic since they consist mostly of proteins and nucleic acids (Loc-Carrillo

and Abedon, 2011). Phages are also capable of causing disruption to bacterial biofilms and confer equal effectiveness against antibiotic-resistant and antibiotic-sensitive bacteria (Lim, et al., 2012). According to Sandeep (2006), a single dose of phage particles is often sufficient to treat a bacterial infection, since phages grow exponentially. Moreover, different phages that target different bacterial species can be mixed as cocktails to obtain a greater spectrum of antibacterial activity (Loc-Carrillo and Abedon, 2011).

The therapeutical usage of bacteriophages also has its limitations in spite of the benefits. These include the presence of bacterial toxins in phage lysate and the presence of phage-resistant bacteria (Lim, et al., 2012). According to Sirsat, Muthaiyan and Ricke (2009), bacteria may develop resistance towards phages just as they become resistant to antibiotics. However, phages may also mutate in step with the bacteria and continue to infect the bacterial cells. Besides that, the causative agent of an infection need to be identified prior to treatment with the appropriate phage due to the high specificity of the phage particles (Inal, 2003). The limited host range of the phage particles is also one of the disadvantages of phage therapy (Sirsat, Muthaiyan and Ricke, 2009).

Apart from that, phages also act as attractive tools for the biocontrol of bacterial contamination of food substances, for controlling environmental microflora, for controlling food-borne and water-borne pathogens and in plant protection (Tiwari, et al., 2011). Figure 2.3 shows the application of phages along the food chain in the “farm to fork” approach.

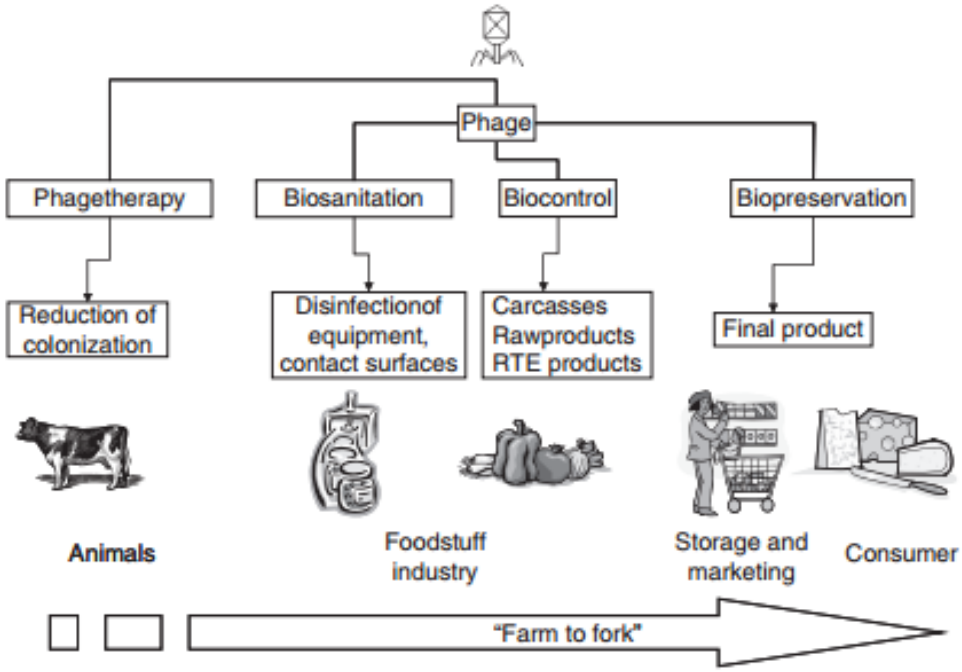

Figure 2.3: Phage applications along the food chain in the classic "farm to fork" approach (Garcia, et al., 2008).

As shown on Figure 2.3, the concept of combating pathogens present in food substance using phages can be addressed at all stages of production in the classic "farm to fork" approach. Phages are suitable agents to reduce or prevent the colonization and diseases in livestock. Besides that, phages can also be used to disinfect contact surfaces and equipments, decontaminate carcasses and other raw products. It may also serve as natural preservatives in extending the shelf life of perishable manufactured foods (Garcia, et al., 2008). Based on a study conducted in 1986, it was shown that beef spoilage caused by *Pseudomonas spp*. can be reduced by the application of phages (Atterbury, et al., 2003). Apart from that, Greer (1986) reported that when *Pseudomonas*-inoculated steaks were treated with high titre of phage lysates (10^8 PFU/ml), 2-log increase in the phage numbers and 1- to 2-log reduction in the level of

bacterial contamination were observed four days after retail display. A marked decrease in the steak surface discoloration accompanied these changes.

Besides that, phages also encode lytic enzymes that aid in the digestion of bacterial cell wall for the release of new phage progeny. These lytic enzymes could act as effective antimicrobial agents as small quantities of purified recombinant lysin added to Gram-positive bacteria result in the immediate lysis of the target bacteria (Fischetti, 2005).

2.2 Endolysin

2.2.1 Characteristics of Endolysin

With the prevalence of antibiotic-resistant bacteria, alternative therapeutic approaches are needed in order to combat the problem. This is where endolysins come into the picture as an effective antimicrobial agent (Lim, et al., 2012). Endolysins are employed by a vast majority of phages to cause enzymatic degradation of the peptidoglycan layer of the host bacterium 'from within' at the last stage of the lytic multiplication cycle (Schmelcher, Donovan and Loessner, 2012). Two different proteins; endolysins and holins usually take part in the release of the progeny phage. Holin is a tunnel-like protein that acts in producing non-specific holes in the bacterial cytoplasmic membrane in order to facilitate the movement of endolysin to the periplasm (Morita, et al., 2001). This enables the endolysins to cleave the bonds which hold the peptidoglycan layer together, leading to the lysis of the host cells (Keary, et al., 2013).

According to Drulis-Kawa, et al. (2012), endolysins, also known as muralytic hydrolases or lysins are highly evolutionarily advanced enzymes encoded by bacteriophages. These enzymes also possess high efficiency in killing target bacterial cells. They possess bactericidal activity and are effective against antibiotic-resistant bacteria. Besides that, endolysins are also target specific and do not disrupt the normal microflora of the body as they are non-toxic to eukaryotic cells (Lim, et al., 2012). The ability of purified endolysins in killing bacteria as recombinant proteins was first reported in 1959 and since then, research on phage-encoded endolysins, particularly those encoded by Gram-positive bacteriophages are highly evolving (Drulis-Kawa, et al., 2012). According to Hermoso, García and García (2007), the development of bacterial resistance is one of the major concerns of the therapeutic use of purified endolysin. However, endolysin-resistant streptococcal bacteria were absent even after repeated exposure of the bacteria to low lysin concentrations either when grown on agar plates or liquid media (Hermoso, García and García, 2007). Hence, it can be concluded that there is a low probability that a target bacteria will develop resistance towards the activity of endolysins as they target highly conserved and unique peptidoglycan bonds (Kaźmierczak, Górski and Dąbrowska, 2014).

2.2.2 Target of Endolysins; General Structure of Bacterial Cell Wall

According to Barton (2005), bacterial cell wall provides the shape and confers osmotic stability to the bacteria. In Gram-positive bacteria, the structure exterior to the plasma membrane is known as the cell wall. On the other hand,

the cell wall of Gram-negative bacteria includes the structural layer of the cell in addition to the outer membrane layer. The region between the outer membrane and the plasma membrane contains a gel-like substance that aids in maintaining turgor pressure. This region is termed as the periplasmic space and plays a very important role in various cellular processes (Barton, 2005). The outer membrane contains porins that are protein channels with size exclusion limit of about 600 Dalton (Hobson, 1988).

Apart from mollicutes, all pathogenic bacteria (Gram-positive and Gram-negative) have cell wall that is termed as peptidoglycan (Paul, 2008). It is composed of several chains of alternating residues of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) held together by β-1, 4 glycosidic bonds, linked to a short stem tetrapeptide (Oliviera, et al., 2012). A thicker cell wall can be observed in Gram-positive bacteria as compared to Gram-negative bacteria. Figure 2.4 shows the schematic representations of the differences in the peptidoglycan structures between Gram-negative and Gram-positive bacteria.

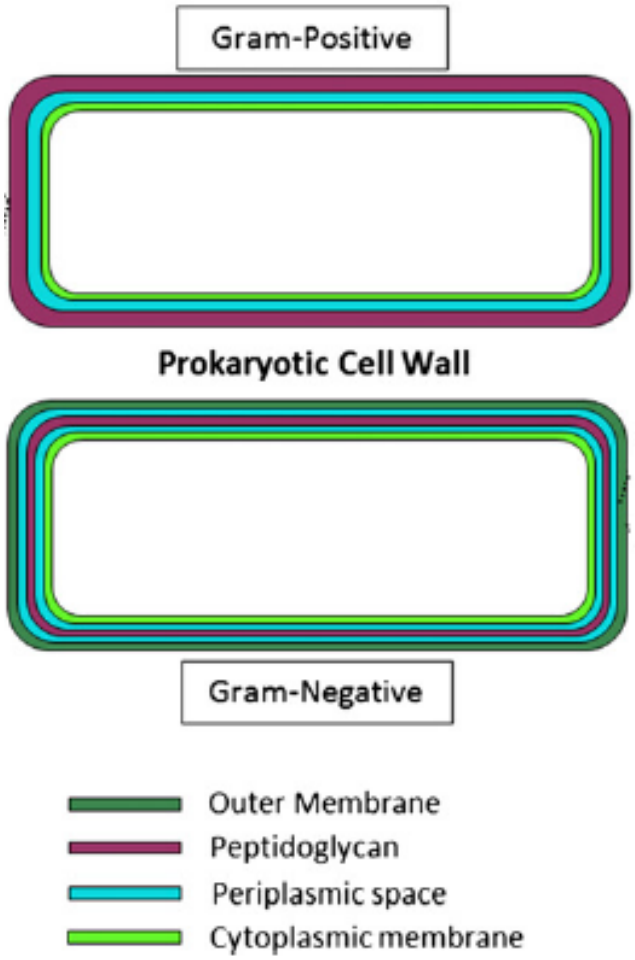

Figure 2.4: Schematic representation of the main peptidoglycan differences between a Gram-negative and Gram-positive bacterium (Oliviera, et al., 2012).

Gram-negative bacteria are less penetrable to antimicrobials as compared to Gram-positive bacteria due to the presence of the outer membrane layer (Seltmann and Holst, 2002). According to Devroy (2008), in comparison to Gram-positive bacteria, Gram-negative bacteria tend to be more resistant towards antimicrobials. This is due to the presence of additional outer membrane permeability barrier that limits or prevents the access of various compounds to their respective targets in the bacteria. Most antibiotic classes that are available in the drug market are only effective against Gram-positive and not Gram-negative bacterial infections (Devroy, 2008).

According to Tišáková and Godány (2014), with respect to Gram-positive bacteria, endolysins can also act as exolysins whereby they can digest the peptidoglycan layer and cause lysis of the bacterial cells when applied exogenously. However, this is not the case for Gram-negative bacteria due to the presence of the outer membrane that prevents the access of the hydrophilic lytic enzymes effectively. According to Lim, et al. (2012), there are studies that showed the lytic activities of purified endolysins as potential therapeutic agents against *Bacillus anthracis, Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, Lactobacillus fermentum, Enterococcus faecalis, Listeria monocytogenes* and *Clostridium perfringens*. However, it was obvious that most studies on purified endolysin focused on Gram-positive bacteria due to the accessibility of the peptidoglycan layer.

Recently, there were many researches done on the effectiveness of endolysins against Gram-negative bacteria obtained from different sources. The potential lytic activities of purified endolysin on Gram-negative bacteria were reported on *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacteria* phage T5 (Lim, et al., 2012). According to Rai and Bai (2014), the outer membrane layer of Gram-negative bacteria can be permeabilized for the entry of exogenously applied endolysin via a variety of compounds which include polymyxin, EDTA, citric acid and chitooligosaccharides. Besides that, non-thermal treatments can also sensitize the Gram-negative bacteria towards the application of endolysin. With the combinations of endolysins and high hydrostatic pressure, a synergetic bactericidal activity can be observed toward different Gram-negative bacteria (Rai and Bai, 2014). A study conducted by

Lim, et al. (2014) on the antimicrobial activity of SPN9CC endolysin against Gram-negative bacteria without the presence of EDTA as an outer membrane permeabilizing agent showed that some Gram-negative endolysins could also lead to bacterial cell destruction without an outer membrane permeabilizer. Besides that, Lai, et al. (2011) conducted a study to analyse the antibacterial activity of LysAB2 endolysin against both Gram-negative and Gram-positive bacteria. The results obtained from the study showed that LysAB2 endolysin possess broad bacteriolytic activity against some Gram-positive and Gram-negative bacteria when applied exogenously.

2.2.3 Biology and Structure of Endolysins

The structure of endolysins generally differs between those targeting Gram-negative and Gram-positive bacteria. This reflects the differences in the cell wall architecture between these two bacterial groups (Tišáková and Godány, 2014). Endolysins from phages against Gram-positive bacteria utilize a modular design by the combination of at least two different polypeptide modules that are dedicated to two basic functions; enzymatic hydrolysis and substrate recognition (Tišáková and Godány, 2014). According to Yuan, Peng and Gao, 2012, the two functional domains of Gram-positive endolysins are the C-terminal cell wall binding domain and the N-terminal catalytic domain. The cell wall binding domain is divergent and is able to differentiate distinct cell wall epitopes. On the other hand, the catalytic domain belongs to one of the four families of peptidoglycan hydrolases which are rightly categorized according to catalytic site-specificity; N-acetylmuramoyl-L-alanine amidases,

N- acetylmuramidases, N-acetylglucosaminidases and endopeptidases (Yuan, Peng and Gao, 2012).

To date, about 723 diverse putative endolysins have been identified with 13 cell binding domain (CBD) and 24 different enzymatic catalytic domain (ECD), which can be further classified based on their structural organization into 89 different types (Plotka, et al., 2013). Endolysins can adopt either a modular or globular structure. Majority of Gram-negative endolysins are globular with only a catalytic domain, although some have a modular structure that are made up of a C-terminal catalytic domain and an N-terminal cell wall binding domain (Walmagh, et al., 2012). According to Keary, et al. (2013), the cell wall binding domain targets the lytic domain to its substrate and tightly secures it in position even after the lysis so that it will not be available to lyse nearby cells that can be infected by phages. The absence of the cell wall binding domain in Gram-negative endolysins may be explained by the fact that the endolysins cannot possibly harm nearby cells that could act as hosts for the replication of phages (Keary, et al., 2013). Briers, et al. (2007) conducted a study on *Pseudomonas aeruginosa* phage endolysins EL188 and KZ144 that are highly lytic on Gram-negative bacteria with permeabilized outer membrane. Site-directed mutagenesis in the study revealed that both the endolysins have C-terminal catalytic domains that are often present in Gram-positive endolysins, in addition to the normally present N-terminal substrate-binding domain. Conserved repeat sequences were present in both the binding domains, consistent with those of some Gram-positive endolysins (Briers, et al., 2007).

2.2.4 Applications of Endolysins

Purified preparation of endolysins can be utilized as therapeutic agents by itself alone or in combination with classical antibiotics that are currently available (Loessner, 2005). According to Fenton, et al. (2010), there are studies that have shown the ability of some endolysins in working synergistically with other lysins or with certain antibiotics *in vivo* and *in vitro*. The pneumococcal enzymes, LytA and Cpl-1 have been used synergistically with some antibiotics including cefotaxime, penicillin, moxifloxacin and gentamicin. Enhanced bactericidal activities were observed when antibiotics were used in combination with the enzymes. Other than that, LysK is a staphylococcal phage-K-derived endolysin that was isolated in 2005. The lysin was shown to be highly lytic against staphylococci of medical importance, which include vancomycin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (Kaur, et al., 2012). Besides that, according to Kaźmierczak, Górski and Dąbrowska (2014), the *in vivo* antibacterial activity of P-27/HP endolysin in mice showed 99.9% elimination of *Staphylococcus aureus* 27/HP present in murine spleens. Treatment with the endolysin saved the mice from death due to *Staphyloccus aureus* infection. The results of the study suggest that P-27/HP endolysin can be used as an alternative to antibiotics in treating staphylococcal infections. Apart from that, reduction in the viability caused by Gram-negative bacterium, *Pseudomonas aeruginosa* that is resistant to most antibiotics was observed with the addition of endolysin from *Bacillus amyloliquefaciens* phage (Morita, et al., 2001). Other lysins that can be used as potential treatment against bacterial infections in humans and animals have also been described, such as Ply3626 from a *Clostridium*

perfringens phage and PlyV12 from an *Enterococcus faecalis* phage (Loessner, 2005). All these studies prove that endolysins may serve as potential therapeutic agents to treat bacterial infections.

According to Oliviera, et al. (2012), endolysins also seem to be good candidates as food antimicrobials due to their selective mode of action and their inability in changing the texture and the organoleptic characteristics of the food products. Besides that, endolysins are also innocuous for human consumption (Oliviera, et al., 2012). According to Fenton, et al. (2010), the lysin Ply3626 showed lytic activity against some strains of *Clostridium perfringens*, which is a common causative agent for food poisoning. The lysin has been proposed to be exploited as a bio-control agent that can be directly added into feed or food (Fenton, et al., 2010). Apart from that, Ply500, Ply511 and Ply118 showed potential as anti-listerial agents on food products which include iceberg lettuce, whereby the viable counts of *Listeria monocytogenes* was reduced by up to 2.4 log units after six days of storage (Schmelcher, Donovan and Loessner, 2012).

According to Khatibi, et al. (2014), *Lactobacillus* species are one of the predominant contaminants that cause the "stuck" fermentation and reduce ethanol yields. Two genes, LysA and LysA2 encoding lytic enzymes were individually expressed and the purified enzymes obtained were found to be lytic against *Lactobacillus* isolates obtained from fermenters including *Lactobacillus brevis, Lactobacillus fermentum* and *Lactobacillus mucosae*.

The acetic and lactic acid levels were reduced in all experimentally infected fermentations.

2.3 *Shigella flexneri*

2.3.1 Characteristics

Shigella spp. are non-spore forming, rod-shaped, non-motile bacteria from the genus of gamma proteobacteria under the *Enterobacteriaceae* family (Todar, 2012). They are very closely related to enteroinvasive *Escherichia coli* (EIEC) with respect to the DNA sequences of the virulence and housekeeping genes (Boulette, 2007). *Shigella flexneri (S. flexneri)* is a Gram-negative, facultative intracellular bacteria that causes acute bacillary dysentery or shigellosis (Waddell, et al., 2014). The pathogenesis of *S. flexneri* is based on the ability of the bacteria to invade and replicate within the host's colonic epithelium that causes epithelial destruction and severe inflammation (Jennison and Verma, 2004).

2.3.2 Shigellosis and Treatment

Shigellosis is a bacterial infection caused by *Shigella spp.* Consumption of faecal-contaminated water is one of the common causes of infection by *Shigella spp.* (Steinhausen, 2008). *Shigella spp*. also appear to be the third most common disease-causing microorganisms that spread through faecal-oral route (Singh and Sharma, 2014). Over 164 million cases, with the majority of cases involving children of developing countries and 1.1 million deaths are

caused by shigellosis yearly (Jennison and Verma, 2004). The symptoms of shigellosis include fever, diarrhoea with or without the presence of mucous or blood in stools and stomach cramps. The onset of the disease occurs typically between 12 hours to 2 days after exposure (Steinhausen, 2008). The plasma carbon dioxide of the infected patient is usually low in response to metabolic acidosis induced by the severe diarrhoea and the white blood cell count often appear to be reduced at onset and then goes up to 13, 000 white blood cells per microliters (Timmreck, 2002).

Medical treatment is required for moderate-to-severe infection and this includes treatments with antibiotics such as ciprofloxacin, azithromycin, and co-trimoxazole for the elimination of the bacteria from the patient's digestive tract. Confirmation of *Shigella spp.* from stool samples enables doctors to choose the right medication to combat against the disease (Krucik, 2012). Over the years, Shigellosis has emerged as one of the major public health concerns in developing regions due to poor sanitation, hygiene and scarcity of water. In current situations, Shigellosis is estimated to cause 108, 000 deaths per year with 90 million episodes (Singh and Sharma, 2014).

Low infectivity dose, high virulence, and development of drug-resistance are major causes that pose Shigellosis as a threat. The absence of an effective vaccine and the increasing in resistance of *Shigella spp.* towards drugs like ampicillin, amoxicillin, chloramphenicol and cotrimoxazole has significantly increased the need for different therapeutic approach against this disease

(Singh and Sharma, 2014). A study conducted by Chu, et al. (1998) on 333 isolates of *S. flexneri* obtained from 1986 to 1995 found that resistance to nalidixic acid emerged in 59.6% of the *S. flexneri* isolates during 1994-1995. Besides that, according to Cheasty, et al. (1998), resistance to ampicillin in *S. flexneri*, *Shigella dysenteriae* and *Shigella boydii* has increased to 65% from 42% in Wales and England since 1983. On the other hand, the bacterial resistance to trimethoprim increased from 6% up to 64% (Cheasty, et al., 1998). In order to overcome the problem in treating Shigellosis, different treatment approaches need to be adapted. According to Westwater, et al. (2003), lytic phages may offer an alternative therapeutic approach for combating the bacterial infections as they kill their hosts following amplification inside the host cell.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strain

The SFN6B phage sample and the clinical strain of *Shigella flexneri* used throughout this project were obtained from Dr. Tan Gim Cheong, Faculty of Science, Universiti Tunku Abdul Rahman.

3.2 Reagents, Chemicals and Equipment

The list of reagents and chemicals used throughout this study are listed in Table 3.1.

Table 3.1: List of reagents and chemicals used.

Reagents/ Chemicals	**Manufacturers, Country**
0.1% Coomassie Blue R-250	Bio Basic Canada Inc., Canada
1 Kb DNA ladder	GeneDirex, S.A.
10X Buffer Tango (with BSA)	Thermo Scientific, USA
6X DNA Loading Dye	Thermo Scientific, USA
6X SDS Sample Buffer	Bioland Scientific LLC, USA
Acetic acid glacial	QRec, Malaysia
Acrylamide	Amersham Biosciences, UK

Table 3.1: List of reagents and chemicals used (continued).

Reagents/ Chemicals	**Manufacturers, Country**
Agarose powder	Choice-Care Sdn. Bhd., Malaysia
Ammonium persulfate	Sigma Aldrich, UK
Ammonium sulphate	R & M Marketing, UK
Benchmark protein ladder	Thermo Fisher Scientific, USA
Bis-acrylamide	Amresco, U.S.
Butanol	QReC, Malaysia
Caesium chloride	Bio Basic Canada Inc., Canada
DNase I	Vivantis Technologies Sdn. Bhd., Malaysia
RNaseA	TIANGEN, China
Ethylenediaminetetraacetic acid (EDTA)	QReC, Malaysia
Glycine	Merck, Germany
Hydrochloric acid	VWR Prolabo, Singapore
Luria Bertani agar	Laboratorios Conda, Spain
Luria Bertani broth	Laboratorios Conda, Spain
Lysozyme	Bio Basic Canada Inc., Canada
Methanol	Merck, Germany
Phenol:Chloroform:Isoamyl Alcohol (25:24:1)	Sigma-Aldrich, USA
Polyethylene glycol 8000 (PEG 8000)	Fisher Scientific, United States

Table 3.1: List of reagents and chemicals used (continued).

Reagents/ Chemicals	**Manufacturers, Country**
Restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Xba*I)	BIORON, Germany
Restriction enzyme (*BgI*II)	New England Biolabs, UK
Sodium acetate	Bio Basic Canada Inc., Canada
Sodium chloride	Bio Basic Canada Inc., Canada
Sodium dodecyl sulphate (SDS)	Bio Basic Canada Inc., Canada
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific, USA
Tris	Bio Basic Inc., Malaysia
Viral Nucleic Acid Extraction Kit II	Geneaid, Taiwan

The list of instruments, apparatus and laboratory wares used throughout this study are listed in Table 3.2.

Table 3.2: List of instruments, apparatus and laboratory wares used.

Instruments/Apparatus/Laboratory wares	**Brand/Model, Country**
Autoclave machine	HIRAYAMA, Japan
Cellulose acetate 0.45 μm syringe filter	Thermo Scientific, USA
Centrifuge tubes (15 mL and 50 mL)	AXYGEN Scientific, USA
Gel electrophoresis set	Major Science, Taiwan

Table 3.2: List of instruments, apparatus and laboratory wares used (continued).

Instruments/Apparatus/Laboratory wares	**Brand/ Model, Country**
High speed centrifuge	Sigma 2-16PK Sartorius, Germany
Media bottles	KIMAX, Germany
Microcentrifuge machine	Thermo Scientific, USA
Microcentrifuge tubes (200 µl and 1.5 mL)	AXYGEN Scientific, USA
Microwave oven	SHARP, Japan
Mini-PROTEAN Tetra System	Bio-Rad, USA
Nano-spectrophotometer	Implen, USA
Petri dishes	Labmart, Pakistan
pH meter	Sartorius, Germany
Vortex mixer	Gemmy Industrial Corp., Taiwan
SnakeSkin Pleated Dialysis Tubing	Thermo Scientific, USA
Shaking incubator	Copens Scientific (M) Sdn. Bhd., Malaysia
Spectrophotometer and cuvettes	Biochrom, USA
Syringe (1 ml/cc)	Muzamal Industries Sdn. Bhd., Malaysia
TH-641 polyallomer tube	Thermo Scientific, USA
UV transilluminator	UVP, Canada
Ultracentrifuge machine	Thermo Scientific, USA
Water bath	Memmert, Germany

Table 3.2: List of instruments, apparatus and laboratory wares used (continued).

Instruments/Apparatus/Laboratory wares	**Brand/ Model, Country**
Weighing balance	Sartorius, Germany

3.3 Methodology

3.3.1 Amplification of Bacteriophage

3.3.1.1 Preparation of Luria Bertani Agar

Luria Bertani (LB) agar was prepared in a 1 L media bottle by adding 17.5 g of LB agar powder and topped up with 500 mL of deionized water. The mixture was mixed well and autoclaved. Then, the medium was poured into sterile Petri dishes and allowed to solidify at room temperature. The prepared agar plates were then stored at 4^{o}C.

3.3.1.2 Preparation of Soft Agar/ Top Agar

Soft agar/ top agar was prepared by adding 1.4 g of LB agar into a media bottle and mixed with 40 mL of deionized water. The mixture was mixed well and autoclaved. Prior to usage, the agar was melted using a microwave oven and 3 mL of the agar was aliquot into each test tube and placed in a water bath at 55^{o}C until use.

3.3.1.3 Preparation of LB Broth

For the preparation of overnight cultures, LB broth was prepared in a media bottle by adding 4 g of LB broth powder and topped up with 200 mL of deionized water. The mixture was mixed well and 10 mL was aliquot in several universal bottles, sealed and autoclaved.

In order to amplify the volume of the phage stock, 2 L of LB broth were prepared in four different 1 L conical flasks, each containing 500 mL of broth. Therefore, 10 g of LB broth were added into each conical flasks and topped up with 500 mL of deionized water. The mixture were mixed well, sealed tightly and autoclaved.

3.3.1.4 Obtaining Single Bacterial Colony

Shigella flexneri bacterial stock was streaked on a LB agar and incubated at 37ºC for 16-18 h. Distinct single colonies can be observed after 16-18 h of incubation.

3.3.1.5 Preparation of Overnight *Shigella flexneri* Bacterial Culture

An overnight bacterial culture was prepared by inoculating a single bacterial colony into 10 mL of LB broth that was prepared in universal bottles. The culture was then incubated at 37ºC for 16-18 h with constant agitation at 200 rpm.

In order to grow the bacteriophage in large scale, a total of 100 mL of overnight bacterial culture was needed hence, 10 different universal bottles of overnight bacterial culture were prepared.

3.3.1.6 Preparation of Log-phase Bacteria

Exponentially growing bacterial culture was prepared at a 1:20 ratio, with 1 part of the overnight bacterial culture and 20 parts of fresh LB broth. In order to grow 10 mL of exponentially growing bacterial culture, 500 µL of overnight bacterial culture was added into 10 mL of LB broth and incubated at 37^{o}C with constant agitation at 200 rpm until the exponential phase (OD_{600} = 0.4-0.6) was attained.

3.3.1.7 Phage Enrichment from Small Volume of Phage Stock

Ten millilitres of log phase *Shigella flexneri* (*S. flexneri*) culture was prepared. Then, 500 µL of the phage stock was inoculated into the log phase bacterial culture and incubated in the shaking incubator at 37^{o}C with constant agitation at 200 rpm until total clearance in turbidity was observed. Next, the culture was centrifuged at 8, 603 ×*g* for 30 min at 4^{o}C and the supernatant was obtained.

3.3.1.8 Plaque Assay

Firstly, the soft/ top agar was melted and 3 mL of agar was aliquot into several test tubes and placed in a water bath at 55^{o}C. Next, 10 mL of log phase *S.*

flexneri culture was prepared and the enriched phage supernatant was serially diluted with LB broth. Then, 200 µL of the log phase bacterial culture and 50 µL of the serially diluted, enriched phage supernatant were added into the soft agar and vortexed. The agar was then overlaid on the base agar and it was ensured that the soft agar covered the entire surface of the base agar by gentle swirling. The plaque assay was done for several dilution factors in order to obtain plates with plaque formation. The agar plates were then incubated for 16-18 h at 37ºC. The titre (pfu/mL) of the phage sample was calculated based on formula (1).

$$\frac{\text{pfu}}{\text{ml}} = \frac{\text{Number of plaques}}{\text{dilution factor} \times \text{volume of diluted virus added (mL)}} \qquad (1)$$

3.3.1.9 Phage Amplification

One hundred millilitres of log phase *S. flexneri* cultures were prepared in 10 different universal bottles, each containing 10 mL of the bacterial culture. Then, a single plaque was picked from one of the plates and transferred into the universal bottle containing 10 mL of the log phase bacterial culture. All the other universal bottles were then inoculated with single plaques in the same way. Then, the cultures were placed in the shaking incubator at 37ºC with constant agitation at 200 rpm until total clearance in turbidity was observed. Then, the cultures were centrifuged at 8, 603 ×*g* for 30 min at 4ºC and the supernatant was obtained.

In order to amplify the bacteriophage, 100 mL of overnight culture of *S. flexneri* was grown. Then, the overnight culture was added into 2 L of fresh LB broth and incubated at 37ºC with constant agitation at 200 rpm. When the bacterial culture reached OD_{600} = 0.4-0.6, 100 mL of the phage supernatant obtained earlier was inoculated into the bacterial culture and incubated in the shaking incubator at 37ºC with constant agitation at 200 rpm until clearance in turbidity was observed. Then, the culture was centrifuged at 8, 603 ×*g* for 30 min at 4ºC and the supernatant was obtained.

3.3.2 Precipitation of Phage

3.3.2.1 Preparation of 50 mM Tris-HCl, 150 mM NaCl Tris-Buffered Saline (TBS) (pH 7.6)

For the preparation of TBS, 6.05 g Tris and 8.76 g of sodium chloride (NaCl) were dissolved in 800 mL of deionized water. Then, the pH of the mixture was adjusted to pH 7.6 using 37% hydrochloric acid (HCl). Once the desired pH was achieved, the volume of the mixture was topped up to 1 L and autoclaved before use.

3.3.2.2 Preparation of 20% (w/v) Polyethylene Glycol 8000 (PEG 8000) containing 2.5 M Sodium Chloride (NaCl).

To prepare 500 mL of 20% (w/v) PEG 8000, 100 g of PEG 8000 and 73.05 g of NaCl were dissolved in 400 mL of deionized water. The final volume was then brought up to 500 mL and the mixture was mixed well and autoclaved.

3.3.2.3 Precipitation of Phage with 20% (w/v) PEG 8000

The supernatant obtained from section 3.3.1.9 was transferred into two 2 L conical flasks, each containing 1 L of the supernatant. Next, 20% (w/v) PEG 8000 was added into the conical flasks. The volume of 20% (w/v) PEG 8000 added was 15% (v/v) of the total volume of the supernatant obtained. The mixture was placed in a chiller at 4^{o}C overnight with constant stirring. The next day, the mixture was aliquot into centrifuge tubes and centrifuged at 8, 603 x*g* for 30 min at 4^{o}C. The pellet was collected and washed with 20 mL of TBS. Then, the suspension was aliquot into 1.5 mL microcentrifuge tubes and centrifuged at 15, 000 x*g* for 10 min at 4^{o}C. Then, the supernatant was collected and 20% (w/v) PEG 8000 was added. The volume of 20% (w/v) PEG 8000 added was 15% (v/v) of the total volume of the supernatant. The mixture was then placed in a chiller at 4^{o}C for approximately 2 h with constant stirring. The mixture was then centrifuged at 8, 603 x*g* for 30 min at 4^{o}C. The pellet was then collected and resuspended with 9 mL of TBS. The phage suspension obtained was then stored at 4^{o}C.

3.3.3 Density Gradient Caesium Chloride Ultracentrifugation

3.3.3.1 Preparation of Caesium Chloride Gradients

Four caesium chloride gradients with densities of 1.3 g/ml, 1.4 g/ml, 1.5 g/ml and 1.7 g/ml were prepared separately by adding 2.988 g, 3.988 g, 4.988 g and 6.988 g into 10 mL of TBS, respectively. The mixtures were mixed well and stored at 4^{o}C until use.

3.3.3.2 Preparation of Phage Sample

Prior to the layering procedure, 3 mL of phage sample was prepared for caesium chloride purification by adding 0.446 g of caesium chloride to 3 mL of PEG-precipitated phage sample so that the density of the phage sample was 1.15 g/mL.

3.3.3.3 Layering of the Densities and Phage Sample

Four different gradients, 1.3 g/ml, 1.4 g/ml, 1.5 g/ml and 1.7 g/ml of caesium chloride were prepared separately. Using a needle and a 1 mL syringe, 1.5 mL of 1.7 g/ml caesium chloride gradient was first placed into the TH-641 polyallomer tube. Next, 2 mL of 1.5 g/ml caesium chloride gradient was layered on top of the 1.7 g/ml gradient slowly and carefully. Then, 2.5 mL of 1.4 g/ml gradient was layered followed by 2.5 mL of 1.3 g/ml caesium chloride gradient. After all the caesium chloride gradients have been layered, the prepared phage sample was layered on the top most part of the tube. During the layering procedure, it was ensured that the surface tensions of the flowing caesium chloride gradients were not disrupted so that the different densities were properly established. The tube was then slowly lowered into the bucket using forceps and screwed shut. A total of two samples were prepared this way and it was ensured that the weight of both the samples were similar prior to ultracentrifugation.

3.3.3.4 Ultracentrifugation

The ultracentrifuge machine was first pre-cooled. Once the temperature has dropped to 4^{o}C, the TH-641 rotor was removed from the machine and the buckets containing the samples were fixed onto the rotor. Then, the rotor was placed back into the machine and the button "vacuum" was pressed to create a vacuum environment inside the ultracentrifuge machine. Lastly, the samples were spun at 280, 000 ×g for 4 h.

3.3.3.5 Obtaining the Purified Phage Sample

Once the ultracentrifugation has completed, the ultracentrifuge machine was first de-vacuumed prior to removing the sample. The rotor was removed from the machine and placed onto the holder. The polyallomer tubes were then removed from their respective buckets and clamped to a retort stand. The distinct bands present were collected separately into 1.5 mL microcentrifuge tubes and stored at 4^{o}C.

3.3.4 Phage Nucleic Acid Extraction

3.3.4.1 Preparation of 3 M Sodium Acetate

Three hundred millilitres of 3 M sodium acetate was prepared by adding 73.83 g of sodium acetate powder into 200 mL of deionized water. Once the sodium acetate powder was completely dissolved, the mixture was topped up to 300 mL by adding deionized water.

3.3.4.2 Phage Nucleic Acid Extraction Using Conventional Method

Two hundred microliters of Phenol:Chloroform:Isoamyl Alcohol (PCI) and 200 μL of the PEG-precipitated phage sample (1:1 ratio) were added into a 1.5 mL microcentrifuge tube. The mixture was then centrifuged at 14, 000 ×g at 4ºC for 10 min. Then, the top aqueous layer was collected and placed into a clean 1.5 mL microcentrifuge tube. Cold isopropanol and 3 M sodium acetate were added into the tube at a 1:1 ratio and 1:10 ratio, respectively. The mixture was then allowed to precipitate at room temperature for 20 min and then centrifuged at 14, 000 ×g at 4ºC for 15 min. Then, the supernatant was discarded. The pellet was washed with absolute ethanol and allowed to air dry. Once dried, the pellet was dissolved in 50 μL of autoclaved distilled water and stored at -20ºC.

3.3.4.3 Phage Nucleic Acid Extraction Using Viral Nucleic Acid Extraction Kit II

The PEG-precipitated phage nucleic acid was then extracted using a kit. Firstly, 200 μL of the phage sample and 400 μL of Lysis Buffer were transferred into a 1.5 mL microcentrifuge tube and vortexed. The mixture was incubated for 10 min at room temperature. Then, 450 μL of AD Buffer was added into the mixture and mixed vigorously.

Six hundred microliters of the prepared lysate was transferred into a VB column and centrifuged at 16, 000 ×g for 1 min. The flow-through was then discarded and the remaining 450 μL of the prepared lysate was transferred into

the VB column and centrifuged at 16, 000 ×g for 1 min. The flow-through was discarded together with the collection tube and a new collection tube was fixed to the VB column.

Four hundred microliters of W1 Buffer was added into the VB column and centrifuged at 16, 000 ×g for 30 sec. The flow-through was discarded and 600 µL of Wash Buffer was added into the VB column and centrifuged at 16, 000 ×g for 30 sec. The flow-through was discarded and the VB column was centrifuged at 16, 000 ×g for 3 min to dry the column matrix. The dried column was then placed in a clean 1.5 mL microcentrifuge tube and 50 µL of RNase-free water was added at the centre of the VB column. The tube was allowed to stand for 10 min and then centrifuged at 16, 000 ×g for 1 min. The eluted purified nucleic acid was stored at -20^{o}C.

3.3.4.4 Nucleic Acid Quantification Using Nano-Spectrophotometer

Quantification of the extracted nucleic acids using both the conventional method and kit were done by measuring the concentration and absorbance of the samples at wavelengths 260 nm and 280 nm. The ratio of the readings obtained at 260 nm and 280 nm shows the purity of the phage nucleic acid. The nano-spectrophotometer was first switched on and calibrated. Then, a submicroliter cell was placed into the cell holder in the correct position. The machine was then blanked using 2 µL of autoclaved distilled water. Then, the measurement window was cleaned prior to placing 2 µL of the phage nucleic

acid on it carefully. A cleaned lid of 1 mm of pathlength was placed on the submicroliter cell and the readings were taken.

3.3.5 Partial Genomic Characterization of Phage Nucleic Acid

3.3.5.1 Preparation of 10X Tris-Acetate-EDTA (TAE) Buffer

To prepare 1 L of 10X TAE buffer, 400 mM (48.4 g) of Tris was added into a media bottle and mixed with 11.4 mL of glacial acetic acid. Next, 20 mL of 0.5 M EDTA at pH 8 and 800 mL of deionized water were added into the mixture. The mixture was mixed well and then topped up to 1 L by adding deionized water. The buffer was then autoclaved before use.

3.3.5.2 Preparation of 1X Tris-Acetate-EDTA (TAE) Buffer

Ten times TAE buffer was diluted with deionized water to prepare 1X TAE buffer. Briefly, 100 mL of 10X TAE buffer was mixed with 900 mL of autoclaved deionized water to obtain 1 L of 1X TAE buffer.

3.3.5.3 DNase and RNase Digestion of Phage Nucleic Acid

The phage nucleic acid extracted via the kit was treated with DNase and RNase to determine the type of the phage nucleic acid. For DNase treatment, 2.0 µL of phage nucleic acid was mixed with 0.5 µL of 10X Buffer Tango (with BSA), 0.2 µL of DNase and 2.3 µL of deionized water. As for the RNase treatment, 2.0 µL of phage nucleic acid was treated with 0.7 µL of RNase and

2.3 µL of deionized water. Lastly for the negative control, another 2 µL of phage nucleic acid was mixed with 3 µL of deionized water. All the prepared samples were incubated for approximately 1 h at room temperature.

3.3.5.4 Preparation of 1% (w/v) Agarose Gel

For the preparation of 1% (w/v) agarose gel, 0.3 g of agarose powder was mixed with 30 mL of 1X TAE buffer in a 100 mL conical flask. The mixture was then heated in a microwave oven for 1-3 min until the agarose powder was completely dissolved. The mixture was first allowed to cool slightly and then poured into the casting tray of the electrophoresis set. The comb was then lowered slowly on to the gel and after securing the position of the comb, the gel was allowed to solidify.

3.3.5.5 Agarose Gel Electrophoresis of DNase and RNase Digested Phage Nucleic Acid

Once the 1% (w/v) agarose gel has solidified and the wells were perfectly formed, the gel was placed in a gel tank and filled with 1X TAE buffer. Two microliters of 1 Kb DNA ladder was loaded into the first well. Next, 5 µL of the treated samples were mixed separately with 1 µL of 6X DNA loading dye and loaded into respective wells. Then, the gel was subjected to electrophoresis at 80 V for 45 min or until the dye front reached the bottom of the gel. The gel was then stained with 0.1 mg/ml of ethidium bromide for approximately 20 min, then de-stained with water for a few seconds. The gel was then viewed to visualize the bands obtained using a UV transilluminator.

3.3.5.6 Restriction Digestion of Phage Nucleic Acid

Six restriction enzymes; *Bam*HI, *BgI*II, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I were used to digest the phage nucleic acid. The reaction mixtures were prepared based on Table 3.3.

Table 3.3: Restriction enzyme digestion reaction mixtures

Components	**Volume (µL)**
Phage nucleic acid	5.0
Restriction enzyme	0.5
Restriction enzyme buffer	1.0
PCR water	3.5
Total	10.0

The reaction mixtures were then incubated at 37^{o}C for 3 h. Then, the reaction mixtures were inactivated by incubating in the water bath at temperatures as shown in Table 3.4.

Table 3.4: Heat inactivation of restriction enzyme mixtures (Adapted from New England Biolabs Inc., 2015).

Restriction enzyme	**Temperature (ºC)**	**Time (min)**
*Bam*HI	-	-
*BglI*I	-	-
*Eco*RI	65	20
*Eco*RV	80	20
*Hind*III	80	20
*Xba*I	65	20

(-) no heat inactivation required.

After heat inactivation, agarose gel electrophoresis was carried out. The prepared agarose gel was placed in the gel tank and filled with 1X TAE buffer. The first well was loaded with 2 µL of 1 Kb DNA ladder. Next, 1 µL of 6X DNA loading dye were separately mixed with each of the heat inactivated sample and loaded into respective wells. The gel was then subjected to electrophoresis at 80 V for 45 min or until the dye front reached the bottom of the gel. Then, the gel was stained with ethidium bromide for approximately 20 min and de-stained with water for a few seconds. The gel was then visualized using the UV transilluminator.

3.3.6 Ammonium Sulphate Precipitation

3.3.6.1 Extraction of Protein Precipitates

Ten millilitres of *Shigella flexneri* overnight culture was grown and inoculated into 200 mL of fresh LB broth. The culture was incubated at 37^{o}C with constant agitation at 200 rpm. When the bacterial culture reached OD_{600} = 0.4-0.6, the bacterial culture was infected with 2 mL of PEG-precipitated phage sample and incubated at 37^{o}C with constant agitation at 200 rpm. The absorbance at OD_{600} was measured every 15 min until total clearance in turbidity was observed. Then, the culture was centrifuged at 8, 603 ×*g* for 30 min at 4^{o}C and the supernatant was obtained for ammonium sulphate precipitation. On the other hand, the pellet was dissolved in 100 µL of TBS and stored at 4^{o}C for further analysis.

Two hundred millilitres of supernatant obtained was transferred into a sterile 1 L conical flask. Solid ammonium sulphate was then added in increasing percentage of saturation to precipitate the proteins present in the lysate. The addition of ammonium sulphate was carried out on ice. To obtain 0-20% saturation, 21.98 g of ammonium sulphate was slowly added into the 200 mL lysate with continuous stirring. The suspension was then placed in a chiller at 4^{o}C with continuous stirring for 2 h. Then, the suspension was centrifuged at 12, 000 ×*g* for 30 min at 4^{o}C. The supernatant was poured back into the conical flask and the pellet obtained was resuspended with 1 mL of TBS.

To obtain 20-40% saturation, 24.51 g of solid ammonium sulphate was slowly added into the supernatant with continuous stirring. The suspension was then placed in the chiller with continuous stirring for 2 h at 4^{o}C. Then, the suspension was centrifuged at 12, 000 ×g for 30 min at 4^{o}C. The supernatant obtained was transferred back into the conical flask and the pellet obtained was resuspended with 1.5 mL of TBS.

Next, in order to obtain 40-60% saturation, 27.37 g of solid ammonium sulphate was added slowly into the supernatant collected while stirring continuously. The suspension was then placed in the chiller with continuous stirring for 2 h at 4^{o}C. Then, the suspension was centrifuged at 12, 000 ×g for 30 min at 4^{o}C. The supernatant obtained was transferred back into the conical flask and the pellet obtained was resuspended with 7 mL of TBS.

Lastly, 30.64 g of solid ammonium sulphate was slowly added into the supernatant while stirring continuously to obtain 60-80% saturation. The suspension was then placed in the chiller with continuous stirring for 2 h at 4^{o}C. Then, the suspension was centrifuged at 12, 000 ×g for 30 min at 4^{o}C. The supernatant obtained was transferred back into the conical flask and the pellet obtained was resuspended with 9 mL of TBS.

3.3.6.2 Dialysis

The protein precipitates obtained were dialyzed overnight against TBS at 4^{o}C using the SnakeSkin Pleated Dialysis Tubing with a molecular weight cut off (MWCO) of 10, 000. The dialyzed protein precipitates obtained were stored at 4^{o}C.

3.3.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.7.1 Preparation of 10% Sodium Dodecyl Sulphate (SDS)

For the preparation of 100 mL of 10% SDS, 10 g of SDS was mixed with 100 mL of deionized water. Once prepared, the solution was stored at room temperature.

3.3.7.2 Preparation of 10% Ammonium Persulfate (APS) Stock Solution

To prepare 1 mL of 10% APS stock solution, 100 mg of ammonium persulfate was dissolved in 1 mL of deionized water and stored at -20^{o}C until use.

3.3.7.3 Preparation of 4X Lower Buffer for Resolving Gel (pH 8.8)

To prepare 100 mL of 4X lower buffer, 1.5 M of Tris-base and 4 mL of 10% SDS were added into 60 mL of deionized water and mixed well. The pH of the mixture was then adjusted to pH 8.8 using 37% hydrochloric acid. Then, the mixture was topped up to 100 mL with deionized water and mixed well. The

mixture was then filtered using a 0.45 μm cellulose acetate syringe filter and stored at 4^{o}C until use.

3.3.7.4 Preparation of 4X Upper Buffer for Stacking Gel (pH 6.8)

To prepare 100 mL of 4X upper buffer, 0.5 M of Tris-base and 4 mL of 10% SDS were added into 60 mL of deionized water and mixed well. The pH of the mixture was then adjusted to pH 6.8 using 37% hydrochloric acid. Then, the mixture was topped up to 100 mL with deionized water and mixed well. The mixture was then filtered using a 0.45 μm cellulose acetate syringe filter and stored at 4^{o}C until use.

3.3.7.5 Preparation of 1X Running Buffer

To prepare 1000 mL of 1X running buffer, 25 mM (3 g) of Tris-base, 10 mL of 10% SDS and 14.4 g of glycine were added into 600 mL of deionized water. The mixture was mixed well and then topped up to 1000 mL with deionized water and mixed well.

3.3.7.6 Preparation of Destaining Solution

To prepare 1000 mL of destaining solution, 10% acetic acid and 40% methanol were added into a Schott bottle. The mixture was then topped up to 1000 mL with deionized water and mixed well.

3.3.7.7 Casting of Gels and Sample Electrophoresis

The Mini-PROTEAN Tetra System was used for running SDS-PAGE. Firstly, the glass plates were set vertically on to the casting frame and fixed to the casting stand. After making sure that the apparatus was not leaking, the resolving gel was first prepared. The components and the volumes required for preparing the resolving gel are shown in Table 3.5.

Table 3.5 Volume of components used in the preparation of resolving gel.

Components	**Volume (µL/gel)**
Autoclaved distilled water	1243
4X Lower buffer (pH8.8) [1.5 M Tris-base, 0.4% SDS]	1243
30% Bis/acrylamide (30% T, 2.67% C)	2479
10% APS	31
TEMED	5

The resolving gel was then pipetted slowly into the assembled SDS-PAGE cast and allowed to set. Butanol was layered on top of the resolving gel to prevent any air bubbles present. After the resolving gel has solidified, the butanol was poured out and the stacking gel was prepared. The components and the volumes required for preparing the stacking gel are shown in Table 3.6.

Table 3.6 Volume of components used in the preparation of stacking gel.

Components	**Volume (µL/gel)**
Autoclaved distilled water	1159.0
4X Upper buffer (pH6.8) [0.5 M Tris-base, 0.4% SDS]	496.0
30% Bis/acrylamide (30% T, 2.67% C)	332.0
10% APS	13.2
TEMED	2.8

The stacking gel was then pipetted slowly on top of the solidified resolving gel and allowed to solidify upon inserting the comb. Once the gel has solidified, the gel plates were removed from the casting apparatus and fixed to the cassette with the short plate facing inwards. The assembled apparatus was then placed inside the gel tank and the inner chamber was filled with 1X running buffer. The comb was then removed carefully without breaking the wells.

The samples that were subjected to SDS-PAGE were the overnight bacterial culture, PEG-precipitated phage sample, the lysate and pellet obtained after centrifugation of the phage-infected bacterial culture, prior to ammonium sulphate addition, and the four protein precipitates obtained at 20% to 80% ammonium sulphate saturation, respectively. Twenty microliters of the samples were aliquot into separate PCR tubes and mixed with 4 µL of 6X SDS sample buffer. The sample mixtures were then boiled for 10 min. Five microliters of protein ladder was loaded into the first well carefully. Then, 20

μL of the prepared samples were loaded into subsequent wells. Next, the inner chamber was topped up with 1X running buffer and the outer chamber was also filled with 1X running buffer so that the bottom of the gel was submerged with the buffer. The gel was then electrophoresed at 200 V and 16 mA per gel for 70 min or until the dye front ran off. Then, the gel was removed from the cast and stained in the staining solution (0.1% Coomassie Blue R-250, 40% methanol, 1% acetic acid) for 10 min. The gel was then de-stained with destaining solution (10% acetic acid, 40% methanol) for 20 min before the bands were visualized.

3.3.8 Lytic Activity Assay

3.3.8.1 Preparation of 0.02 M Tris-HCl

Three hundred millilitres of 0.02 M Tris-HCl was prepared by dissolving 0.946 g of Tris-HCl in 200 mL of deionized water. The mixture was thoroughly mixed and the pH was adjusted to pH 8.0 using 1 M sodium hydroxide (NaOH). The volume of the mixture was then topped up to 300 mL. The mixture was mixed well and autoclaved.

3.3.8.2 Preparation of 0.02 M Tris-HCl Containing 0.1 M EDTA

Three hundred millilitres of 0.02 M Tris-HCl containing 0.1 M EDTA was prepared by dissolving 0.946 g of Tris-HCl and 11.405 g of EDTA into 200 mL of deionized water. The mixture was mixed well and the pH was adjusted to pH 8.0 using 37% hydrochloric acid. Then, the mixture was topped up to

300 mL by adding deionized water. The mixture was mixed well and autoclaved.

3.3.8.3 Preparation and Lawning of Bacterial Culture

Ten millilitres of log phase bacterial culture was prepared. When the bacterial culture reached OD_{600} = 0.4-0.6, the culture was transferred into two 1.5 mL microcentrifuge tubes (labelled A and B), each containing 1 mL of culture. The cultures were then centrifuged at 10, 000 ×g for 1 min and the supernatant was discarded. The resulted bacterial pellet in microcentrifuge tube A was resuspended with 1 mL of 0.02 M Tris-HCl (pH 8.0). The suspension was then used to lawn two agar plates. The resulted bacterial pellet in microcentrifuge tube B was resuspended with 0.02 M Tris-HCl with 0.1 M EDTA (pH 8.0). The mixture was allowed to stand for 5 min and then centrifuged at 10, 000 ×g for 1 min. The resulted pellet was then resuspended with 1 mL of 0.02 M Tris-HCl (pH 8.0). The suspension was then used to lawn two agar plates.

3.3.8.4 Lytic Activity Assay of Protein Precipitates

The agar plates lawned were air dried before performing the lytic activity assay. TBS was the negative control in this study whereas, different concentrations of lysozymes (25 µg/ml, 50 µg/ml and 100 µg/ml) acted as the positive controls. All the agar plates were divided into four quadrants. Each quadrant of the first agar plate lawned with the bacterial culture from microcentrifuge tube A was spotted respectively with TBS, 25 µg/ml, 50 µg/ml

and 100 µg/ml of lysozyme. The second agar plate was spotted with the four protein fractions obtained from ammonium sulphate precipitation.

Each quadrant of the first agar plate lawned with the bacterial culture from microcentrifuge tube B was spotted with TBS, 25 µg/ml, 50 µg/ml and 100 µg/ml of lysozyme, respectively. Apart from performing the negative and positive controls of this study, this step also enabled the determination of the effectiveness of 0.1 M EDTA as a permeabilizing agent. The second agar plate was spotted with the four protein fractions obtained from ammonium sulphate precipitation. The plates were then incubated at 37ºC for 16-18 h. The next day, the plates were observed for clear zones which may indicate the presence of endolysin.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Amplification of Bacteriophage

This study began with the enrichment and amplification of the bacteriophage SFN6B against *Shigella flexneri (S. flexneri)* followed by double precipitation of the phage with polyethylene glycol (PEG) in order to increase the titre of the phage. Plaque assays were then performed over several dilution factors and the respective phage titres were calculated based on formula (1). The average phage titre obtained after double precipitation with PEG was 3.66×10^{11} pfu/mL.

According to Boulanger (2009), harvesting of lytic phage progeny is performed after they have been released from their host cells following cell lysis. Apart from the phage themselves, the other major components that are normally present in the crude phage lysate are bacterial debris with ribosomes, bacterial proteins and nucleic acids. The debris also includes lipopolysaccharides (LPS) and endotoxins in the lysates from Gram-negative bacteria. In order to obtain a highly purified phage sample, all these contaminants need to be removed (Boulanger, 2009). According to Yamamoto, et al. (1970), by adding the PEG, phages may be readily concentrated from crude phage culture of infected bacteria. PEGs are non-ionic, water-soluble

polymers with a wide range of different molecular weights (Vajda, 1978). Even though different phages need relatively different concentrations of PEG to attain maximal degree of pelleting, PEG concentrations of 10% or higher enables the recovery of at least 90% of the infective titre of phages in the pellet (Yamamoto, et al., 1970). Precipitation of phages using chemicals such as PEG is relatively simple and renders it easy to be concentrated. However, some phages may lose infectivity through this process as polymers may co-precipitate along with the phage particles. Hence, two different purification methods can be performed sequentially to overcome the problem (Hutornojs et al., 2012). In this study, the SFN6B phage infectivity was not lost, as the lytic activity of the phage was still clearly detectable in plaque assays performed after PEG precipitation.

4.2 Caesium Chloride Purification

The PEG-precipitated phage sample was subjected to density gradient caesium chloride ultracentrifugation to determine the effectiveness of caesium chloride ultracentrifugation in the purification of phage particles and to determine the buoyant density of the SFN6B phage in caesium chloride gradients. Four different gradients; 1.3 g/ml, 1.4 g/ml, 1.5 g/ml and 1.7 g/ml were used. Figure 4.1 shows the presence of two distinct bands after the ultracentrifugation step was carried out. Both the bands were extracted to determine the presence of phage and the respective phage titres.

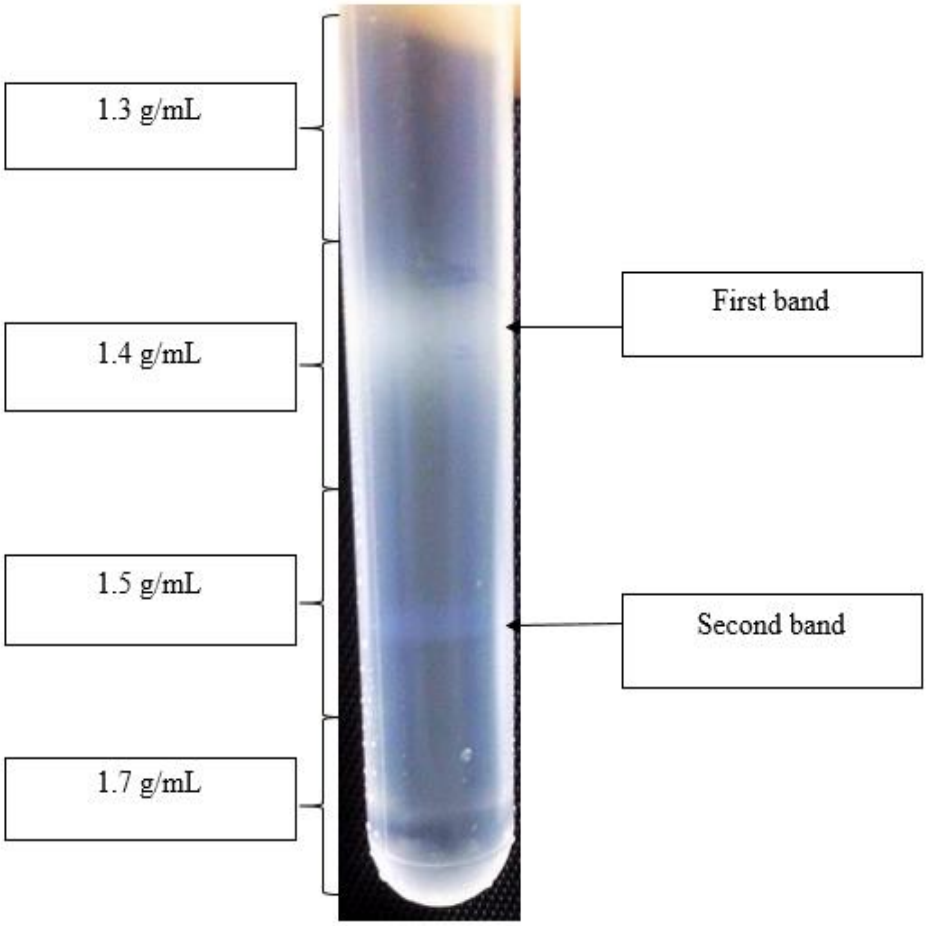

Figure 4.1: Image showing the results of density gradient caesium chloride purification after ultracentrifugation. The approximate densities of the first and second bands were 1.4 g/mL and 1.5 g/mL, respectively.

The first band was white in colour and present at a density of about 1.4 g/mL. When plaque assay was performed for the band, no phage was detected as there were no lyses observed in any of the plates. A study conducted by Osborn, Weiner and Weber (1970) emphasized that phage particles may be contaminated with bacterial proteins and the increasing caesium chloride gradients will allow the sedimentation of the phage particles together with the contaminating proteins. The presence of high salt (CsCl) aids in the removal of any protein contaminants that are bound to the phage particles as the protein contaminants with a density less than that of the phage particles would be

stripped away from the sedimenting phage (Osborn, Weiner and Weber, 1970). Besides that, according to a study conducted by Bachrach and Friedmann (1971), phage ghosts and bacterial debris can also be recovered from zones of different densities. Hence, it was concluded that the first white band contained contaminating proteins, phage ghosts and bacterial debris due to the absence of phage lytic activity.

On the other hand, the second band was bluish in colour and was present at a density of about 1.5 g/mL with an average phage titre of 7.80×10^{13} pfu/mL. The average phage titre of the second band obtained after caesium chloride ultracentrifugation was approximately 200 times higher than the average phage titre obtained after PEG precipitation. Sufficient gravitational force provided by the ultracentrifugation leads to efficient sedimentation even for the smallest viral particles. Ultracentrifugation of viral particles in density gradients also allows both the purification and concentration of the viral particles (Lawrence and Steward, 2010). Moreover, density gradient ultracentrifugation yields a greater degree of purification of the phage sample as the phage particles can be separated from other contaminants by sedimentation through different gradients of caesium chloride solutions as observed in this study (Hutornojs, et al., 2012).

When comparison was made between the bacteriophage R17 specific to *Escherichia coli (E.coli)* S26 or D10 as reported by Osborn, Weiner and Weber (1970) and the SFN6B phage in this study, the density obtained for both

phages was almost similar. Both the phages banded at approximately 1.5 g/mL. According to Goodridge (2013), the similarities between *E. coli* and *Shigella spp.* provide intriguing possibilities in analysing the relationship between phage particles that infect both species. Multiple reports of phages that infect *Shigella spp.* and *E. coli* are present (Goodridge, 2013). One of these reports includes a study conducted by Goodridge, Gallaccio and Griffiths (2003), whereby it was reported that two coliphages, LG1 and AR1 were able to lyse *Shigella dysenteriae.* This report provided insights on the nature of somatic antigens on the surface of the bacteria. *Shigella spp.* and *E. coli* are also very closely related in terms of similarities in genetic characteristics and physico-chemical properties (Goodridge, 2013). Besides that, according to Murphy, et al. (1995), the buoyant density of coliphage-T7 was 1.50 g/mL, which is approximately similar to the phage SFN6B against *S. flexneri* in this study. Although coliphage-T7 was not shown to cause the lysis of *S. flexneri*, it was shown to cause lysis of *S. sonnei* D(2) which is of the same genus as *S. flexneri* with an average burst size of 0.1 phage per cell (Hausmann, Gomez and Moody, 1969).

4.3 Partial Genomic Characterization

4.3.1 Phage Nucleic Acid Extraction

The PEG-precipitated phage sample was subjected to nucleic acid extraction using both the conventional method and Viral Nucleic Acid Extraction Kit II. Subsequently, the concentration and purity of the extracted nucleic acids were determined using the Nano-spectrophotometer. The purity was determined by

the ratio of the readings obtained at 260 nm and 280 nm. The concentration and purity of the phage nucleic acid extracted via the conventional method were 6.000 ng/µL and 1.463, respectively. On the other hand, the concentration and purity of the nucleic acid extracted using the kit were 0.300 ng/µL and 3.000, respectively.

The conventional method of extracting phage nucleic acid yielded a relatively better concentration of nucleic acid, however, the nucleic acid obtained via the kit seemed to be purer. According to Desjardins and Conklin (2010), pure nucleic acids normally yield A260/280 ratio of approximately 1.8 and 2.0 for DNA and RNA, respectively. The presence of phenol, protein or other contaminants may yield significantly different purity ratios due to strong absorptions near or at 280 nm. The nucleic acid extracted using the conventional method had a purity that was much lower than that of the nucleic acid extracted using the kit. This may be due to the phenol component present in the phenol:chloroform:isoamyl alcohol used in the first step of the conventional nucleic acid extraction method. Further optimization may be required for the nucleic acid isolation technique if the purity ratios yield is significantly lower than the expected value (Desjardins and Conklin, 2010). Therefore, the phage nucleic acid extracted using the kit was used for further analysis due to the higher purity.

4.3.2 DNase and RNase Digestion of Phage Nucleic Acid

The size of the SFN6B phage nucleic acid extracted using the kit was estimated via agarose gel electrophoresis using a 1 Kb DNA ladder. Besides that, the nucleic acid was also subjected to digestion by DNase and RNase to determine the nature of the nucleic acid. If the nucleic acid was digested by DNase, no bands would be observed after gel electrophoresis. Likewise, if the phage nucleic acid was of RNA genome, the nucleic acid would be digested by the RNase and no bands would be observed after the gel electrophoresis. Figure 4.2 shows the gel image obtained after agarose gel electrophoresis of the digested nucleic acid.

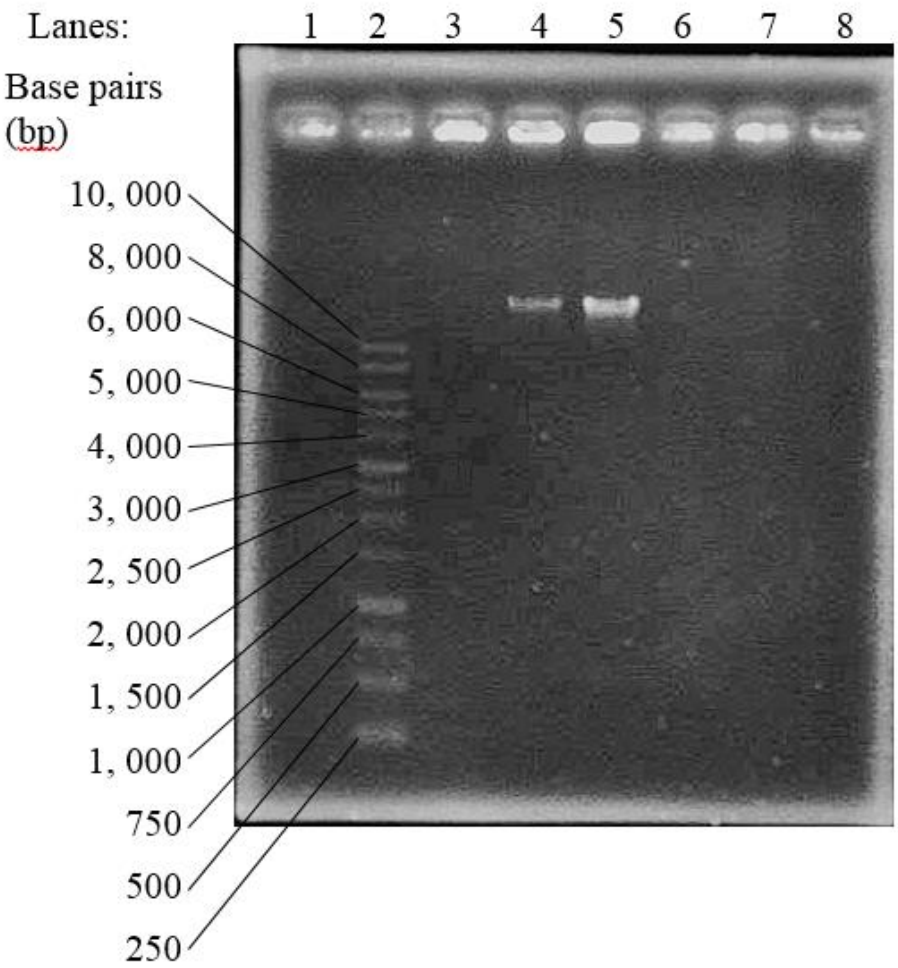

Figure 4.2: Gel image of the digested phage nucleic acid after being subjected to digestion by DNase and RNase. Lane 2: 1 kb DNA Ladder; Lane 3: Phage nucleic acid digested with DNase; Lane 4: Phage nucleic acid digested with RNase; Lane 5: Phage nucleic acid without any digestion that act as negative control.

No band was present in Lane 3 that was loaded with the phage nucleic acid digested with DNase. Lanes 4 and 5 that was loaded with phage nucleic acid digested with RNase and nucleic acid without any digestion, respectively showed the presence of single bands. Therefore, the nature of the SFN6B phage nucleic acid was determined to be DNA because the digestion of the nucleic acid by the DNase resulted in the absence of the band in the gel. Since the SFN6B phage used in this study was a lytic phage, the genome of this

phage was compared with the genomes of other lytic phages. Based on a study conducted by Tremblay and Moineau (1999), a lytic phage named DT1 that targets *Streptococcus thermophiluslytic* isolated from a mozzarella whey has a linear double-stranded DNA genome with the presence of cohesive ends at its extremities. Besides that, a T3-related lytic phage of *Yersinia enterocolitica* serotype O:3 also has a double-stranded DNA genome (Pajunen, et al., 2001). These studies showed that the lytic phages studied were of DNA genome, similar to the lytic phage in this study, SFN6B which targets *S. flexneri*.

The results also showed that the size of the phage genome was more than 10, 000 base pairs (bp). Although the exact size of the SFN6B phage genome in this study was unable to be determined, comparisons can be made to see the similarities and differences of the phage genome in this study with phages of different sources reported in other studies. To date, all known *S. flexneri* phages are lambdoid phages (Jakhetia and Verma, 2015). A study conducted by Jakhetia and Verma (2015) revealed that the genome of phage SfMu that targets *S. flexneri* composed of 37, 146 bp. Besides that, a study conducted by Jakhetia, Talukder and Verma (2013) on a novel serotype converting temperate bacteriophage SfIV targeting *S. flexneri* revealed that the size of the phage genome was 39, 758 bp. Another study conducted by Casjens, et al. (2004) revealed that the genome size of the temperate bacteriophage Sf6 that targets *S. flexneri* is 39, 044 bp. Complete sequencing of the phage genome revealed that Sf6 has a highly mosaic genome. All these studies showed that the genome size of the phages against *S. flexneri* were larger than 10, 000 bp. However, the phages used in these studies were temperate phages but not lytic phages.

Since the SFN6B phage used in this study is a lytic phage, comparison of the genome size with other lytic phages were made. The genome of a lytic virus, cyanophage P60 which infects marine *Synechococcus* WH7803 was completely sequenced and it was found that the genome composed of 47, 872 bp (Chen and Lu, 2002). On the other hand, according to Kim, et al. (2010), pyrosequencing the genome of a virus called SP18 that shows morphology characteristic of the *Myoviridae* family revealed a 170 kb length sequence. However, this phage was lytic towards *Shigella sonnei* but not to *S. boydii*, *S. flexneri* or the members of the genera *Salmonella* and *Escherichia* (Kim, et al., 2010). These studies showed that the size of the different lytic phages studied were also above 10, 000 bp.

4.3.3 Restriction Digestion of Phage DNA

The phage genome was then digested using six different restriction enzymes to identify those that are able to digest the phage DNA and subsequently produce fragments (Kutter and Sulakvelidze, 2004). The principle biological function of restriction endonucleases is to protect the host genome against foreign DNA, particularly bacteriophage DNA. Therefore, the substrate of restriction endonucleases is foreign DNA (Pingoud and Jeltsch, 2001). Restriction endonucleases present in the cells not only destroy the invading phage DNA, but also the DNA transferred via transduction, conjugation and transformation (Kasarjian, Iida and Ryu, 2003). Restriction endonucleases occur ubiquitously among prokaryotic organisms and they are parts of the restriction-modification

(RM) systems which comprise of a methyltransferase activity and an endonuclease (Pingoud and Jeltsch, 2001).

Restriction enzymes cleave specific sequence called the recognition sequence at specific sites and the length of the recognition sequence varies. Some restriction enzymes have a recognition sequence of only 4 bp whereas others may recognize 6 bps and 8 bp sequence of DNA (Kumar and Garg, 2005). According to Skiena (2001), restriction endonucleases cut double-stranded DNA. Hence, it can be further confirmed that the phage used in this study is a DNA phage. Figure 4.3 shows the gel image after the digested nucleic acids were electrophoresed on an agarose gel.

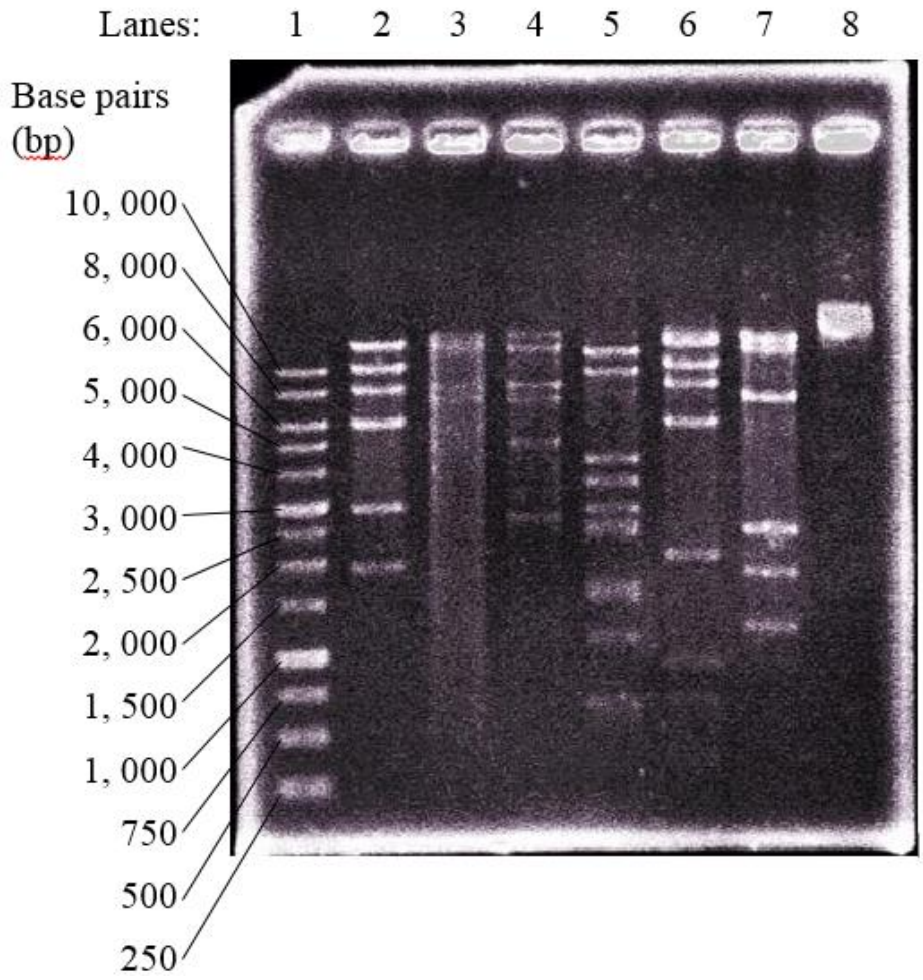

Figure 4.3: Gel image of digested phage nucleic acid after digestion with different restriction enzymes. Lane 1: 1 kb DNA Ladder; Lane 2: Phage nucleic acid digested with *Bam*HI; Lane 3: Phage nucleic acid digested with *Bgl*II; Lane 4: Phage nucleic acid digested with *Eco*RI; Lane 5: Phage nucleic acid digested with *Eco*RV; Lane 6: Phage nucleic acid digested with *Hind*III; Lane 7: Phage nucleic acid digested with *Xba*I; Lane 8: Undigested phage nucleic acid that acts as negative control.

Six different bands can be observed in Lane 2 and all the bands present were above 2, 000 bp. The first band in Lane 2 was observed to be above 10, 000 bps. Only four faint bands can be observed in Lane 3 containing DNA fragments after digestion by *Bgl*II. The first and second bands were present above 10, 000 bps and the third band was observed at 8, 000 bps. The presence of smear can be clearly detected in Lanes 3 and 7. According to Promega

(2015), for an efficient digestion by restriction endonuclease, a highly purified DNA is required. Contaminants that are commonly used in the purification steps such as phenol, protein, sodium chloride (NaCl) and caesium chloride may interfere with the performance of restriction endonuclease if they are not properly removed before digestion. Moreover, the presence of nuclease activity may result in degradation and loss of the substrate DNA which is indicated by the presence of smears as seen upon electrophoresis. Smear of DNA can be observed from a point at the estimated size of the substrate DNA to the bottom of the gel due to incomplete digestion (Promega, 2015). The SFN6B phage sample used in the present study from which the nucleic acid was extracted from was precipitated with PEG in the presence of 2.5 M sodium chloride. The high concentration of sodium chloride present during the precipitation of phage particles may interfere with the performance of restriction enzyme. As observed in Figure 4.3, smears were seen to be present in Lanes 3 and 7. In Lane 3, the smear was observed from the top to the bottom of the gel. These smears may be due to incomplete digestion caused by the presence of nuclease activity.

Six faint bands can be observed with *Eco*RI digestion and all the bands were above 2, 500 bp. A study by Jamal, et al. (2015) showed that *Eco*RI digestion of *S. dysenteriae* produced three bands of different sizes. Even though the phage used in this study and the one by Jamal, et al. (2015) targeted the bacteria of the same genus, restriction analysis revealed that both the phages were different in terms of the number of restriction sites. On the other hand, digestion with *Eco*RV yielded the most DNA fragments ranging from

approximately 500 bp to 10, 000 bp as shown in Lane 5. According to Kumar and Garg (2005), the length of the recognition sequence is a measure of how frequently the enzyme will cleave the DNA. The greater the number of occurrences of the given endonuclease sequence on the phage genome or the number of restriction sites, the more vulnerable they are to the restriction endonuclease (Skiena, 2001). Hence, the endonuclease sequence of *Eco*RV occurred more frequently in the SFN6B phage genome compared to the endonuclease sequence of the other restriction enzymes, as observed in Lane 5. However, in another study conducted by Goodridge, Gallaccio and Griffiths (2003), it was shown that *Eco*RV was able to digest the genome of phage LG1 completely. The coliphage LG1 is capable to induce lysis in *S. dysenteriae*, a bacteria of the same genus as *S. flexneri*.

Two very faint bands can be observed in Lane 6. These bands were present at approximately 750 to 1, 000 bps. In addition to that, five other thicker bands can be observed in Lane 6, which were above 2, 000 bp. On the other hand, restriction digestion with *Xba*I yielded five distinct, bright bands as observed in Lane 7. Lane 8 shows the phage DNA without any digestion and this acted as the negative control in this study.

All the restriction endonucleases used in this study are type II restriction endonucleases. They cleave specifically close to or within their recognition sites and do not require the presence of ATP for their nucleolytic activity (Pingoud and Jeltsch, 2001). Type II restriction endonucleases recognize

continuous sequences of 4-8 bps continuously on DNA. However, some may recognize discontinuous sites where a defined length of nonspecific DNA interrupts the specific sequence (Gormley, Bath and Halford, 2000). Type II restriction endonucleases were used in this study because they are commonly used for cloning and DNA analysis (Pingoud, Wilson and Wende, 2014). Besides that, type II restriction endonucleases are able to recognize their respective substrate sequences so accurately that they seem to be attractive subjects for the study of the mechanism of recognition (Pingoud, Wilson and Wende, 2014).

4.4 Ammonium Sulphate Precipitation

4.4.1 SDS-PAGE

According to Grodzki and Berenstein (2010), "salting out" proteins from solutions is where the principle of ammonium sulphate precipitation lies. The proteins present in the solution are prevented from forming hydrogen bonds with water and the salt (ammonium sulphate) added facilitates their interaction with each other leading to the formation of aggregates. These aggregates are then precipitated out of the solution. In this study, proteins precipitated at four different percentage of ammonium sulphate saturations were obtained and subjected to SDS-PAGE along with the overnight bacterial culture and PEG-precipitated phage sample. Besides that, the bacterial pellet obtained after centrifugation of the phage-infected bacterial culture and the lysate obtained after centrifugation of the phage-infected bacterial culture, prior to ammonium

sulphate addition were also subjected to SDS-PAGE. Figure 4.4 shows the resulted gel image.

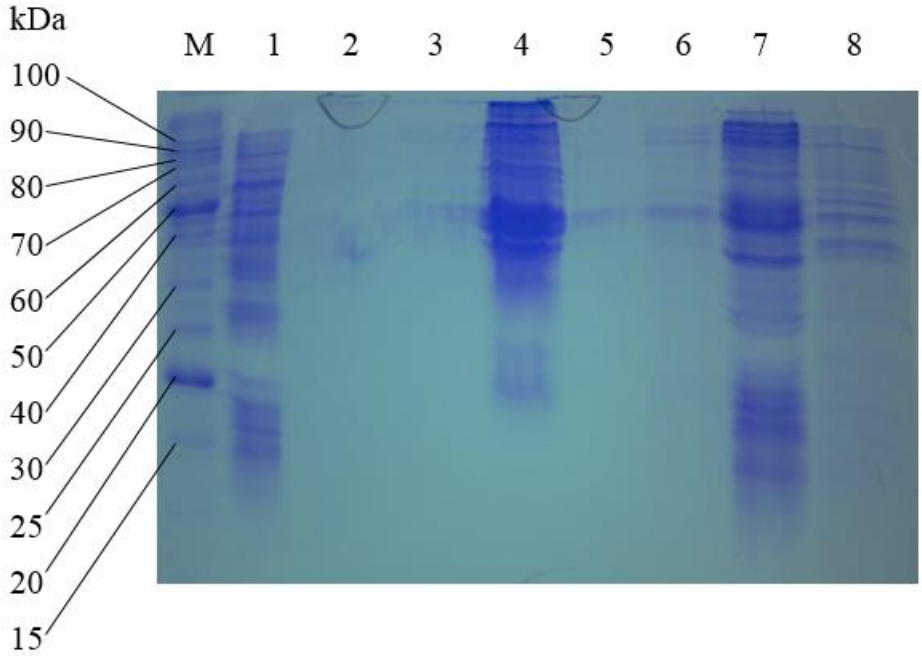

Figure 4.4: SDS-PAGE gel image. M: Protein ladder; Lane 1: Overnight bacterial culture; Lane 2: PEG-precipitated phage sample; Lane 3: Lysate prior to ammonium sulphate addition; Lane 4: Bacterial pellet prior to ammonium sulphate addition; Lane 5: Protein precipitate obtained at 0-20% saturation; Lane 6: Protein precipitate obtained at 20-40% saturation; Lane 7: Protein precipitate obtained at 40-60% saturation; Lane 8: Protein precipitate obtained at 60-80% saturation.

The protein profile of the overnight bacterial culture indicated in Lane 1 showed more bands compared to the PEG-precipitated phage sample in Lane 2. Lanes 2 and 3 which were loaded with the PEG-precipitated phage sample and cell lysate obtained after centrifugation of the phage-infected bacterial culture, prior to ammonium sulphate addition, respectively yielded very faint bands. The faint bands were almost undetectable and this may be due to the low protein concentrations present in the samples. The PEG-precipitated phage

sample was subjected to SDS-PAGE because this technique enables the determination of the nature of the phage proteins' molecular weights. However, SDS-PAGE could not detect active lytic cycles (Elshayeb, et al., 2011). Hence, the comparison of protein profiles between the overnight bacterial culture and the phage sample to that of the protein precipitates obtained after ammonium sulphate precipitation may allow the detection of an extra band. This extra band may indicate the endolysin, as endolysin is only produced at the end stage of the lytic replication cycle. On the other hand, Lane 4 that was loaded with the bacterial pellet obtained after centrifugation of the phage-infected culture, before addition of ammonium sulphate showed the presence of many thick bands that were poorly resolved. This may be due to the very high protein concentrations present in the protein precipitate.

When the protein profiles of the precipitates obtained at different percentage of ammonium sulphate saturations were compared, the proteins precipitated at 0-20% saturation showed the faintest and the least number of protein bands. This indicates that low concentrations of proteins were present in this precipitate. On the other hand, the proteins precipitated at 40-60% saturation showed the presence of more bands that were thicker. This showed that higher concentrations of proteins were precipitated at 40-60% saturation of ammonium sulphate compared to the other three percentages of saturations. According to Nybo (2009), small proteins will be precipitated at higher concentrations of ammonium sulphate whereas bigger proteins will be precipitated earlier in the ammonium sulphate precipitation step whereby, lower percentages of saturations of the salt are used. Therefore, the proteins

precipitated at 40-60% of ammonium sulphate saturation were low molecular weight proteins. Besides that, according to Nelson, et al. (2012), most Gram-negative endolysins have a molecular weight of 15 to 20 kDa. Since low molecular weight proteins precipitated at 40-60% saturation of ammonium sulphate, there is a high chance that the SFN6B phage endolysin is present in proteins precipitated at 40-60% saturation.

Ammonium sulphate precipitation was used in this study as the technique to precipitate the endolysin because several other studies implemented the same method to obtain endolysin from the crude lysate. For instance, a study conducted by Rawat and Mishra (2010) used the ammonium sulphate precipitation technique followed by high speed centrifugation in order to eliminate the bacterial cell population from the crude lysate. Likewise in this study, ammonium sulphate precipitation was performed followed by high speed centrifugation to eliminate the *S. flexneri* population from the lysate. However, Rawat and Mishra (2010) subsequently conducted Sephadex G200 filtration to further purify the lysate, which was not done in this study. Besides that, Mishra, et al. (2013) also used ammonium sulphate precipitation of fresh phage-bacteria culture lysate, followed by high speed centrifugation. Apart from that, a study conducted by Gilmer, et al. (2013) also used ammonium sulphate precipitation for the purification of PlySs2, a novel bacteriophage lysin with a broad lytic activity derived from *Streptococcus suis* phage. There are also other reported ways to extract the endolysin from crude lysate. For instance, a study conducted by Mindich and Lehman (1979) used Triton X-100

to extract lysin from ø6 by suspending the virus in solutions containing 2.5% Triton X-100 at 0ºC for 1 h and centrifuged.

4.4.2 Lytic Activity Assay

The proteins precipitated at each percentage of saturation were spotted on lawns of *S. flexneri* to determine the percentage of ammonium sulphate saturation that was able to extract endolysin and subsequently caused lysis of the bacterial cells. Two types of bacterial cultures were prepared to perform the lytic activity assay. One bacterial culture was treated with an outer membrane permeabilizer, EDTA and the other was not treated with EDTA. The bacterial cultures were prepared as such to determine the ability of the protein precipitates to cause lysis on the bacterial cells with and without an outer membrane permeabilizer.

According to Ibrahim, Matsuzaki and Aoki (2001), lysozyme is a powerful antibacterial protein that belongs to the class of enzymes that cause the lysis of certain Gram-positive bacterial cell walls. Lysozyme splits the bond between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan. Lysozymes are widely distributed in various tissues and biological fluids including animal secretions, avian egg, bacteria and plants. Apart from the antimicrobial activity, lysozymes also play important roles in surveillance of mammalian cell membranes, inactivation of certain viruses and enhance the phagocytic activity of polymorphonuclear leukocytes (Ibrahim, Matsuzaki and Aoki, 2001). Hence, it was obvious that both lysozyme and endolysin function

similarly by causing the lysis of bacterial cell walls and that was the reason why lysozyme was used as the positive control in this study. Different concentrations of lysozymes were spotted on different quadrants in this study to determine the lowest concentration of lysozyme that was sufficient to lyse the bacterial cells.

The outer membranes of Gram-negative bacteria such as *S. flexneri* act as effective permeability barriers against external noxious agents such as antibiotics. However, there are antibacterial agents including chelators and polycations that target these outer membranes by weakening the molecular interactions of the lipopolysaccharide constituent of the outer membrane (Vaara, 1992). Nevertheless, there are also some agents which are better permeabilizers than others such as nitrilotriacetic acid, sodium hexametaphosphate and EDTA. These agents break down the outer membrane by removing the Ca^{2+} and Mg^{2+} ions (Vaara, 1992). A study by Son, et al. (2012) reported the antimicrobial activity of LysB4, an endolysin from *Bacillus cereus (B. cereus)* phage B4 against several Gram-negative and Gram-positive bacteria. The enzyme did not show any lytic activity against Gram-negative bacteria but when the bacterial cells were washed with 0.1 M EDTA to increase the permeability of the cell wall, LysB4-mediated cell lysis was detected for all the Gram-negative bacteria tested including *S. flexneri*. Therefore, 0.1 M EDTA was chosen as the outer membrane permeabilizer in this study. Figure 4.5 shows the results obtained from the lytic activity assay.

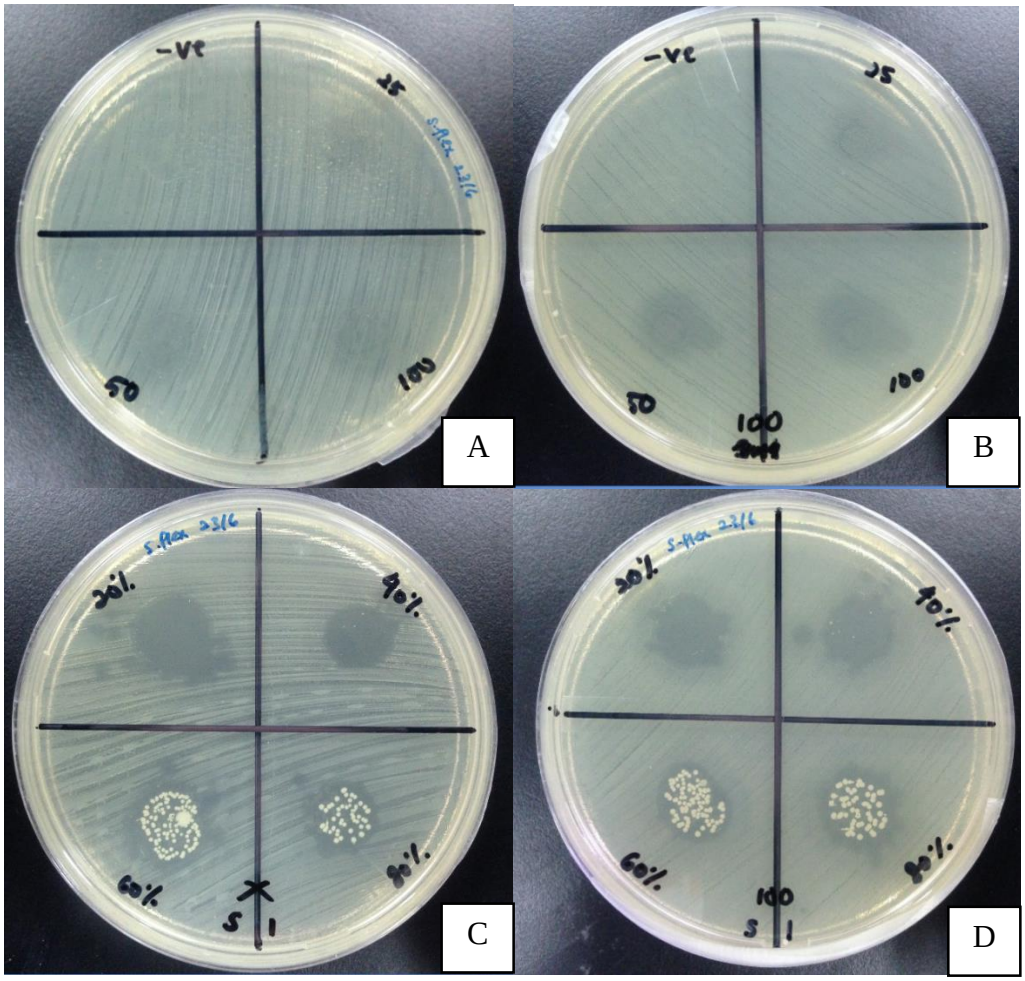

Figure 4.5: Results of lytic activity assay. (A) Bacterial culture without 0.1 M EDTA treatment spotted with TBS and different concentrations of lysozyme (25 µg/ml, 50 µg/ml and 100 µg/ml); (B) Bacterial culture treated with 0.1 M EDTA spotted with TBS and different concentrations of lysozyme (25 µg/ml, 50 µg/ml and 100 µg/ml); (C) Protein precipitates spotted on the bacterial culture without 0.1 M EDTA treatment; (D) Protein precipitates spotted on bacterial culture treated with 0.1 M EDTA.

Figure 4.5(A) does not show area of lysis in any of the quadrants but Figure 4.5(B) shows area of lysis in three of the quadrants that were spotted with different concentrations of lysozyme. This showed that lysozyme was unable to lyse the *S. flexneri* bacterial cells in this study without an outer membrane permeabilizing agent. On the other hand, no cell lysis can be observed on the first quadrant that was spotted with TBS in both Figure 4.5(A) and 4.5(B). This

showed that TBS did not play any role in causing bacterial cell lysis and hence, acted as negative control in this study.

Figure 4.5(C) shows bacterial culture that was not treated with EDTA and Figure 4.5(D) shows bacterial culture that was treated with EDTA. The expected result was to observe a single quadrant containing a lytic area indicating the presence of endolysin in one or both of the agar plates. However, clear lytic zones were observed in all the quadrants. Hence, the presence of phage particles in the proteins precipitated at different percentage of ammonium sulphate saturations was confirmed using plaque assay. Plaque assays performed for the precipitated proteins at all the percentage of saturations showed the presence of phage particles. The average phage titres of the co-precipitated phage particles at 0-20% and 20-40% saturations were 2.22×10^{11} pfu/mL and 2.09×10^{11} pfu/mL, respectively. The average phage titre of the co-precipitated phage particles at 40-60% saturation was the highest among the four percentages of saturations with a value of 4.51×10^{12} pfu/mL. At 60-80% saturation, the average phage titre obtained was 4.31×10^{11} pfu/mL. This showed that ammonium sulphate precipitation technique was able to co-precipitate phage particles together with other proteins.

Several reported studies have used ammonium sulphate precipitation method as a technique for the purification of bacteriophages as highlighted by Hotchin (1954). Ammonium sulphate has also been useful in promoting viral aggregation for some viruses followed by removal using low-speed

ultracentrifugation (Hutornojs, et al., 2012). Hence, this showed that phage particles are capable of reacting to ammonium sulphate. The co-precipitation of phage particles may be due to the presence of protein coats encapsulating the phage genomes, which interacted with ammonium sulphate and precipitated together with other proteins present in the crude lysate.

However, there are several studies that used ammonium sulphate precipitation technique to isolate endolysin from crude lysate. For example, Gupta and Prasad (2011) used repetitive ammonium sulphate precipitation to obtain the endolysin. However, Gupta and Prasad (2011) performed gel filtration chromatography on the proteins precipitated via ammonium sulphate precipitation using Sephadex G-200 matrix and the resulting eluted fractions obtained were then analysed for the presence of endolysin using spectrophotometer at 280 nm. The protein fractions obtained in this way would be more purified and free of phage particles as the phage particles would be eluted as different fractions. Besides that, Vipra, et al. (2012) also used ammonium sulphate precipitation technique with solid ammonium sulphate to obtain a purified preparation of P128 protein, a chimeric protein that possessed anti-staphylococcal activity. The protein precipitates were further purified through an anion exchange column to obtain P128 protein, which was free from phage particles (Vipra, et al., 2012).

4.5 Limitations of Study

The proteins precipitated at different percentage of ammonium sulphate saturations may have precipitated the endolysin however, the fraction that contained the endolysin was unable to be determined in this study due to the co-precipitation of the phage particles together.

4.6 Future Studies

Since the percentage of ammonium sulphate saturation at which endolysin may be precipitated was unable to be determined, different techniques can be used to overcome the problem. For instance, the protein precipitates obtained from ammonium sulphate precipitation in this study can be subjected to anion-exchange chromatography to further purify the protein precipitates. Gel filtration chromatography can also be performed so that the co-precipitated phage particles can be removed from the protein samples and the purity of the endolysin obtained may be enhanced and concentrated.

Once the purified endolysin is obtained, characterization of the endolysin can be performed to determine the lytic activity range and the susceptibility of different host range towards the endolysin. The identified endolysin can then be sent for sequencing to determine the amino acid sequence of the protein.

CHAPTER 5

CONCLUSIONS

Density gradient caesium chloride ultracentrifugation technique using densities 1.3 g/mL, 1.4 g/mL, 1.5 g/mL and 1.7 g/mL yielded more concentrated phage particles, compared to precipitation of phage particles using polyethylene glycol. The phage titre was increased by approximately 200 times after caesium chloride purification compared to the phage titre obtained after PEG precipitation. Besides that, the density of SFN6B phage was also determined to be approximately 1.5 g/mL.

Extraction of the PEG-precipitated phage genome using the commercialized kit yielded a purer form of phage nucleic acid compared to the extraction using conventional method. This could be due to the presence of phenol components in the reagent used in the conventional technique of nucleic acid extraction that compromised the purity of the phage nucleic acid. In addition, it was found that the genome of the SFN6B phage was made up of DNA. The size of the phage genome was also found to be approximately above 10, 000 bps. Digestion of the phage genome using six different restriction enzymes showed that all the six restriction enzymes used had different number of occurrence of their respective endonuclease sequences. This was indicated by the different number of fragments produced for the respective restriction endonucleases.

Apart from that, SDS-PAGE revealed that most proteins were precipitated at 40% to 60% saturations of ammonium sulphate. However, the presence of endolysin in the protein precipitates obtained at different percentage of ammonium sulphate saturations was unable to be determined due to the co-precipitation of phage particles. In future studies, additional steps such as gel-filtration chromatograpy are definitely necessary to remove the co-precipitated phage particles prior to performing lytic activity assay. This way, the exact protein precipitate obtained at different percentage of ammonium sulphate saturations that contain the endolysin can be determined for further analysis of the endolysin.

REFERENCES

Abedon, S., Kuhl, S., Blasdel, B. and Kutter, E., 2011. Phage treatment of human infections. *Bacteriophage*, 1(2), pp. 66-85.

Alisky, J., Iczkowski, K., Rapoport, A. and Troitsky, N., 1998. Bacteriophages show promise as antimicrobial agents. *Journal of Infection*, 36(1), pp. 5-15.

Almeida, D.R., Trevilato, P.B., Bartoleti, L.A., Proença-Módena, J.L., Hanna, E.S., Gregoracci, G.B. and Brocchi, M., 2004. Bacteriophages and insertion sequences of *Chromobacterium violaceum* ATCC 12472.*Genetics and Molecular Research*, 3(1), pp. 76-84.

Atterbury, R.J., Connerton, P.L., Dodd, C., Rees, C.E.D. and Connerton, I.F., 2003. Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. *Applied and Environmental Microbiology*, 69(10), pp. 6302-6306.

Bachrach, U. and Friedmann, A., 1971. Practical procedures for the purification of bacterial viruses. *Applied Microbiology*, 22(4), pp. 706-715.

Barton, L., 2005. *Structural and functional relationships in prokaryotes*. New York: Springer.

Birge, E., 2000. *Bacterial and bacteriophage genetics*. 4th ed. New York: Springer-Verlag.

Borysowski, J. and Gorski, A., 2008. Is phage therapy acceptable in the immunocompromised host? *International Journal of Infectious Diseases*, 12(5), pp. 466-471.

Borysowski, J., Weber-Dabrowska, B. and Gorski, A., 2006. Bacteriophage endolysins as a novel class of antibacterial agents. *Experimental Biology and Medicine*, 231(4), pp. 366-377.

Boulanger, P., 2009. Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. *Methods in Molecular Biology*, 502, pp. 227-238.

Boulette, M., 2007. *Shigella flexneri ArcA and Fnr regulate iron acquisition and contribute to plaque formation under anaerobic conditions*. Texas: University of Texas.

Briers, Y., Volckaert, G., Cornelissen, A., Lagaert, S., Michiels, C., Hertveldt, K. and Lavigne, R., 2007. Muralytic activity and modular structure of the endolysins of *Pseudomonas aeruginosa* bacteriophages φKZ and EL. *Molecular Microbiology*, 65(5), pp. 1334-1344.

Burrowes, B., Harper, D.R., Anderson, J., McConville, M. and Enright, M.C., 2011. Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. *Expert Review of Anti-infective Therapy*, 9(9), pp. 775-785.

Casjens, S., Winn-Stapley, D.A., Gilcrease, E.B., Morona, R., Kühlewein, C., Chua, J.E., Manning, P.A., Inwood, W. and Clark, A.J., 2004. The chromosome of *Shigella flexneri* bacteriophage Sf6: complete nucleotide sequence, genetic mosaicism, and DNA packaging. *Journal of Molecular Biology*, 339(2), pp. 379-394.

Chart, H., 1994. *Methods in practical laboratory bacteriology*. Boca Raton, FL: CRC Press.

Cheasty, T., Skinner, J.A., Rowe, B. and Threlfall, E.J., 1998. Increasing incidence of antibiotic resistance in *Shigellas* from humans in England and Wales: recommendations for therapy. *Microbial Drug Resistance*, 4(1), pp. 57-60.

Chen, F. and Lu, J., 2002. Genomic sequence and evolution of marine cyanophage P60: a new insight on lytic and lysogenic phages. *Applied and Environmental Microbiology*, 68(5), pp. 2589-2594.

Chu, Y.W., Houang, E.T., Lyon, D.J., Ling, J.M., Ng, T.K. and Cheng, A.F., 1998. Antimicrobial resistance in *Shigella flexneri* and *Shigella sonnei* in Hong Kong, 1986 to 1995. *Antimicrobial Agents and Chemotherapy*, 42(2), pp. 440-443.

Delcour, A.H., 2008. Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta*, 1794(5), pp. 808-816.

Denyer, S., Hugo, W. and Russell, A., 2011. *Hugo and Russell's pharmaceutical microbiology*. Oxford: Wiley-Blackwell.

Deresinski, S., 2009. Bacteriophage therapy: exploiting smaller fleas. *Oxford Journals*, 48(8), pp.1096-1101.

Drulis-Kawa, Z., Majkowska-Skrobek, G., Maciejewska, B., Delattre, A. and Lavigne, R., 2012. Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Current Protein and Peptide Science*, 13(8), pp. 699–722.

Desjardins, P. and Conklin, D., 2010. NanoDrop microvolume quantitation of nucleic acids. *Journal of Visualized Experiments*, 45, p. 2565.

Devroy, V., 2008. *Structure-function aspects of three component multidrug transporters in gram-negative bacteria*. PhD. University of Oklahoma Graduate College.

Elbreki, M., Ross, R., Hill, C., O'Mahony, J., McAuliffe, O. and Coffey, A., 2014. Bacteriophages and their derivatives as biotherapeutic agents in disease prevention and treatment. *Journal of Viruses*, 2014, pp. 1-14.

Elshayeb, A.A., Yagoub, S.O., Yousif, A.S., Abedalkareem, E.A., Hag, S.M.E. and Elagib, A.A., 2011. Identification of protein profiles of *Escherichia coli*, *Staphylococcus aureus* and their corresponding phages. *American Journal Biotechnology and Molecular Sciences*, 1(2), pp. 39-44.

Fenton, M., McAuliffe, O., O'Mahony, J. and Coffey, A., 2010. Recombinant bacteriophage lysins as antibacterials. *Bioengineered Bugs*, 1(1), pp. 9-16.

Fischer, E. and Braun, V., 1981. Permeability barrier of bacterial cell envelopes as cause of resistance to antibiotics. *Immunitat und Infektion*, 9(3), pp. 78-87.

Fischetti, V.A., 2005. Bacteriophage lytic enzymes: novel anti-infectives. *Trends in Microbiology*, 13(10), pp. 491-496.

Fry, J. and Day, M., 1992. *Release of Genetically Engineered and Other Micro-organisms*. New York: The Press Syndicate of the University of Cambridge.

Garcia, P., Martinez, B., Obeso, J.M. and Rodriguez, A., 2008. Bacteriophages and their application in food safety. *Letters in Applied Microbiology*, 47(6), pp. 479–485.

Gilmer, D.B., Schmitz, J.E., Euler, C.W. and Fischetti, V.A, 2013. Novel bacteriophage lysin with broad lytic activity protects against mixed infection by *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 57(6), pp. 2743-2750.

Goodridge, L.D., 2013. Bacteriophages for managing *Shigella* in various clinical and non-clinical settings. *Bacteriophage*, 3(1), pp. e25098.

Goodridge, L., Gallaccio, A. and Griffiths, M.W., 2003. Morphological, host range, and genetic characterization of two coliphages. *Applied and Environmental Microbiology*, 69(9), pp. 5364-5371.

Gormley, N.A., Bath, A.J. and Halford, S.E., 2000. Reactions of *BgI*I and other type II restriction endonucleases with discontinuous recognition sites. *Journal of Biological Chemistry*, 275(10), pp. 6928-6936.

Greer, G.G., 1986. Homologous bacteriophage control of *Pseudomonas* growth and beef spoilage. *Journal of Food Protection*, 49(2), pp. 104-109.

Grodzki, A.C. and Berenstein, E., 2010. Antibody purification: ammonium sulfate fractionation or gel filtration. *Methods in Molecular Biology*, 588, pp. 15-26.

Gupta, R. and Prasad, Y., 2011. P-27/HP Endolysin as antibacterial agent for antibiotic aesistant *Staphylococcus aureus* of human infections. *Current Microbiology*, 63(1), pp. 39-45.

Haq, I.U., Chaudhry, W.N., Akhtar, M.N., Andleeb, S. and Qadri, I., 2012. Bacteriophages and their implications on future biotechnology: a review. *Virology Journal*, 9(9), pp. 1-2.

Hausmann, R., Gomez, B. and Moody, B., 1969. Physiological and genetic aspects of abortive infection of a *Shigella sonnei* strain by coliphage T7. *Journal of Virology*, 2(4), pp. 335-345.

Hermoso, J.A., García, J.L. and García, P., 2007. Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Current Opinion in Microbiology*, 10(5), p.9.

Hobson, P., 1988. *The Rumen microbial ecosystem*. London: Elsevier Applied Science.

Hotchin, J.E., 1954. The purification and electron microscopical examination of the structure of staphylococcal bacteriophage K. *Journal of General Microbiology*, 10(2), pp. 250-260.

Hutornojs, V., Niedre-Otomere, B., Kozlovska, T. and Zajakina, A., 2012. Comparison of ultracentrifugation methods for concentration of recombinant alphaviruses: sucrose and iodixanol cushions. *Environmental and Experimental Biology*, 10, pp. 117–123.

Hycult Biotech, 2010. *Troubleshooting sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*. [online] Available at: <https://www.hycultbiotech.com/media/wysiwyg/Troubleshooting_SDS-PAGE_1.pdf> [Accessed 26 August 2015].

Hyde, R., 1987. *Microbiology and immunology*. New York: Springer-Verlag.

Hyman, P. and Abedon, S., 2012. *Bacteriophages in health and disease*. Wallingford, Oxfordshire: CABI.

Ibrahim, H.R., Matsuzaki, T. and Aoki, T., 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic

function. *Federation of European Biochemical Societies Letters*, 506(1), pp. 27-32.

Inal, J.M., 2003. Phage therapy: a reappraisal of bacteriophages as antibiotics. *Archivum Immunologiae et Therapiae Experimentalis*, 51, pp. 237-244.

Jakhetia, R. and Verma, N.K., 2015. Identification and Molecular Characterisation of a novel Mu-Like bacteriophage, SfMu, of *Shigella flexneri. Public Library of Science ONE*, 10(4), p. e0124053.

Jakhetia, R., Talukder, K.A. and Verma, N.K., 2013. Isolation, characterization and comparative genomics of bacteriophage SfIV: a novel serotype converting phage from *Shigella flexneri*. *BMC Genomics*, 14(1), p.677.

Jamal, M., Chaudhry, W.N., Hussain, T., Das, C.R. and Andleeb, S., 2015. Characterization of new *Myoviridae* bacteriophage WZ1 against multi-drug resistant (MDR) *Shigella dysenteriae*. *Journal of Basic Microbiology*, 55(4), pp. 420-431.

Jennison, A.V. and Verma, N.K., 2004. *Shigella flexneri* infection: pathogenesis and vaccine development. *FEMS Microbiology Reviews*, 28(1), pp. 43-58.

Kasarjian, J.K.A., Iida, M. and Ryu, J., 2003. New restriction enzymes discovered from *Escherichia coli* clinical strains using a plasmid transformation method. *Nucleic Acids Research*, 31(5), p. e22.

Kaur, T., Nafissi, N., Wasfi, O., Sheldon, K., Wettig, S. and Slavcev, R., 2012. Immunocompatibility of bacteriophages as nanomedicines. *Journal of Nanotechnology*, 2012, p. 5.

Kaźmierczak, Z., Górski, A. and Dąbrowska, K., 2014. Facing antibiotic resistance: *Staphylococcus aureus* phages as a medical tool. *Viruses*, 6(7), pp. 2551-2570.

Keary, R., McAuliffe, O., Ross, R.P., Hill, C., O'Mahony, J. and Coffey, A., 2013. Bacteriophages and their endolysins for control of pathogenic bacteria. *Microbial pathogens and strategies for combating them: science, technology and education*, pp. 1028-1035.

Khatibi, P.A., Roach, D.R., Donovan, D.M., Hughes, S.R. and Bischoff, K.M., 2014. *Saccharomyces cerevisiae* expressing bacteriophage endolysins reduce *Lactobacillus* contamination during fermentation. *Biotechnology for Biofuels*, 7(1), p. 104.

Kim, K.H., Chang, H.W., Nam, Y.D., Roh, S.W. and Bae, J.W., 2010. Phenotypic characterization and genomic analysis of the *Shigella sonnei* bacteriophage SP18. *The Journal of Microbiology*, 48(2), pp. 213-222.

Krucik, G., 2012. *Shigellosis*. [online] Available at: <http://www.healthline.com/health/shigellosis#Overview1> [Accessed 28 July 2015].

Kumar, A. and Garg, N., 2005. *Genetic engineering*. New York: Nova Science Publishers, Inc.

Kutter, E. and Sulakvelidze, A., 2004. *Bacteriophages*. Boca Raton, FL: CRC Press.

Lai, M.J., Lin, N.T., Hu, A., Soo, P.C., Chen, L.K., Chen, L.H. and Chang, K.C., 2011. Antibacterial activity of *Acinetobacter baumannii* phage ϕAB2 endolysin (LysAB2) against both Gram-positive and Gram-negative bacteria. *Applied Microbiology and Biotechnology*, 90, pp. 529-539.

Lawrence, J.E. and Steward, G.F., 2010. *Manual of aquatic viral ecology*. American Society of Limnology and Oceanography, Inc.

Lim, J.A., Shin, H., Heu, S. and Ryu, S., 2014. Exogenous lytic activity of SPN9CC endolysin against gram-negative bacteria. *Journal of Microbiology and Biotechnology*, 24(6), pp. 803-811.

Lim, J.A., Shin, H., Kang, D.H. and Ryu, S., 2012. Characterization of endolysin from a *Salmonella Typhimurium*-infecting bacteriophage SPN1S. *Research in Microbiology*, 163(3), pp. 233-241.

Lima, A.A., Sidrim, J.J., Lima, N.L., Titlow, W., Evans, M.E. and Greenberg, R.N., 1997. Molecular epidemiology of multiply antibiotic-resistant *Shigella flexneri* in Fortaleza, Brazil. *Journal of Clinical Microbiology*, 35(5), pp. 1061-1065.

Liu, D. ed., 2011. *Molecular detection of human bacterial pathogens*. Boca Raton, FL: Taylor & Francis/CRC Press.

Loc-Carrillo, C. and Abedon, S.T., 2011. Pros and cons of phage therapy. *Bacteriophage*, 1(2), pp. 111-114.

Loessner, M.J., Maier, S.K., Daupek-Puza, H., Wendlinger, G. and Scherer, S., 1997. Three *Bacillus cereus* bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different bacilli. *Journal of Bacteriology*, 179(9), pp. 2845–2851.

Loessner, M.J., 2005. Bacteriophage endolysins — current state of research and applications. *Current Opinion in Microbiology*, 8(4), pp. 480-487.

Mathur, M.D., Vidhani, S. and Mehndiratta, P.L., 2003. Bacteriophage therapy: an alternative to conventional antibiotics. *Journal of the Association of Physicians of India*, 51, p. 594.

Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Imai, S., Ikeuchi, M., Tani, T., Fujieda, M. and Wakiguchi, H., 2005. Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. *Journal of Infection and Chemotherapy*, 11(5), pp. 211-219.

Mc Grath, S. and Sinderen, D., 2007. *Bacteriophage*. Norfolk, UK: Caister Academic Press.

Mindich, L. and Lehman, J., 1979. Cell wall lysin as a component of the bacteriophage ø6 virion. *Journal of Virology*, 30(2), p. 492.

Mishra, A.K., Sharma, N., Kumar, A., Kumar, N., Bayyappa, M.R.G., Kumar, S. and Kumar, N., 2013. Isolation, characterization and therapeutic potential assessment of bacteriophages virulent to *Staphylococcus aureus* associated with goat mastitis. *Iranian Journal of Veterinary Research*, 15(4), pp. 320-325.

Morita, M., Tanji, Y., Orito, Y., Mizoguchi, K., Soejima, A. and Unno, H., 2001. Functional analysis of antibacterial activity of *Bacillus amyloliquefaciens* phage endolysin against Gram-negative bacteria. *Federation of European Biochemical Societies Letters*, 500(1-2), pp. 56-59.

Murphy, F., Fauquet, C., Bishop, D., Ghabrial, S., Jarvis, A., Martelli, G., Mayo, M. and Summers, M., 1995. *Virus Taxonomy*. Wien: Springer-Verlag.

Nelson, D.C., Schmelcher, M., Rodriguez-Rubio, L., Klumpp, J., Pritchard, D.G., Dong, S. and Donovan, D.M., 2012. Endolysins as antimicrobials. *Advances in Virus Research*, 83, p. 307.

New England Biolabs Inc., 2015. *Heat inactivation | NEB*. [online] Available at: <https://www.neb.com/tools-and-resources/usage-guidelines/heat-inactivation> [Accessed 1 August 2015].

Nikaido, H., 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science*, 264(5157), p. 383.

Nybo, K., 2009. Protein methods: recovery of a secreted protein. *BioTechniques*, 47(6), pp. 1009-1011.

Oliveira, H., Azeredo, J., Lavigne, R. and Kluskens, L.D., 2012. Bacteriophage endolysins as a response to emerging foodborne pathogens. *Trends in Food Science & Technology*, 28(2), pp. 103-115.

Orlova, E., 2012. *Bacteriophages and their structural organisation*. [online] Intech. Available at: <http://cdn.intechopen.com/pdfs-wm/32276.pdf> [Accessed 1 August 2015].

Osborn, M., Weiner, A.M. and Weber, K., 1970. Large scale purification of A-protein from bacteriophage R17. *European Journal of Biochemistry*, 17(1), pp. 63-67.

Pajunen, M.I., Kiljunen, S.J., Soderholm, M.E. and Skurnik, M., 2001. Complete genomic sequence of the lytic bacteriophage Â YeO3-12 of *Yersinia enterocolitica* serotype O:3. *Journal of Bacteriology*, 183(6), pp. 1928-1937.

Paul, W., 2008. *Fundamental Immunology*. 6th ed. Philadelphia: Lippincott Williams & Wilkins.

Parija, S., 2009. *Textbook of microbiology & immunology*. Haryana: Elsevier.

Passarge, E., 2007. *Color atlas of genetics*. 3rd ed. Stuttgart: Thieme.

Paterson, D.L., 2008. Impact of antibiotic resistance in Gram-negative bacilli on empirical and definitive antibiotic therapy. *Oxford Journals*, 47(1), pp. 14-20.

Pingoud, A. and Jeltsch, A., 2001. Structure and function of type II restriction endonucleases. *Nucleic Acids Research*, 29(18), pp. 3705-3727.

Pingoud, A., Wilson, G.G. and Wende, W., 2014. Type II restriction endonucleases--a historical perspective and more. *Nucleic Acids Research*, 42(12), pp. 7489-7527.

Plotka, M., Kaczorowska, A.K., Stefanska, A., Morzywolek, A., Fridjonsson, O.H., Dunin-Horkawicz, S., Kozlowski, L., Hreggvidsson, G.O., Kristjansson, J.K., Dabrowski, S., Bujnicki, J.M. and Kaczorowski, T., 2013. Discovery and characterization of a novel highly thermostable endolysin from Thermus scotoductus MAT2119 bacteriophage Ph2119 that shows amino-acid sequence similarity to eukaryotic peptidoglycan recognition proteins (PGRPs). *Applied Environmental Microbiology*, 80(3), pp. 886-895.

Promega, 2015. *Applications and reaction conditions for restriction enzymes*. [online] Available at: <https://worldwide.promega.com/resources/product-guides-and-selectors/restriction-enzyme-resource/applications-and-reaction-conditions-for-restriction-enzymes/> [Accessed 20 August 2015].

Rai V, R. and Bai, J., 2014. *Beneficial Microbes in Fermented and Functional Foods*. Hoboken: Taylor and Francis.

Rawat, A.M. and Mishra, A., 2010. Purification and activity of phage induced lysin against mastitogenic strains of *Staphylococcus aureus. Biotechnology International*, 3(1), pp. 18-25.

Sandeep, K., 2006. Bacteriophage precision drug against bacterial infections. *Current Science*, 90(5), pp. 631-633.

Schmelcher, M., Donovan, D.M. and Loessner, M.J., 2012. Bacteriophage endolysins as novel antimicrobials. *Future Microbiology*, 7(10), pp. 1147-1171.

Seltmann, G. and Holst, O., 2002. *The bacterial cell wall*. NY: Springer-Verlag Berlin Heidelberg.

Silhavy, T.J., Kahne, D. and Walker, S., 2010. The bacterial cell envelope. *Cold Spring Harbor Perspectives in Biology*, 2(5), p. 4.

Singh, P. and Sharma, V., 2014. *Water and Health*. New Delhi: Springer.

Sirsat, S.A., Muthaiyan, A. and Ricke, S.C., 2009. Antimicrobials for foodborne pathogen reduction in organic and natural poultry production. *The Journal of Applied Poultry Research*, 18(2), pp. 379-388.

Skiena, S.S., 2001. Designing Better Phages. *Bioinformatics*, 17(1), pp. S253-S261.

Son, B., Yun, J., Lim, J., Shin, H., Heu, S. and Ryu, S., 2012. Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4. *BMC Microbiology*, 12(1), p. 33.

Steinhausen, J.J., 2008. *A Space-time Analysis of Shigellosis and Salmonellosis Cases in Texas from 2000 to 2004*. Michigan: ProQuest LLC.

Sulakvelidze, A., Alavidze, Z. and Morris, J.G., 2001. Bacteriophage therapy. *Antimicrobial Agents and Chemotherapy*, 45(3), pp. 649-659.

Sundar, M.M., Nagananda, G.S., Das, A., Bhattacharya, S. and Suryan, S., 2009. Isolation of host-specific bacteriophages from sewage against human pathogens. *Asian Journal of Biotechnology*, 1(4), pp. 163-170.

Tan, L.T., Chan, K. and Lee, L.H, 2014. Application of bacteriophage in biocontrol of major foodborne bacterial pathogens. *Journal of Molecular Biology and Molecular Imaging*, 1(1), pp. 1-6.

Tillotson, G.S. and Theriault, N., 2013. New and alternative approaches to tackling antibiotic resistance. *F1000Prime Reports*, 5(51), p. 1.

Timmreck, T., 2002. *An Introduction to Epidemiology*. 3rd ed. London: Jones and Bartlett Publishers International.

Tišáková, L. and Godány, A., 2014. Bacteriophage endolysins and their use in biotechnological processes. *Journal of Microbiology, Biotechnology and Food Sciences*, 3(2), pp. 164-170.

Tiwari, R., Dhama, K., Wani, M.Y., Verma, V., Vaid, R.K. and Chauhan, R.S., 2011. Bacteriophage therapy: a novel tool for combating bacterial diseases of poultry - A Review. *Journal of Immunology and Immunopathology*, 13(2), pp. 55-66.

Todar, K., 2012. *Bacteriophage*. [online] Available at: <http://textbookofbacteriology.net/phage_2.html> [Accessed 18 July 2015].

Todar, K., 2012. *Shigella and Shigellosis*. [online] Available at: <http://textbookofbacteriology.net/Shigella.html> [Accessed 23 May 2015].

Tremblay, D.M. and Moineau, S., 1999. Complete genomic sequence of the lytic bacteriophage DT1 of *Streptococcus thermophilus*. *Virology*, 255(1), pp. 63-76.

Vaara, M., 1992. Agents that increase the permeability of the outer membrane. *Microbiology and Molecular Biology Reviews*, 56(3), pp. 395-411.

Vajda, B.P., 1978. Concentration and purification of viruses and bacteriophages with polyethylene glycol. *Folia Microbiologica*. 23(1), pp. 88-96.

Vipra, A.A., Desai, S.N., Roy, P., Patil, R., Raj, J.M., Narasimhaswamy, N., Paul, V.D., Chikkamadaiah, R. and Sriram, B., 2012. Antistaphylococcal activity of bacteriophage derived chimeric protein P128. *BMC Microbiology*, 12(1), p. 41.

Waddell, C.D., Walter, T.J., Pacheco, S.A., Purdy, G.E. and Runyen-Janecky, L.J., 2014. NtrBC and Nac contribute to efficient *Shigella flexneri* intracellular replication. *Journal of Bacteriology*, 196(14), pp. 2578-2586.

Walmagh, M., Boczkowska, B., Grymonprez, B., Briers, Y., Drulis-Kawa, Z. and Lavigne, R., 2013. Characterization of five novel endolysins from Gram-negative infecting bacteriophages. *Applied Microbiology and Biotechnology*, 97(10), pp. 4369-4375.

Westwater, C., Kasman, L.M., Schofield, D.A., Werner, P.A., Dolan, J.W., Schmidt, M.G. and Norris, J.S., 2003. Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for

treatment of bacterial infections. *Antimicrobial Agents and Chemotherapy*, 47(4), pp. 1301-1307.

Wittebole, X., Roock, D.S. and Opal, S.M., 2014. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence*, 5(1), pp. 226-235.

Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L. and Treiber, G., 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology*, 40(3), pp. 734-744.

Yuan, Y., Peng, Q. and Gao, M., 2012. Characteristics of a broad lytic spectrum endolysin from phage BtCS33 of *Bacillus thuringiensis*. *BMC Microbiology*, 12(297).

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