CHARACTERIZATION OF BACTERIOPHAGE LT-B ISOLATED FROM LUBUK TIMAH HOT SPRING, PERAK

By

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ABSTRACT

CHARACTERIZATION OF BACTERIOPHAGE LT-B ISOLATED FROM LUBUK TIMAH HOT SPRING, PERAK

Chang Phooi Li

Bacteriophages are the obligate intracellular parasite that target specific group of bacteria. It can be found mostly in aquatic environment. They contain genetic material that facilitates their replication. Various researches have investigated on thermophiles isolated from Malaysia's hot spring. However, very limited study was carried out on bacteriophages isolated from Malaysia's hot spring. Therefore, this study was aimed to characterize the isolated LT-B bacteriophage from Lubuk Timah hot spring, Perak based on its physiological and partial genomic properties. One-step growth curve and adsorption rate of phage LT-B were determined using multiplicity of infection (MOI) of 0.00007 and 0.002, respectively. Phage infectivity was investigated at different temperatures, pH and concentrations of chloroform. Moreover, the stability of the phage at various storage temperatures (-80˚C, -20˚C, 4˚C and 25˚C) was evaluated weekly for 28 days. The phage host range was determined by using ten different types of Gram-negative bacteria. In addition, DNase, RNase and restriction enzyme digestions

analyses were carried out to characterize the phage genome. Based on the results, one-step growth curve of this phage was completed within 50 min with a burst size of 22 phages per bacterial cell. The phage achieved its adsorption rate of 91.3% within 2 min post infection. The phage was inactivated at 70˚C after 20 min post treatment. Furthermore, this phage exhibited an optimal growth at pH 7 and its infectivity was not affected after chloroform treatment with concentrations ranging from 2% to 10% (v/v). The optimum storage temperature for phage LT-B was at 4˚C after 28 days in comparison to storage temperatures at -80˚C, -20˚C and 25˚C. Moreover, this study also showed that this phage contains DNA genome and all the restriction enzymes tested failed to digest the phage genome. Therefore, phage LT-B can be a potential antimicrobial agent to combat *E. coli* infections.

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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.

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CHANG PHOOI LI

APPROVAL SHEET

This project report entitled "**CHARACTERIZATION OF BACTERIOPHAGE LT-B ISOLATED FROM LUBUK TIMAH HOT SPRING, PERAK**" was prepared by CHANG PHOOI LI 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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FACULTY OF SCIENCE

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SUBMISSION OF FINAL YEAR PROJECT

It is hereby certified that **CHANG PHOOI LI (ID No: 15ADB02974)** has completed this final year project entitled "**CHARACTERIZATION OF BACTERIOPHAGE LT-B ISOLATED FROM LUBUK TIMAH HOT SPRING, PERAK**" under the supervision of **DR. MICHELLE NG YEEN TAN** (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my final year project in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

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(CHANG PHOOI LI)

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LIST OF ABBREVIATIONS

x *g*	Time gravity (acceleration due to gravity)
˚C	Degree Celcius
µL	Microliter
%	Percentage
A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
g	Gram
µg/ mL	Microgram per milliliter
HCl	Hydrochloric acid

h	Hour
kb	kilo base pair
K. pneumonia	*Klebsiella pneumoniae*
L	Liter
LB	Luria Bertani
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
min	Minutes
mM	Millimolar
mL	Milliliter
MOI	Multiplicity of infection
NaCl	Sodium chloride
nm	Nanometer
OD_{600}	Optical density measure at wavelength 600 nm
pfu/ mL	Plaque forming unit per millimeter
PEG	Polyethylene glycol
pH	Power of hydrogen

RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
S. flexeri	*Shigella flexneri*
S. dysenteriae	*Shigella dysenteriae*
S. sonnei	*Shigella sonnei*
S. typhi	*Shigella typhi*
TAE	Tris-Acetate EDTA
TBS	Tris-buffered saline
UV	Ultraviolet
V	Volt
w/v	Weight per volume
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

Bacteriophage is a unique group of bacterial viruses that target and infect specific group of bacteria. Bacteriophages contain genetic materials (DNA or RNA) that are enclosed by protein that forms the nucleocapsid, or a lipoprotein coat. All viruses are known as obligate intracellular parasites due to their inability to replicate outside the host cells. They only have own genetic information to control their replication process in the host cells. However, they are lacking of replication machinery and important proteins such as ribosomes to synthesize proteins (Guttman et al., 2005).

Bacteriophage can be found abundantly in aquatic environment (sewage, deep thermal vents, oceans, etc) of which give rise to the most abundant biological entities on Earth, with a total of 10^{30} or more. Thus, it is claimed that bacteriophages are crucial in maintaining the balance of bacterial species in microbial ecology with their existence in the environment (Guttman et al., 2005; Mann, 2005).

Antibiotics are commonly being used in treating bacterial infections in human. However, antibiotic resistance, especially penicillin, threatened the public health due to overconsumption, inappropriate prescription and usage in livestock. Thus,

beta-lactam antibiotics were developed to overcome the problem. Shortly after this, methicillin-resistant *Staphylococcus aureus* (MRSA) was emerged to be resistant to beta-lactam antibiotics, followed by other antibiotic-resistant strains. Now, bacterial infections that were common and easily treatable previously have constitute a danger in worldwide at an alarming state due to the emerging of antibiotic-resistant strains (Ventola, 2015).

To combat the antibiotic resistance crisis, phage therapy has been proposed as the "key" to overcome the problem due to its host specificity, self-replication, biofilm control ability and it causes less toxicity to human. Phage therapy can be applied to patients with only the whole bacteriophage or phage-derived lytic proteins (endolysin). However, phage therapy in human trials are limited and therefore more efforts are needed to understand about bacteriophage and phage therapy (Lin et al., 2017).

Hot spring, which is also known as thermal spring, is an area with water at higher temperature than the surrounding air temperature. Hot springs are divided into two types, which are located in volcanic areas and the area that heat up the water via convective circulation. Study on the thermophiles from hot spring have been the interest of researchers for many years because of their useful thermostable enzymes in different fields (Encyclopaedia Britannica, n.d.; Beal, n.d.). Examples of thermostable enzymes are the thermostable DNA polymerases isolated from *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis*. It can be named as *Taq*, *Pfu* and *Vent* DNA polymerases, respectively.

These polymerases are important in polymerase chain reaction (PCR) as they contain proofreading ability and survival ability beyond the DNA denaturation temperature (Rigoldi et al., 2018). In Malaysia, various findings have been reported on thermophiles diversity in hot spring or the activity of the thermostable enzymes, but it is less common for the study on bacteriophage in hot spring (Goh et al., 2011; Norashirene et al., 2013).

Therefore, the main objectives of this study were to characterize phage LT-B isolated from Lubuk Timah hot spring, Perak based on its physiological and partial genomic properties. Physiological properties such as one step growth curve, adsorption rate, pH and organic solvent stability test, storage temperature assessment, heat inactivation assay and host range were investigated. On the other hand, partial genomic characterization which consisted of DNAse and RNAse digestion of phage nucleic acid, and restriction enzyme digestion of phage nucleic acid were carried out in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Bacteriophages Discovery

Bacteriophage was first observed by Frederick W. Twort (1915) when he was trying to culture Vaccinia virus without other living cells. Twort initially interpreted it as bacterial contamination. However, microscopic examination of the "glassy" colonies showed bacterial degeneration in the form of small rounded "granules" when stained with Giemsa stain. Thus, he expressed the possibility of the existence of ultra-microscopic viruses that are in a lower organization than bacteria or amoeba, which infected the micrococci. Later, Felix d'Herelle (1917) observed some lyses, which appeared as clear spots on the confluent bacterial culture. Furthermore, he found that the invisible agent proliferated limitlessly only in the living cells and caused lysis in order to proliferate. He was also able to enumerate the number of invisible agents by plaque counting and demonstrated that these agents proliferated in a cycle, which involves infection, proliferation, release and reinfection stages. The invisible agent was then named as bacteriophage (Summers, 2005).

2.2 General Characteristics of Bacteriophages

2.2.1 Morphology of Bacteriophages

Previous classification has divided bacteriophages based on the type of nucleic acid and capsid, presence of lipid envelope and the number of capsomeres, which were abbreviated in 1965 as LTH. This classification was accepted by Provisional Committee on Nomenclature of Viruses (PCNV). Later, Bradley (1967) introduced another classification of bacteriophages as shown in Figure 2.1 (Novik et al., 2017):

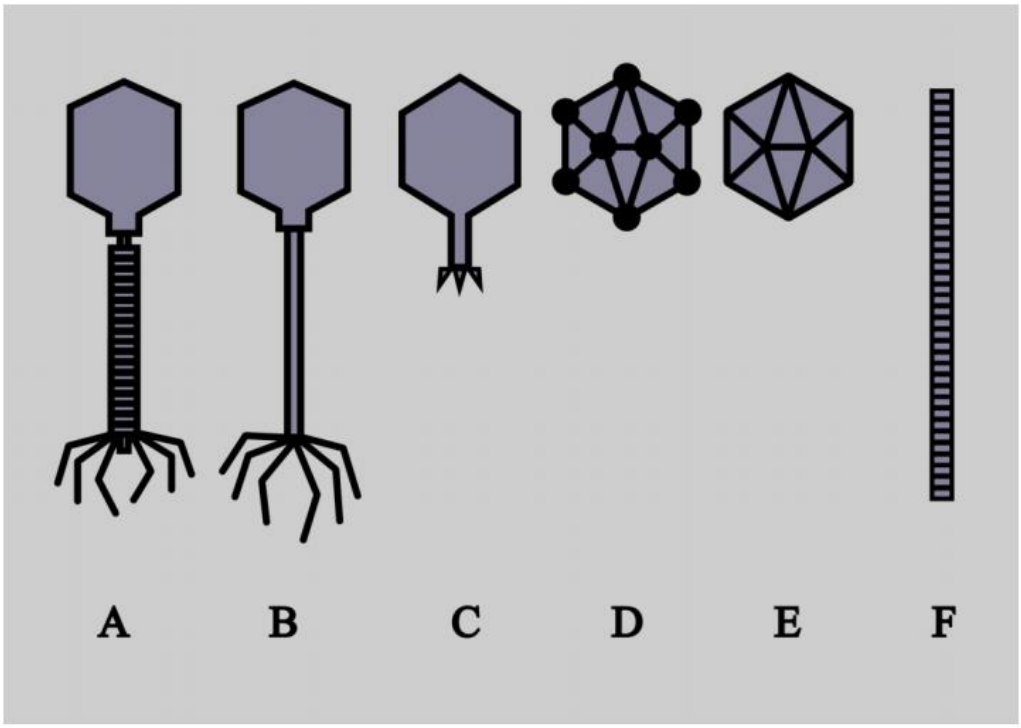

Figure 2.1: Bradley's classification of bacteriophage in 1967 (A: phages with capsid and contractile tail sheath; B: phages with capsid and rigid tail sheath; C: phages with capsid and short tail; D: phages with fibrous or spiky nucleocapsid; E: phages including one nucleocapsid; F: filamentous phages) (Adapted from Novik et al. 2017).

Soon after this, Bradley's scheme was expanded in inclusion of cubic, filamentous, and pleomorphic phages from recent discovery. In 1966, PCNV was converted into International Committee for Nomenclature of Viruses (ICNV) and subsequently, International Committee on Taxonomy of Viruses (ICTV) in 1973, which it remained most of the taxonomy classification in LTH scheme (Ackermann, 2005).

The ICTV is paying effort in classifying viruses into orders, families and genera. Current classification of bacteriophages by ICTV consists of 1 order, 13 families and 34 genera of bacteriophages based on the types of nucleic acid and morphology (Novik et al., 2017). Most of the bacteriophages are in the order of *Caudovirales*, which are also known as tailed phages. They are high in diversity, populations and distribution at different locations. They can be further classified into three families such as *Myoviridae*, *Siphoviridae* and *Podoviridae*. They consist of 2 – 3 nm thick capsid which is in icosahedral (regular or prolate) and tails that are helical or stacked disks (six-fold symmetry). Additionally, tailed phages also consist of base plates, spikes or fibers. However, there are differences between families of *Caudovirales. Myoviridae* is the only family with contractile tail sheath, while *Siphoviridae* and *Podoviridae* have long and short non-contractile tails, respectively. The tails are crucial in DNA injection into bacteria in which the DNA will travel down the tail. Other additional features that are present in some bacteriophages are collars, head or collar appendages and transverse tail disks (Ackermann, 2005; Lavigne et al., 2012).

On the other hand, tailless phage is a minor group of bacteriophage which is consisted only 4% or less among the reported bacteriophages, and it can be further divided into 10 families. Three types of tailless phages that are well-known based on their morphology including polyhedral, filamentous and pleomorphic phages, with cubic, helical and asymmetry, respectively. Interestingly, some tailless phages consist of lipids that lead to low buoyant density and low sensitivity towards ether and chloroform (Ackermann, 2005; Novik et al., 2017).

Polyhedral phages can be further divided into *Microviridae, Corticoviridae, Tectiviridae, Leviviridae* and *Cystoviridae*. *Microviridae* phages are non-enveloped phages, while phages in *Corticoviridae* and *Tectiviridae* contain phospholipoprotein vesicle located within the capsid. *Tectiviridae* phage has a unique characteristic whereby its lipoprotein vesicle will transform into a tail-like tube after adsorption of phage to the host bacteria or after incubation with chloroform (Ackermann, 2005). This plays a role in nucleic acid ejection similar to the tailed phages. Next, phages in the family *Leviviridae* are similar to polioviruses in terms of their morphology and they are mostly known as plasmid-specific coliphages in which they will adsorb to the sex pili of the host bacteria. Lastly, *Cystoviridae* phages are the enveloped phages with lipid-containing envelopes surrounding icosahedral capsids (Ackermann, 2005).

Filamentous phages are composed of the family *Inoviridae*, *Lipothrixviridae* and *Rudiviridae*. Family *Inoviridae* can be categorized into two genera: *Inovirus*

which have filaments that are long, rigid or high flexibility; *Plectrovirus* which have filaments that are short and straight. *Lipothrixviridae* phages are in rod shape and composed of lipoprotein envelope and nucleosome-like core. *Rudiviridae* phages are non-enveloped phages with straight, rigid rod structure and noticeable fixation components at one end of the rod structure similar to tobacco mosaic virus (Ackermann, 2005).

Last but not least, pleomorphic phages can be classified into family *Plasmaviridae*, *Fuselloviridae* and *Sulfolobus* SNDV-like viruses. *Plasmaviridae* phages are composed of envelopes and a dense nucleoprotein granule without capsid, while *Fuselloviridae* phages are presented in spindle-shaped structure with attachment of short spikes at one end. Lastly, *Sulfolobus* SNDV-like viruses found in New Zealand are described as droplet-shaped and beehive-like structure with attachment of beard-like fibers at its pointed end (Ackermann, 2005). According to Stedman (2012), *Guttaviridae* and *Guttavirus* were assigned as family and genus names, respectively for *Sulfolobus* SNDV-like viruses.

2.2.2 Genome

Bacteriophages have a wide variety of genome size, which varies from approximately 3300 nucleotides to approximately 500 kilo base pair (kbp). Larger genome size results in larger capsid size in order to be packaged in the capsid for successful infectivity. However, too less or too much genomic content in the capsid will affect the virion stability. Therefore, the selection for genome

size will exert evolutionary pressure to the bacteriophage to either obtain or lose nucleic acid to determine virion stability (Hatfull and Hendrix, 2011).

Most of the bacteriophages are double-stranded DNA (dsDNA) phages, but some of the phages contain single-stranded DNA (ssDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Moreover, there are also a wide variety of nucleic acid conformation in each bacteriophage (Ackermann, 2005). The overview of the types of nucleic acid in bacteriophages is presented in Table 2.1. In the case of thermophilic bacteriophage, filamentous phages are the most common phages found to be resistant to high temperature. Within this group of bacteriophage, *Lipothrixviridae* and *Rudiviridae* were reported to be found in hot spring. Moreover, *Fuselloviridae* that is under pleomorphic phages were also found in hot spring. These three families of bacteriophages share a similarity of having DNA genome (Prangishvili et al., 2001).

Based on a study by Schoenfeld et al. (2008), most of the bacteriophages isolated from Yellowstone hot spring contain dsDNA, except inoviruses which are *Thermus*-specific contain ss DNA. Moreover, RNA-digestible substance was found in Yellowstone hot spring during preparation of phage nucleic acid, suggested that RNA phage may also be found in hot spring. Another study by Tamakoshi et al. (2011) had investigated phage ϕTMA isolated from Atagawa hot spring, Japan which is also a double-stranded DNA phage. In addition, phage TSP4 isolated from hot spring in Tengchong, China also contains double-stranded DNA genome (Lin et al., 2010).

Table 2.1: Overview of the types of nucleic acid in each family of bacteriophage (Ackermann, 2005).

Shape of Bacteriophage	Family	Types of Nucleic Acid
Tailed	*Myoviridae*	Linear dsDNA
	Siphoviridae	
	Podoviridae	
Polyhedral	*Microviridae*	Circular ssDNA
	Corticoviridae	Circular or supercoiled dsDNA
	Tectiviridae	Linear dsDNA
	Leviviridae	Linear ssRNA
	Cystoviridae	Linear or multipartite dsRNA
Filamentous	*Inoviridae*	Circular ssDNA
	Lipothrixviridae	Linear dsDNA
	Rudiviridae	
Pleomorphic	*Plasmaviridae*	Circular or supercoiled dsDNA
	Fuselloviridae	
	Guttaviridae	

2.2.3 Life Cycle

Bacteriophages are obligate intracellular parasites that infect only bacteria to amplify themselves by injecting their nucleic acid into the host bacteria. Interestingly, they are able to maintain their infectivity even in the absence of hosts, as long as they are not affected by external agents such as temperature and pH. Generally, bacteriophages can be categorized according to their amplification method, which are virulent or temperate phages. Virulent and temperate phages undergo lytic and lysogenic cycle, respectively as shown in Figue 2.2 (Guttman et al., 2005).

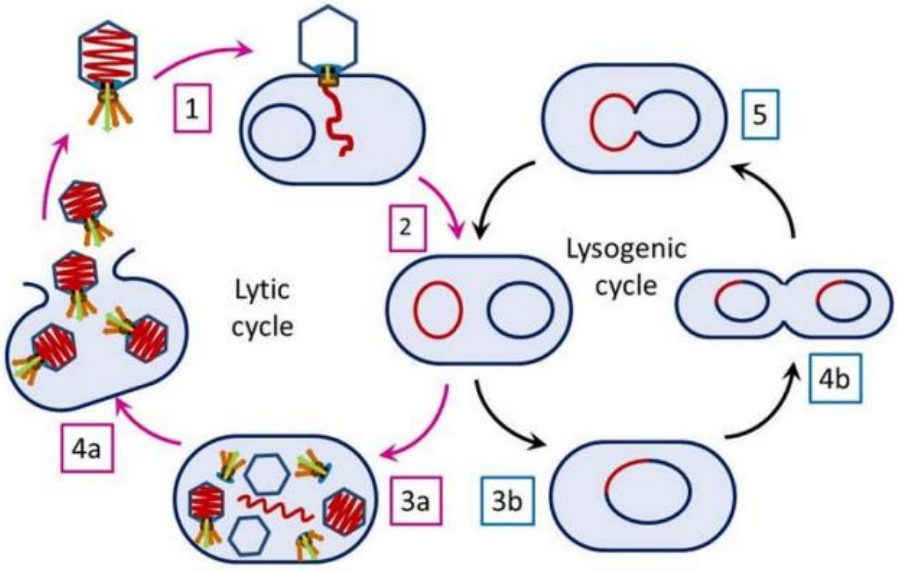

Figure 2.2: The overview of lytic and lysogenic cycle of bacteriophage (Adapted from Orlova, 2012).

Lytic cycle is the life cycle for virulent phage which it infects the host bacteria and causes cell lysis to release the progeny phages. It starts with adsorption

phase, whereby the phages are adsorbed to the surface molecules or capsules of the host bacteria by random collision using the tail fibers or spikes. For example, proteins, oligosaccharides or lipopolysaccharides of gram-negative bacteria can serve as the binding site for phages' tail fibers or spikes (Guttman et al., 2005; Kokare, 2008). When the attachment becomes irreversible, penetration phase occurs in which the tail tip of the phage penetrates through the peptidoglycan layer or even cell membrane of the host bacteria for injection of nucleic acid. This process may be facilitated by lysozyme found on the phage tail that digests a part of the bacterial cell membrane to aid the entry of the phage nucleic acid. Several defense mechanisms acquired by the phage are aiming to prevent the nucleic acid degradation by the bacterial host exonucleases and restriction enzymes. These including circularization of phage nucleic acid through protection of the two ends of nucleic acid, inhibition of bacterial host nucleases, presence of unique nucleotide and selection towards phage nucleic acid with the absence of restriction sites (Guttman et al., 2005; Kokare, 2008).

Subsequently, phage promoters stimulate the binding of host RNA polymerase to initiate transcription of the immediate early genes, which is useful in the prevention of degradation to phage nucleic acid. This prevention included degradation of host DNA, inactivation of host proteases and restriction enzymes. Next, middle genes are transcribed and translated into the proteins responsible in synthesizing new phage genome. Lastly, late genes undergo transcription and translation into phage components for the preparation of virion assembly (Guttman et al., 2005; Kokare, 2008). Overall, the transcription of phage genes

causes the host cell machinery to be transformed into phage-directed metabolism machinery. In the maturation phase, virion assembly occurs when the nucleic acid is packaged into procapsids and tail structure are then added to the phage "head" containing nucleic acid. Finally, the progeny phages are released from the bacterial cells via cell lysis. This process is aided by the lysozyme which is synthesized within the bacterial cell. Subsequently, a new lytic cycle starts again via the released phages that infect other bacterial cells (Guttman et al., 2005; Kokare, 2008).

Lysogenic cycle is used by temperate phage in which the integration of the phage genome into host genome is the key difference with the lytic cycle. A prophage is formed after the integration. It replicates simultaneously with the host genome in the bacterial host, which is now known as lysogenic bacterium. Lysogenic bacterium is highly resistant to the reinfection by the same or similar phages. However, this life cycle can be transformed into lytic cycle through induction by external agents such as ultraviolet light or certain chemicals that excise the phage genome and initiate the lytic cycle (Guttman et al., 2005; Kokare, 2008; Howard-Varona et al., 2017). During the transition to lytic cycle, the host DNA may be excised together with prophage genome and assembled into new virion for subsequent infection. This may cause evolutionary changes to the phage when the phage genome includes genome from bacterial host cells. The phage involved is known as transducing phage, which initiates a specialized transduction by mutating other cells with the host DNA. Generalized transduction can also occur when the phage is integrated into host genome

randomly and excise different parts of host genome in each infection (Guttman et al., 2005; Kokare, 2008; Howard-Varona et al., 2017).

Recently, a group of researchers suggested an inter-virus communication system named as arbitrium system, which is used by the phages for decision making of their fate in life cycle. This communication system was investigated using phage phi3T to infect *Bacillus subtilis*. It allows phages to predict the number most recent previous infections and choose whether to enter into lytic or lysogenic cycle. With that, phages can preserve their population by choosing lysogenic cycle since lytic cycle causes bacterial cell death, which eventually reduces the number of hosts for the phages (Erez et al., 2017; Harada et al., 2018).

2.3 Physiological Characteristics

2.3.1 One Step Growth Curve

Soon after the discovery of bacteriophage, the interactions between bacteriophage and its host have been demonstrated using one step growth curve as shown in Figure 2.3. This curve allows the understanding of bacteriophage life cycle starting from the entry of nucleic acid into the host until the release of new virions. The curve is divided into four phases: eclipse, latent, release and plateau phase (Guttman et al., 2005; Pelczar et al., 2010).

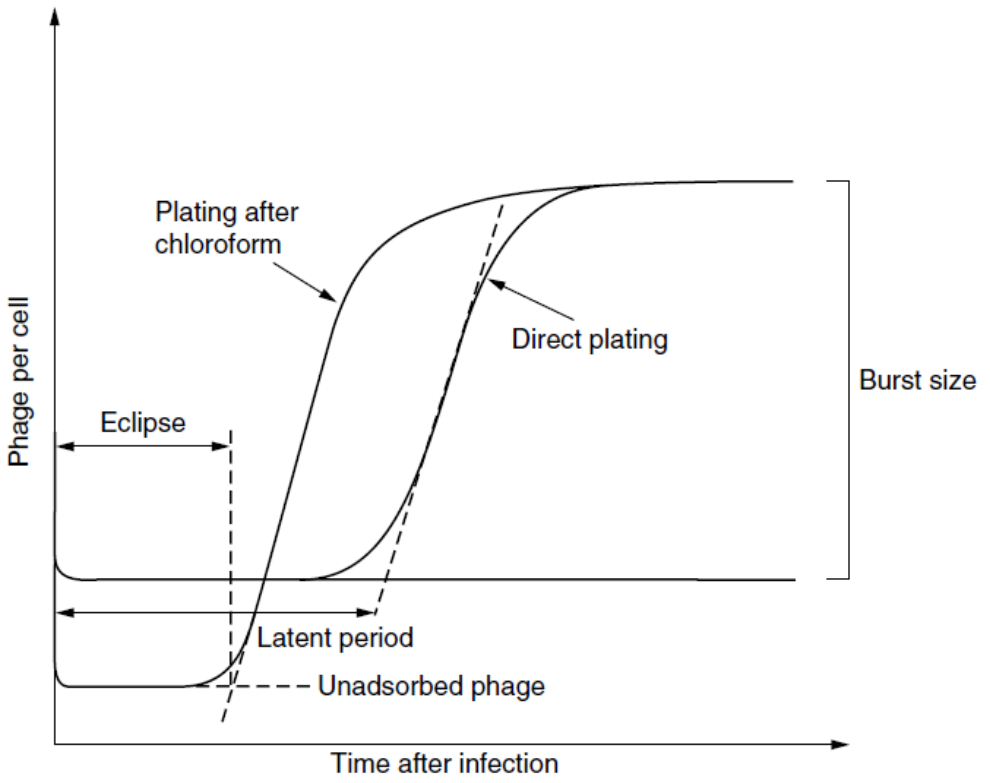

Figure 2.3: One step growth curve of bacteriophage (Adapted from Guttman et al., 2005).

Generally, phages are added into their growing host at a low multiplicity of infection (MOI) in order to make sure that one bacterial cell is infected with only one phage particle. After the phages are adsorbed to the host, the samples are removed at different time interval to perform plaque assay. This is to identify infective centers, whereby the plaques shown on the plate are caused by the burst of one infected host. At the end of the process, a graph is plotted to elaborate the production of virions against the duration of infection (Guttman et al., 2005; Pelczar et al., 2010).

For the first few minutes or longer, eclipse phase occurs whereby the number of plaques remain constant and the plaques formed at this phase are solely by the infected cells (one plaque equals to one infected cell). No phage particles are produced at this phase. After that, virions begin to accumulate inside the cell and ready to be released by cell lysis. The combination of eclipse phase and the moment before cell lysis occur is known as latent phase. When cell lysis occurs, the number of phage will increase drastically, thus this phase is known as the release phase because mature phages are released from the cell. Lastly, the number of phage reaches a constant count after the release phase, which is known as the plateau phase. Apart from that, burst size can be determined from one step growth curve by determining the ratio between the plaque-forming unit before and after cell lysis (Guttman et al., 2005; Pelczar et al., 2010).

Some bacteriophages have shorter latent phase compared to others which give more rapid cell lysis and infect other cells faster (Shao and Wang, 2008; Lau et al., 2012). A study conducted by Abedon et al. (2001) used mutant phage to investigate the relationship between host density and phage latent period. It was found that high host density was selective to phage RB69 that possess shorter latent period rather than longer latent period. Despite from this, it may cause insufficient burst size which is insufficient to infect other host bacteria (Shao and Wang, 2008; Lau et al., 2012). Abedon et al. (2001b) also showed that sta5 mutant phage which possessed shorter latent period has resulted in smaller burst size, quantitatively around 30% of the burst size of wild type phage.

In order to increase burst size, bacteriophage is expected to have longer latent phase to readily synthesize phage elements and assemble into mature phages (Shao and Wang, 2008; Lau et al., 2012). However, another study by Abedon et al. (2001a) showed that the use of different growth media affected the doubling time of the hosts, which in turn prolonged the latent phase. Luria-Bertani broth medium with added glucose (LBG) medium was used as a rich medium containing 0.4% glucose, while 0.4% glucose-based medium (GLU), 0.4% glycerol-based medium (GLY) and 0.4% acetate-based medium (ACET) were used as the poorer media to grow the hosts. Optimum latent phase associated with LBG medium was the shortest compared to other three media that produced longer latent phase. The results drew a conclusion that host quality has the direct effect on the optimum latent phase of phages, which affect the phage maturation and its productive infection. Thus, optimal latent phase and lysis time are ideal for eradication of targeted bacteria with optimal lysis time and number of mature phages (Shao and Wang, 2008; Lau et al., 2012).

Apart from that, the type of genome of the phage may give a different burst size. DNA phages produce burst size ranges from 10 to 100 while the burst size of RNA phages may reach 20,000 pfu (Biotech Khan, 2014). Based on a previous study by Sakaki and Oshima (1975), a hot spring DNA phage øYS40 exhibited a burst size of 80 phage particles per bacterial cell. Another study conducted by Lin et al. (2011) showed that DNA phage MMP17 isolated from hot spring had a burst size of 15 phage particles per bacterial cell.

2.3.2 Adsorption Rate

The first step of the infection cycle of a bacteriophage is the adsorption of phage onto the bacterial cell surface by phage tail fiber recognizing the receptors on bacteria cell surface. Adsorption rate is the characteristic of phage-host relationship on binding and it highly depends on the concentration of phage and bacterial host (Shao and Wang, 2008). Adsorption is crucial in determining the specificity of bacteriophage based on the receptors recognition by the phage tail fiber. It consists of two stages, which are reversible (positioning the baseplate) and irreversible (attachment to secondary receptor molecule) interactions. Irreversible binding is a crucial step for the nucleic acid to penetrate into host cells (Kutter et al., 2005; Rakhuba et al., 2010). Previous study by Baptista et al. (2008) demonstrated that binding of phage SPP1 to glucosylated techoic acid initiates the reversible interaction, which triggers the binding of the phage to membrane protein YueB for irreversible interaction. Absence of glucosylated techoic acid would not inhibit the irreversible binding, but lower rates of DNA injection into the host would occur. This shows the importance of both reversible and irreversible interaction between phage and host cell in yielding productive infection.

High host density in the environment results in a shorter search time for the phage to look for a host and attach to it. Since bacteriophages do not have independent motion characteristic, they adsorb to the host via random collision between phage and host, which can be explained by active mass law. The frequency of random collision increases when the concentration of phage and

host bacteria increases, and in turn raising the adsorption rate of bacteriophage. High adsorption rate will then cause shorter optimal lysis time needed by the phage (Shao and Wang, 2008; Rakhuba et al., 2010)

Gram-negative bacteria has a unique layer, which is lipopolysaccharide (LPS) that serves as bacteriophage receptor most of the time. Bacteriophages that recognize O-antigen of LPS contain enzyme that is localized at the end of the tail, which hydrolyzes a bond in O-antigen polysaccharide chain (Rakhuba et al., 2010). Phage φ1 is an example of the phage containing endo-1,3-N-galactoseaminidase that lyses the bond between polysaccharide chain on the cell surface of *Salmonella johannesbury* (Chaby and Girard, 1980). Phage SSU5 recognized core oligosaccharide (OS) antigen on *Salmonella* spp. whereby the truncation of the antigen caused decrease in phage infectivity (Kim et al., 2014). Apart from that, proteins on outer membrane of gram-negative bacteria can also serve as the receptor for bacteriophage (Rakhuba et al., 2010). For example, OmpC-LPS complex is the receptor for phage T4. It was shown by the absence of one or all receptors on *Escherichia coli* that led to increase resistance towards bacteriophage (Skurray et al., 1974; Yu and Mizushima, 1982).

On the other hand, cell wall of gram-positive bacteria is mainly composed of peptidoglycan, contributing to 40 to 90% of its dry weight. Apart from this, teichoic acids are also the hallmark components of gram-positive bacteria. They are hydrophilic polymers located perpendicularly to the plasma membrane and passing through the peptidoglycan layer (Rakhuba et al., 2010). For example,

phage SPP1 was found to attach to glucosylated poly(glycerolphosphate) cell wall techoic acid for reversible interaction. It was shown by the decline in phage infection on *Bacillus subtilis* mutant which has defected glucosylation of techoic acid (Baptista et al., 2008).

In some cases, phage receptors can be bacterial pili, flagella and capsular polysaccharides (Rakhuba et al., 2010). Flagellum-dependent phage iEPS5 was proven to bind to flagellum of *Salmonella* spp., which the mutated host on the gene encoding for flagellum led to unsuccessful adsorption of the phage (Choi et al., 2013). Phages that recognize pili as the receptor are the Ff class of filamentous bacteriophages including f1, M13, and fd phages that infect *E. coli* (Click and Webster, 1997). Another study conducted by Sørensen et al. (2011) showed that carbohydrate moiety of *Campylobacter jejuni* (O-methyl phosphoramidate) is the receptor of phage F336, with the use of resistant bacterial strain that is lack of O-methyl phosphoramidate.

2.3.3 Temperature

Temperature is an important factor for the survivability and growth of bacteriophages. Bacteriophage viability is highly dependent to temperature which affects adsorption, penetration, replication and perhaps the latent period of bacteriophage (Jończyk et al., 2011).

Phage capsid plays a vital role in protecting the phage genome from destruction in the environment (White et al., 2012). Disulfide bond between protein dimers on the capsid was found to be the major factor in maintaining phage stability under high temperature. For example, when PP7 virus-like particles (VLP) were treated with high temperature, one with reducing reagent dithiothreotol (DTT) and another one without reducing reagent. Virus-like particle that was treated with DTT showed reduced half-life to approximately 5 min at 67˚C, compared to untreated VLP that remained stable for 30 min (Caldeira and Peabody, 2007). Phages that were isolated from California hot spring also demonstrated the reduction of phage titer at boiling temperature, with approximately 18 to 30% of the phage particles remained stable after the treatment. In contrast, California hot spring phages were stable at 0˚C, with more than 75% of the phages conserved its infectivity (Breitbart et al., 2004).

Temperature that is lower than the optimal temperature will slow down the movement of phage, affecting the phage adsorption onto the host cell surface. Subsequently, lesser phages will be able to inject their genome successfully into the host for replication. Slower movement of phage can also prolong the latent phase and therefore lengthen the lysis time (Tey et al., 2009). A previous study by Tey et al. (2009) which fusion M13 phage had lower phage titer at 27˚C compared to 37˚C. Apart from that, lower temperature can cause phage tail contraction which affects the adsorption to the host cell surface. It was proven by Thorne and Holt (1974) which showed that phage CP-51 was unstable at 2 to 4˚C. In addition, rapid inactivation of phage CP-51 was observed at 0˚C. Tail

contraction of the phage was observed using electron microscopy after treatment with freezing temperature for 25 h and 144 h.

One of the bacteriophage group that could survive under harsh environment is *Thermus* phages. Their higher thermal stability and optimal survival temperature are the results of their adaptation to the high temperature environment. Inovirus PH75 was investigated by heat treatment at 75˚C for 24 h, which resulted in 0.0014% of phages remained after the treatment (Yu et al., 2006). Apart from that, two tectiviruses P37-14 and P78-76 were highly resistant to heat, whereby they survived after heat treatment at 80˚C for 24 h (Yu et al., 2006). Another study by Nagayoshi et al. (2016) demonstrated the thermal stability of phage φOH3 that infects *Thermus thermophiles* HB8. It was shown that phage φOH3 reached its highest survival rate at 70˚C for 1 h, followed by drastic reduction in the survival rate with increasing temperature. At the end of the experiment, the phage completely lost its viability at 100˚C.

Apart from *Thermus* phages that infect *Themus* strains, *Meiothermus* strains are closely related with *Thermus* genus. For instance, phage MMP17. The phage was highly stable at 55 to 60˚C, followed by a drastic reduction in infectivity with heat treatment at 70˚C (Lin et al., 2011). On the other hand, some phages are said to be intrinsically resistant to high temperature compared to *Thermus* phages, mainly due to their adaptations to heat. Example of intrinsically heat-resistant phage is filamentous phage PH65 isolated from mesophilic

environment. Although it infects mesophiles, it showed stability after heat treatment at 80˚C (Yu et al., 2006).

2.3.4 pH

pH is another environmental factor that affects bacteriophage stability. It is believed to disrupt electrostatic interaction that is involved in phage adsorption, by changing the charge state of the capsid. It will then resulted in lower propagation rate of bacteriophages (Tey et al., 2009; Nap et al., 2014).

The common pH range for most phages to obtain highest stability is between pH 5 to 9 (Hoa et al., 2014). T7 phage has the highest stability at pH 7, however it lost its infectivity at pH 3 after 1 h. In alkaline environment, T7 phage was highly unstable with very low infectivity at pH 10 compared to pH 9. For T2 phage, its infectivity reached the highest point around pH 5 to 6. Approximately 15% of the phages survived at pH 3 for 24 h. It was demonstrated that increase in acidity could cause coagulation of phage, whereas the condition was reversed with increase in alkalinity. Therefore, it was suggested that the irreversible coagulation at lower pH might affect the phage activity (Jończyk et al., 2011).

Several bacteriophages are stable across wide pH range. A study showed that P100 phage was stable over a wide pH range. It was only deactivated at pH less than 2 and pH higher than 12. Therefore, this phage is very useful in treating

food borne disease caused by *Listeria monocytogenes* (Fister et al., 2016). On the other hand, a T4-like phage which underwent pH treatment has resulted in less statistically significant of differences in phage titer. The less differences of phage titer across the pH gradient indicated the resistance of the phage against wide range of pH. Overall, the optimum pH for the T4-like phage viability and infectivity falls around pH 6 to 8, which is around neutral pH (Silva et al., 2013). A previous study by Yu et al. (2006) which investigated on pH sensitivity of *Thermus* phages demonstrated that myovirus P78-77 and inovirus PH75 were stable over a wide pH range from pH 2 to pH 13 and pH 3 to pH 13, respectively.

Hot spring phages possess a different pH sensitivity and optimal pH for survivability. Phage φOH3 and TSP4 showed that pH 7 was their optimum pH, with gradual loss of viable phages with increasing acidity and alkalinity of growth medium (Lin et al., 2010; Nagayoshi et al., 2016). On the other hand, phage MMP17 was found to survive over the pH range between pH 5 and pH 11. The optimum pH for the phage was from pH 6 to pH 7 (Lin et al., 2011). In summary, most of the hot spring phages were reported the most stable at neutral pH.

2.3.5 Effect of Organic Solvents on Phage

Organic solvent, which is hydrophobic and has hydrogen-bonding capacity, could affect phage's protein structure and stability. Specifically, the lipid framework of the phage will be altered and the van der Waals forces between

the genome and the coat protein will be weakened. As a result, phages could undergo denaturation when they are subjected to pure organic solvents treatment (Turkyilmaz et al., 2009; Moghimian et al., 2016).

For example, Pf1 phage, a filamentous phage was inactivated by diethyl ether, acetone, methanol and chloroform. On the contrary, *fd* phage, another type of filamentous phage was not affected by any of the solvents above except chloroform (Olofsson et al., 2001). M13 phage was also tested on its stability under different organic solvents, and it was found that the phage can only be denatured by polar organic solvents such as chloroform and tetrahydrofuran (Moghimian et al., 2016). KHP30 phage targeting *Helicobacter pylori* is highly sensitive to chloroform and diethyl ether, which the researchers proposed that the phage contains lipids (Uchiyama et al., 2013)

Apart from the alteration of lipid framework, chloroform-water interface causes contraction of filamentous phage's filament into a short and compact rod, known as I-form which is more sensitive to detergent compared to S-form (Olofsson et al., 2001). Phage M13 is a great example for filamentous phage, which I-form was observed using electron microscopy after chloroform treatment, indicating the contraction of phage particles occurred (Moghimian et al., 2016).

However, some phages may be resistant to organic solvents. Phage øZE1 is resistant to chloroform, with the observation of constant phage titer before and

after treatment (Askora et al., 2015). In another finding, hot spring phage TSP4 was found to be resistant to chloroform and sensitive to 0.3% Triton X-100. This outcome suggested the absence of lipid component in the phage capsid (Lin et al., 2010).

2.3.6 Stability

Phage activity is highly dependent on the storage temperature in order to achieve its maximum level after long term storage for further use in laboratory. Tailed phages including T4, T5 and T7 can survive for the longest period among other phages under permanent storage, which viability can still be observed after 10 to 12 years of storage at 4˚C. This statement is in line with a study on T4-like *Shigella* phage C16 which showed the remaining titer up to 10^3 after 32 years of storage at 4˚C (Ackermann et al., 2004). However, lipid-containing phages are sensitive to 4˚C storage but not -80˚C storage. For example, tectiviruses Bam35 and AP50 lost 8 logs of phage titer within one year of storage at 4˚C (Ackermann et al., 2004).

Storage at 4˚C and -80˚C has been suggested as the optimum temperatures for short term and long term storage (Jończyk et al., 2011). A common phage, λ phage, possessed a good stability at 4˚C for 6 months, but not at 42˚C and 37˚C (Jończyk et al., 2011). On the other hand, 80% and 43% of MS2 phages remained stable after 8 days of storage at 4˚C and -80˚C, respectively. However, after 290 days, phages stored at -80˚C experienced lower extent of reduction of

phage particles compared to 4˚C storage (Olson et al., 2004). A study by Mendez et al. (2002) was investigated on phage MS2, B40-8, 196 and (PHI)X174 in 5% to 10% (v/v) glycerol storage. After 30 days of storage at −70˚C and −20˚C, there was little or no change in phage infectivity.

2.3.7 Host Range

The host specificity of a bacteriophage is highly dependable on the binding proteins on the host bacterial cell surface, biochemical interactions between phage and host bacteria, involvement of prophage or plasmids, phage resistance mechanisms mediated by host bacteria and the physiological state of host bacteria. Generally, the factors above can determine the host bacteria's susceptibility to certain phage infections and the productivity of the infection (Marcó et al., 2015; Ross et al., 2016).

As mentioned earlier, bacteria can develop resistance towards phage. This can be divided into adsorption resistance and abortive infections. Adsorption resistance uses a mechanism whereby the phage adsorption process is blocked by reducing the number of receptors on bacteria. On the other hand, abortive infections happen when both phage and bacterial host die after infection. It was also shown that resistance mechanisms developed in bacteria could limit the phage host range over time (Hyman and Abedon, 2010).

Some of the phages have wide host range (polyvalence), which can infect various bacterial species or bacterial strains. One of the well-known example of phage with wide host range, phage Mu, which infect *Escherichia coli*, *Shigella sonnei*, *Citrobacter freundii*, *Enterobacter* and *Erwinia*. In this case, phage resistance mechanism mediated by the bacteria may cause the phage to undergo evolution to overcome the resistance. Thus, each phage may not have a constant host range over a long period of time (Ross et al., 2016). As mentioned earlier, involvement of plasmids in phage infection also greatly influence the broad host range of certain phage. Phage-plasmid interaction may cause horizontal gene transfer (HGT) between bacterial species in a population, which resulted in more bacteria to become more susceptible to phage infection (Koskella and Meaden, 2013).

In contrast, phages with a narrow host range could only infect a bacterial species with few different strains only. There is an evidence stated that phage's narrow host range is determined by the bacterial population in a certain environment that allows "adaptations" made by phages for survival (Koskella and Meaden, 2013). For example, phage AZ1 is only effective against *Pseudomonas aeruginosa*-2995, *Pseudomonas aeruginosa*-2949, *Pseudomonas aeruginosa*-37, *Pseudomonas aeruginosa*-2941, *Escherichia coli* CR-061 and *Achromobacter xylosoxidans*, which generally only infect strains within species. Moreover, phage AZ1 is also known as highly specific phage that shows lowest affinity to other receptors not found on the strains above (Jamal et al., 2017).

2.4 Applications of Bacteriophage

Phage has been widely studied to explore the possible applications in various fields. This included different biotechnological applications such as human anti-biotherapy and environment disinfection. The unique characteristics of phage including high specificity towards specific bacterial species and insertion of phage genome into host genome, that allows various applications of the phage in different fields (Harada et al., 2018).

In human, phages can be used in phage therapy to replace conventional antibiotics, which no longer effective against many bacterial species. Phage therapy provides several benefits to human over antibiotic treatment. First and foremost, unlike antibiotics, phage is only targeting the bacteria, which are within its host range, thus results in fewer side effects to the patient. Next, the administration of phage into the body does not require booster dose or continuous administration as it will replicate and produce large amount of progeny phages, as long as its host bacteria are still present. Phages can be administered to human via oral, topical or inhalation, depending on the phage dynamic kinetics (similar to pharmacodynamics and pharmacokinetic) (Harada et al., 2018). In Georgia, phage therapy is common in healthcare field. One of the phage involved in phage therapy is *Intestiphage* that is specific to 23 enteric bacterial species, *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from the gastrointestinal tract. It is widely used to treat traveler's diarrhea and other gastrointestinal complications (Kutter et al., 2010).

Bacteriophage display technique has been used to display exogenous peptides or proteins. This technique is commonly used in various researches on vaccine development, novel therapeutics research on various diseases, bioimaging and biosensing, nanotechnology and delivery of target particles (such as antigen) to a target site. There are three methods to display the desired protein on the surface of phage particle such as molecular manipulation, bioconjugation and competitive phage display (Harada et al., 2018). Molecular manipulation involves splicing of exogenous gene into phage genome encoding for structural protein. Subsequently, the gene fusion will occur and target protein will be displayed on the phage particle Phage M13 is an example of phage display which targets tumor cells and identify the peptides that are specific to the tumour (Henry and Debarbieux, 2012). For bioconjugation, target proteins are displayed by attachment onto surface of phage particle without engineering the phage genome. Phage MS2 was shown to be an ideal delivery agent for hybrid drug attached on the surface (Kovacs et al., 2007). Lastly, competitive phage display involves infection of engineered host cells by the phage, which synthesizes both wild type and fusion proteins. In the end, both types of proteins are incorporated into phage capsid randomly (Harada et al., 2018).

Phage can also acts as a vaccine carrier which can be divided into phage display vaccine and bacteriophage DNA vaccine. Phage display vaccine involves surface-coated immunogenic phage particles with foreign antigens. Phage T4 was investigated for the ability to induce antibodies in murine model, with its phage particle coated with proteins gp23, gp24, Hoc and Soc. Antibodies was successfully induced after around 4 weeks of phage administration (Majewska

et al., 2015). On the other hand, phage DNA vaccine involves incorporation of foreign DNA into the phage and allows phage to transfer the foreign gene to the target cells. Subsequently, the expression of gene encoding immunogenic antigen will be carried out and may induce immune responses (Harada et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Phage Sample and Bacterial Strains

Bacteriophage LT-B isolate used throughout this study was isolated from Lubuk Timah hot spring, Perak (Coordinates: N04 33.51' E101 9.89'). The temperature of the hot spring is at 50˚C and the pH level is at 7.4. *Escherichia coli* BL21 was used as the bacterial host for phage LT-B throughout this study. The other bacterial samples including Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E.coli* (EPEC), *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella sonnei*, *Shigella flexneri* and *Shigella dysenteriae* were obtained from Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman.

3.2 Amplification and Growth of Bacteriophage

3.2.1 Preparation of Luria Bertani (LB) Agar Plates

The LB agar plates were prepared by dissolving 7 g of LB agar powder in 200 mL of distilled water. The mixture was then mixed well and autoclaved. Then, the sterile LB agar was poured into sterile petri dishes and allowed to solidify at room temperature. The solidified LB agar plates were stored at 4˚C until further use.

3.2.2 Preparation of Luria Bertani (LB) Broth

The LB broth was prepared by dissolving 8 g of LB broth powder in 400 mL of distilled water. The mixture was then mixed well and autoclaved. The sterile LB broth was stored at room temperature for further use.

3.2.3 Preparation of Top Agar

The top agar was prepared by dissolving 1.4 g of LB agar powder and 4 g of LB broth powder in 200 mL of distilled water. The mixture was then mixed well and autoclaved. The sterile top agar was stored at room temperature. Prior to use, the top agar was heated using a microwave oven until the solution become liquefied. Then, 3 mL of the top agar was aliquoted into each 15 mL centrifuge tubes and placed into 60˚C water bath until use.

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

Tris-buffered saline (TBS) was prepared by dissolving 4.84 g of Tris powder and 7.008 g of NaCl in 600 mL distilled water. The pH was adjusted to pH 7.6 by using 1 M HCl. Next, the solution was topped up to 800 mL with distilled water and autoclaved.

3.2.5 Preparation of Log-phase *Escherichia coli* (*E. coli*) Culture

The *E. coli* glycerol stock was streaked onto a LB agar plate and incubated for 16-18 h at 37˚C. Next, a single colony was picked and inoculated into 4 mL LB broth, followed by incubation at 37˚C for 16-18 h with agitation speed at 200 rpm. On the next day, 100 µL of overnight *E. coli* culture was inoculated into 4 mL LB broth (1:40 ratio) and incubated for 2.5 h at 37˚C with agitation speed at 200 rpm until OD_{600} reached between 0.6 and 0.8.

3.2.6 Plaque Assay

Plaque assay was carried out by first preparing several 15 mL centrifuge tubes with 3 ml top agar in each tube and placed them in a 60˚C water bath. Then, phage stock was serially diluted with 10-fold dilution by using TBS buffer. A volume of 10 µL diluted phage and 200 µL of log phase *E. coli* culture were added into the melted top agar. The mixture was mixed by a vortex mixer and poured onto the bottom of LB agar plate as base. The agar was swirled gently to ensure a complete coverage of top agar on the agar plate and then allowed to

solidify. Next, the agar plate was sealed with parafilm and incubated with inverted position at 37˚C for 16-18 h. On the next day, the number of plaques formed on the agar was calculated and the titer was recorded as plaque forming unit per mL (pfu/ mL) using the following formula:

$$Pfu/mL = \frac{Number\ of\ plaques}{Dilution \times volume\ of\ diluted\ phage\ added\ (mL)}$$

3.2.7 Phage Amplification

A single plaque was picked from the agar plate of plaque assay using sterile micropipette tip and the plaque was resuspended in 1 mL of TBS buffer in sterile microcentrifuge tube. The sample was kept at 4˚C for approximately 3 h. Next, 3 mL of the top agar was aliquoted into sterile 15 mL centrifuge tubes and placed in 60˚C water bath. Then, 200 µL of log-phase *E. coli* culture and 500 µL of phage-containing TBS were added into each tube containing melted top agar. The mixture were mixed with a vortex mixer before pouring the mixture onto the LB agar plate. The agar plates were swirled gently and allowed to solidify. The agar plates were incubated at 37˚C for 16-18 h. In this step, a negative control plate was prepared with 200 µL of log-phase *E. coli* culture and 3 mL top agar onto the base agar plate.

On the next day, 5 mL of TBS buffer was added onto the agar plates and stored at 4˚C for approximately 3 h to allow diffusion of the phage from the agar medium into TBS. In the meantime, 2 mL of overnight *E. coli* culture was added into 40 mL of LB broth (1:20 ratio) and incubated with agitation speed of 200

rpm at 37˚C until it reached log phase. After obtaining the log phase culture, the TBS buffer containing diffused phages from two agar plates was pipetted into 1.5 mL microcentrifuge tubes and centrifuged at 17000 x *g*, 4˚C for 5 min. Then, the supernatant was pipetted into the log phase *E. coli* culture and incubated it with agitation speed of 200 rpm at 37˚C until the OD_{600} value become constant or white clumps of dead cell debris is present in the culture. Next, the culture was centrifuged at 8600 x *g* for 15 min and the supernatant was collected into sterile centrifuge tubes and stored at 4˚C.

For large scale amplification, 20 mL of overnight *E. coli* culture was added into 500 mL of LB broth and incubated at 37˚C with 200 rpm agitation speed until it reached log phase. Next, the log-phase *E. coli* culture was added with the supernatant containing the phage collected and incubated at 37˚C with 200 rpm agitation speed. After the OD_{600} reached its constant value at 0.5, the culture was centrifuged at 8600 x *g* for 15 min. The supernatant was then collected to perform phage precipitation with PEG as described in Section 3.3.2.

3.3 Phage Precipitation

3.3.1 Preparation of 20% (w/v) Polyethylene Glycol 8000 (PEG 8000) containing 2.5 M NaCl

The PEG 8000 containing 2.5 M NaCl was prepared by dissolving 80 g of PEG 8000 and 58.44 g of NaCl in 200 mL of distilled water. The mixture was then

stirred and heated gently using a hot plate stirrer to dissolve the PEG powder. Next, the solution was topped up to 400 mL before sending it for autoclave.

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

The PEG 8000 with 2.5 M NaCl and phage-containing supernatant from Section 3.2.7 were added into a beaker. The volume of PEG 8000 with 2.5 M NaCl added was 20% of total volume of the supernatant. Then, the mixture was stirred at 4˚C by using a magnetic stirrer for 16 to 18 h. The next day, the mixture was centrifuged at 8600 x *g* at 4˚C for 30 min to collect the pellet. The pellet was washed with 10 mL of TBS and centrifuged with the same setting as before. Lastly, the pellet collected was dissolved in 1 mL of TBS and filtered with 0.45 µm syringe filter into a sterile 1.5 mL centrifuge tube. The sample was stored at 4˚C until further use. Plaque assay was performed by using serially diluted PEG-precipitated phage to determine the phage titer.

3.4 Physiological Characterization of Bacteriophage

3.4.1 One Step Growth Curve of Bacteriophage

The MOI of 0.00007 was used to study the one step growth curve of the phage. A volume of 10 µL of PEG-precipitated phage (serially diluted to 10^{-5} dilution) was added to 200 µL of log-phase *E. coli* culture and incubated at 37˚C with agitation speed of 200 rpm. Next, 10 µL of the sample was aliquoted every 5 min interval and added into 3 mL of top agar and 200 µL of log-phase *E. coli*

culture to perform plaque assay. The burst size of the phage was calculated using the following formula:

$$Burst\ size\ (phages\ per\ cell) = \frac{Final\ phage\ titer\ of\ release\ phase}{Initial\ phage\ titer\ of\ release\ phase}$$

3.4.2 Adsorption Rate of Bacteriophage

The MOI of 0.002 was used to study the adsorption rate of the phage. A volume of 10 µL of PEG-precipitated phage (serially diluted to 10^{-3} dilution) was inoculated into 1 mL of log-phase *E. coli* culture and incubated at 37˚C with agitation speed of 200 rpm. A volume of 100 µL of the sample was aliquoted from the culture into a sterile 1.5 mL microcentrifuge tube at 2nd, 5th, 8th, 12th, 16th and 20th min. The sample was centrifuged at 13,000 rpm for 2 min to collect the supernatant. Lastly, 10 µL of the supernatant was added into 3 mL of top agar and 200 µL of log-phase *E. coli* culture to perform plaque assay. The adsorption rate was calculated using the following formula:

$$Adsorption\ rate\ (\%) = \frac{Initial\ phage\ titer - Final\ phage\ titer}{Initial\ phage\ titer} \times 100\%$$

3.4.3 Effect of Temperature on Phage Infectivity

A volume of 200 µL of log-phase *E. coli* culture was added into 3 mL top agar, and then poured onto the LB agar plates to solidify. Fifty microliters of PEG-precipitated phage was aliquoted into three microcentrifuge tubes and incubated at different temperatures: 60˚C, 65˚C and 70˚C for 1 h. A volume of 2 µL from

each tube was aliquoted every 10 min and spotted onto the LB agar plates. The spotted agar plates were then incubated at 37˚C for 16 – 18 h.

3.4.4 Effect of pH on Phage Infectivity

The TBS buffer with pH 3 to 11 were prepared in universal bottles. Next, 10 µL of PEG-precipitated phage was added into microcentrifuge tubes containing 90 µL of TBS at pH 3, 5, 7, 9 and 11 respectively, followed by incubation at room temperature for 1 h. Ten microliters of mixture from each tube was inoculated into 3 mL top agar containing 200 µL of log-phase *E. coli* culture to perform plague assay. The agar plates were incubated at 37˚C for 16 – 18 h.

3.4.5 Effect of Organic Solvent on Phage Infectivity

Chloroform with the concentrations of 2% to 10% (v/v) were prepared in five microcentrifuge tubes. Two hundred microliters of log-phase *E. coli* culture was added into 3 mL of top agar and poured onto the LB agar plate. Then, 6 µL of PEG-precipitated phage was added to each microcentrifuge tubes containing 54 µL of chloroform with concentrations of 2%, 4%, 6%, 8% and 10%, followed by incubation at room temperature for 5 min. Two microliters of mixture from each tube was aliquoted and spotted on the solidified agar plate for 16 – 18 h of incubation at 37˚C.

3.4.6 Stability of Phage

Fifty microliters of PEG-precipitated phage was transferred into four 1.5 mL microcentrifuge tubes and placed at different temperatures: -80˚C, -20˚C, 4˚C and 25˚C. Phage titer was accessed via plaque assay, which was carried out every week for a duration of 1 month. This was done by adding 10 µL of sample at different temperatures and 200 µL of log-phase *E. coli* culture into 3 mL of top agar. The solidified agar plates were incubated at 37˚C for 16 – 18 h.

3.4.7 Host Range

A single colony from ten different culture plates: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E.coli* (EPEC), *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella sonnei*, *Shigella flexneri* and *Shigella dysenteriae* were inoculated into 4 mL of LB broth and grown for 16 – 18 h. On the next day, 100 µL of overnight cultures of each bacterial strain was added into 4 mL of LB broth and incubated at 37˚C with agitation speed of 200 rpm until OD_{600} reached between 0.6 and 0.8. Ten microliters of PEG-precipitated phage and 200 µL of log-phase culture of each bacterial strain was added into 3 mL top agar. The mixture was mixed well and poured onto the LB agar plates and followed by incubation at 37˚C for 16 – 18 h.

3.5 Genomic Characterization of Bacteriophage

3.5.1 Preparation of 0.5 M Ethylenediaminetetraacetic Acid (EDTA)

The EDTA solution was prepared by dissolving 3.722 g of EDTA powder in 10 mL of distilled water. The pH of the solution was adjusted to pH 8 and topped up to 20 mL with distilled water.

3.5.2 Preparation of 10% Sodium Dodecyl Sulfate (SDS)

Ten percent SDS solution was prepared by dissolving 2 g of SDS powder in 10 mL of distilled water. The solution was heated gently and stirred by using magnetic stirrer until it dissolved. Lastly, the solution was topped up to 20 mL with distilled water.

3.5.3 Preparation of 3 M Sodium Acetate

Sodium acetate solution was prepared by dissolving 4.922 g of sodium acetate powder in 10 mL of distilled water. The pH of the solution was then adjusted to pH 8 using 1 M glacial acetic acid. Lastly, the solution was topped up to 20 mL with distilled water prior to autoclave.

3.5.4 Preparation of 1 M Magnesium Chloride ($MgCl_2$)

The solution was prepared by dissolving 1.9042 g of $MgCl_2$ powder in 10 mL of distilled water. Then, the solution was mixed well and topped up to 20 mL with distilled water prior to autoclave.

3.5.5 Preparation of 10X and 1X Tris-Acetate EDTA (TAE) Buffer

The 1X TAE buffer was prepared by dissolving 48.4 g of Tris base powder, 11.4 mL of glacial acetic acid and 20 mL of 0.5 M EDTA in 800 mL of distilled water. The solution was mixed well and topped up to 1 L with distilled water. To obtain 1X TAE buffer, 100 mL of 10X TAE buffer was added into 900 mL of distilled water.

3.5.6 Phage Nucleic Acid Extraction Using Phenol-Chloroform-Isoamyl Alcohol (PCI) Method

One milliliter of PEG-precipitated phage was transferred into a sterile 15 mL centrifuge tube, followed by addition of 12.5 µL of 1 M of $MgCl_2$. After the mixture was mixed well, 0.4 µL of DNAse I and 1 µL of RNAse A were added to the mixture. The mixture was mixed briefly using a vortex mixer and incubated at room temperature for 30 min. Then, 40 µL of 0.5 M EDTA, 5 µL of Proteinase K and 50 µL of 10% SDS were added according to the order, followed by mixing it vigorously with a vortex mixer. The mixture was incubated in a 55˚C water bath for 1 h and mixed vigorously at every 20 min interval. Next, the mixture was transferred into two 1.5 mL microcentrifuge tubes with each tube containing 500 µL of the mixture. The tubes were added with 500 µL of PCI solution each and mixed well by inverting the tubes. The mixtures were centrifuged at 13,000 rpm at room temperature for 5 min to collect the top aqueous layer into sterile 1.5 mL microcentrifuge tubes. The aqueous layer was added with 1 mL of 95% ethanol and 50 µL of 3 M sodium acetate, followed by incubation on ice for 5 min. After the incubation on ice, the mixture

was mixed gently before centrifugation at room temperature at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed by 500 µL of 70% ethanol. The mixture was centrifuged once again at 13,000 rpm at room temperature for 10 min. The supernatant was discarded and the pellet was air dried. The pellet was then dissolved in 50 µL TE buffer and stored at -20˚C until use.

3.5.7 Nucleic Acid Quantification Using Nano Spectrophotometer

Nano spectrophotometer was used to measure the nucleic acid concentration and absorbance at wavelengths of 260 nm and 280 nm. The nano spectrophotometer was first calibrated and the cell holder was inserted with submicroliter cell. Next, 1 µL of TE buffer was used as a blank before wiping the measurement window for the loading of 1 µL nucleic acid sample into it. The lid with 1 mm of path length was placed on top of the measurement window before the reading was recorded.

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

The agarose gel was prepared by dissolving 1.5 g of agarose powder in 15 mL of 1X TAE buffer and heated it in a microwave oven until the powder dissolved completely. The mixture was poured into the casting tray after its temperature has slightly reduced. The comb was inserted gently and the gel was allowed to solidify. After the gel has solidified, the comb was removed carefully and the gel was placed in the gel tank filled with 1X TAE buffer.

3.5.9 DNAse and RNAse Digestion of Phage Nucleic Acid

The nucleic acid digestion with DNAse and RNAse was performed to determine the genome type of the phage. The reaction mixture for DNAse digestion, RNAse digestion and negative control were prepared based on Table 3.1.

Table 3.1: DNAse and RNAse digestion reaction mixtures.

Components	DNAse Digestion	RNAse Digestion	Negative Control
Bacteriophage Nucleic Acid (µL)	2.0	2.0	2.0
10X Buffer Tango with BSA (µL)	0.5	-	-
DNAse (µL)	0.2	-	-
RNAse (µL)	-	0.7	-
Sterile Distilled Water (µL)	2.3	2.3	3.0
Total (µL)	5.0	5.0	5.0

The reaction mixtures were incubated at 37˚C for 1 h. Five microliters of 1 kb DNA ladder and each of the reaction mixtures were each mixed with 1 µL of novel juice before loading them into their respective wells. The gel was subjected to electrophoresis at 80 V for 40 min and it was then visualized using UV transilluminator.

3.5.10 Restriction Enzyme Digestion of Phage Nucleic Acid

The extracted phage nucleic acid was subjected to enzymatic digestions by five different restriction enzymes: *Bam*HI, *Eco*RI, *Eco*RV, *Sac*I and *Xba*I. Plasmid pBR322 was used as positive control of *Bam*HI, *Eco*RI and *Eco*RV digestion, while plasmid pUC19 was used for positive control of *Sac*I and *Xba*I digestion. The reaction mixtures for each restriction enzyme digestion were prepared according to Table 3.2.

Table 3.2: Restriction enzyme digestion reaction mixtures.

Components	Volume (µL)
Bacteriophage Nucleic Acid	2.0
Restriction Enzyme	0.5
Restriction Enzyme Buffer	1.0
Sterile Distilled water	6.5
Total	10.0

The reaction mixtures were incubated at 37˚C for 1 h and gel electrophoresis was then carried out. Five microliters of 1 kb DNA ladder and each of the reaction mixtures was mixed with 1 µL of novel juice before loading them into their respective wells. The voltage used for the gel electrophoresis was 80 V for a duration of 40 min. The results of the electrophoresis was viewed using UV transilluminator.

CHAPTER 4

RESULTS

4.1 Amplification and Precipitation of Bacteriophage

Phage LT-B was successfully amplified and precipitated from phage stock. The resulted phage titer after amplification and precipitation process was 2.57 $x10^{10}$ pfu/ mL which was further diluted for subsequent analysis.

4.2 Physiological Characterization of Bacteriophage

4.2.1 One step Growth Curve of Bacteriophage

One step growth curve of phage LT-B was obtained and plotted as depicted in Figure 4.1. The graph is similar to the classic one step growth curve, which also consists of eclipse, release and plateau phases. The MOI used in the assay was 0.00007. The whole process of one step growth of phage LT-B took 50 min to complete.

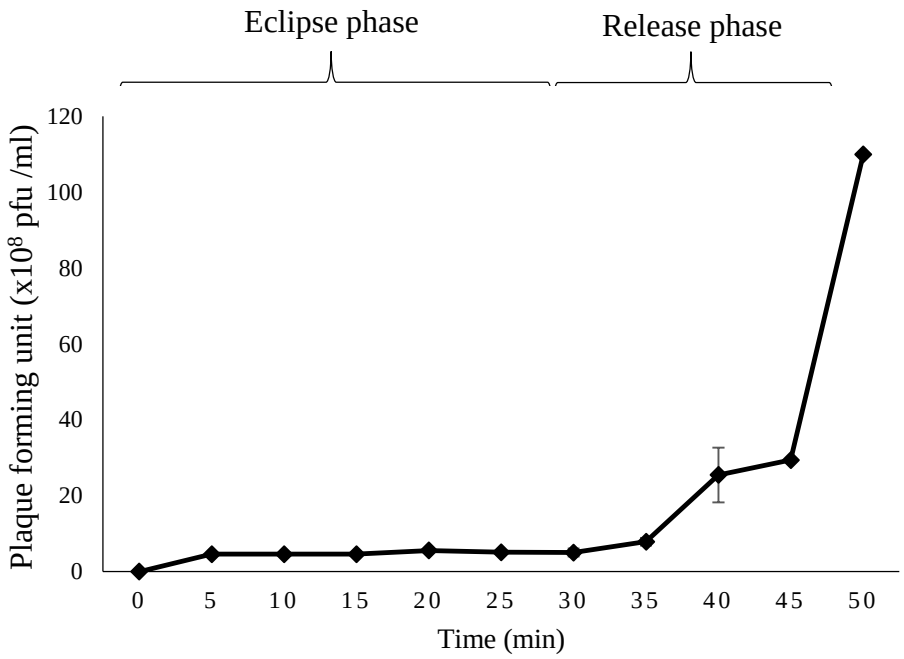

Figure 4.1: One step growth curve of phage LT-B. Error bars represent the standard deviation of data.

Based on Figure 4.1, the eclipse phase of the phage took 30 min before proceeded to release phase. After 30 min, the phage titer increased drastically, which indicated the release of progeny phages from the host cells. The burst size for phage LT-B was 22 phages per bacterium during the release phase.

4.2.2 Adsorption Rate of Bacteriophage

The adsorption rate of phage LT-B at MOI of 0.002 was obtained and a line graph was plotted, as shown in Figure 4.2, to summarize the adsorption rate of the phage at different time interval. Most of the phages were attached to the surface of host cells within the first 2 min of incubation with 91.3% of adsorption rate, followed by gradual increase of adsorption rate to 95.76% at the 20th min.

Generally, Figure 4.2 shows a rapid adsorption of phages to the surface of host cells soon after the phages were added into the host cell culture.

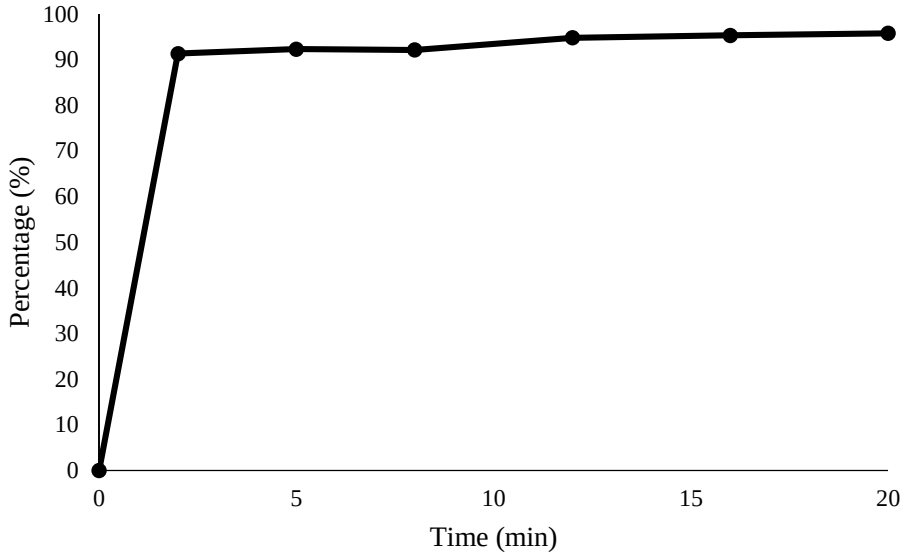

Figure 4.2: Adsorption rate of phage LT-B at different time interval. Error bars represent the standard deviation of data.

4.2.3 Effect of Temperature on Phage Infectivity

Phage LT-B was incubated separately at 60˚C, 65˚C and 70˚C and their viability was tested at 10-min interval. Table 4.1 illustrates the results of this assay, whereby the phage could survive up to 10-min of incubation at 70˚C. The subsequent spotting of phage on the bacterial lawn did not result in lysis as no clear plaque was observed, indicating a complete loss of viability of phage LT-B after 20 min of heat treatment at 70˚C.

Table 4.1: Effect of temperature on phage LT-B infectivity.

Temperature	**Time (min)**					
(˚C)	**10**	**20**	**30**	**40**	**50**	**60**
60	√	√	√	√	√	√
65	√	√	√	√	√	√
70	√	X	X	X	X	X

(√) presence of phage infectivity
(X) absence of phage infectivity

4.2.4 Effect of pH on Phage Infectivity

Phage LT-B was incubated at room temperature at different pH, ranging from pH 3 to 11. Figure 4.3 shows there was no significant difference in phage titer obtained from each pH. The highest phage titer was observed at pH 7 (9.55×10^9 pfu/ mL), followed by pH 3, pH 9, pH 5 and pH 11 (6.23×10^9 pfu/ mL). This results indicated that pH 7 is the optimal pH for phage LT-B, instead of acidic or alkaline environment. There was a slight decrease in phage titer when the pH became more acidic or alkaline. A drop of 9.97% and 23.5% in phage titer was observed at pH 3 and pH 5 in comparison to pH 7. For alkaline pH, a reduction of 30.0% and 35.2% in phage titer was observed at pH 9 and pH 11. Overall, phage infectivity in alkaline medium was lower compared to their infectivity in acidic medium as the lowest phage titer was observed at pH 11, which was 6.23 $\times 10^9$ pfu/ mL.

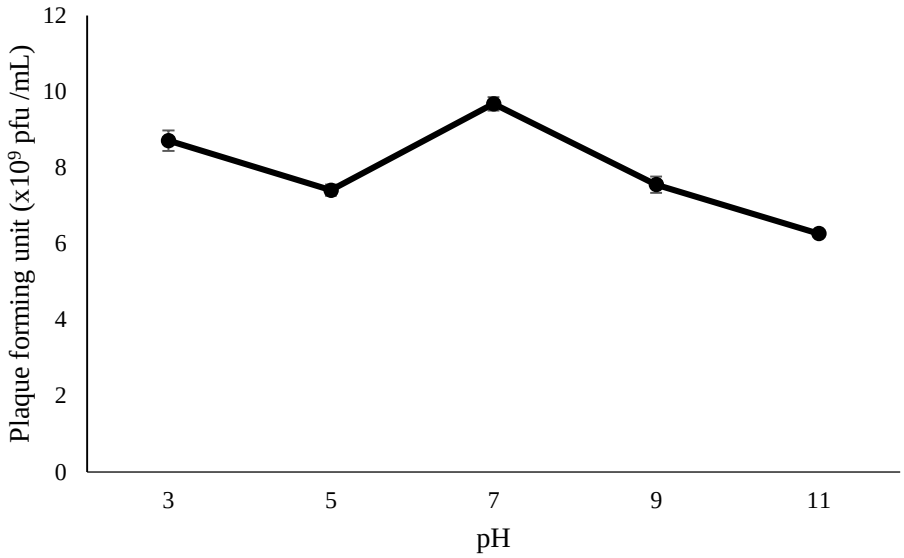

Figure 4.3: Effect of pH on phage LT-B infectivity. Error bars represent the standard deviation of data.

4.2.5 Effect of Organic Solvents on Phage Infectivity

Spot lytic assay was carried out to observe the infectivity of phage LT-B after incubation in different concentrations of chloroform. Based on Table 4.2, phage LT-B could survive up to 10% (v/v) chloroform as spot lysis could be seen on the bacterial lawn. Negative control showed no spot lysis and this has proven that the lyses formed by chloroform-treated phage but not due to chloroform. The images of plaque assay for this assay are located in Appendix C.

Table 4.2: Effect of chloroform on phage LT-B infectivity.

Concentration of chloroform (v/v)	Infectivity
2%	+
4%	+
6%	+
8%	+
10%	+

(+) presence of phage infectivity
(-) absence of phage infectivity

4.2.6 Stability of Phage

The stability of phage LT-B was evaluated by using different storage temperatures, which were at -80˚C, -20˚C, 4˚C and 25˚C for 28 days. The results obtained throughout the duration are shown in Figure 4.4. Storage at 4˚C showed the highest stability, followed by -80˚C, -20˚C and 25˚C. A significant decline in phage titer stored at 25˚C was observed whereby no plaque was observed after 1 week of storage. The decrease in phage titer was less significant for storage at -20˚C compared to storage at 25˚C. After 1 week of storage at -20˚C, the phage titer has dropped 9.4 times compared to initial phage titer, which was 1.88 x10^{10} pfu/ mL. At the second week, the phage titer has dropped 6.7 times compared to previous phage titer. Next, the phages stored at -80˚C showed higher phage titer than storage at 4˚C after 1 week. However, the titer dropped lower than the one obtained at 4˚C after 2 weeks of storage. The phage

titer at -80˚C decreased by 4.3 times compared to initial titer after 2 weeks, while phage titer at 4˚C decreased by 3.4 times compared to initial titer after 2 weeks. After 28 days of storage, phage LT-B stored at 4˚C showed a remaining phage titer of 1 x10^9 pfu/ mL, which was the highest remaining phage titer among phage titers of other storage temperatures. No phage titer was obtained after storage at -80˚C, -20˚C and 25˚C for 28 days.

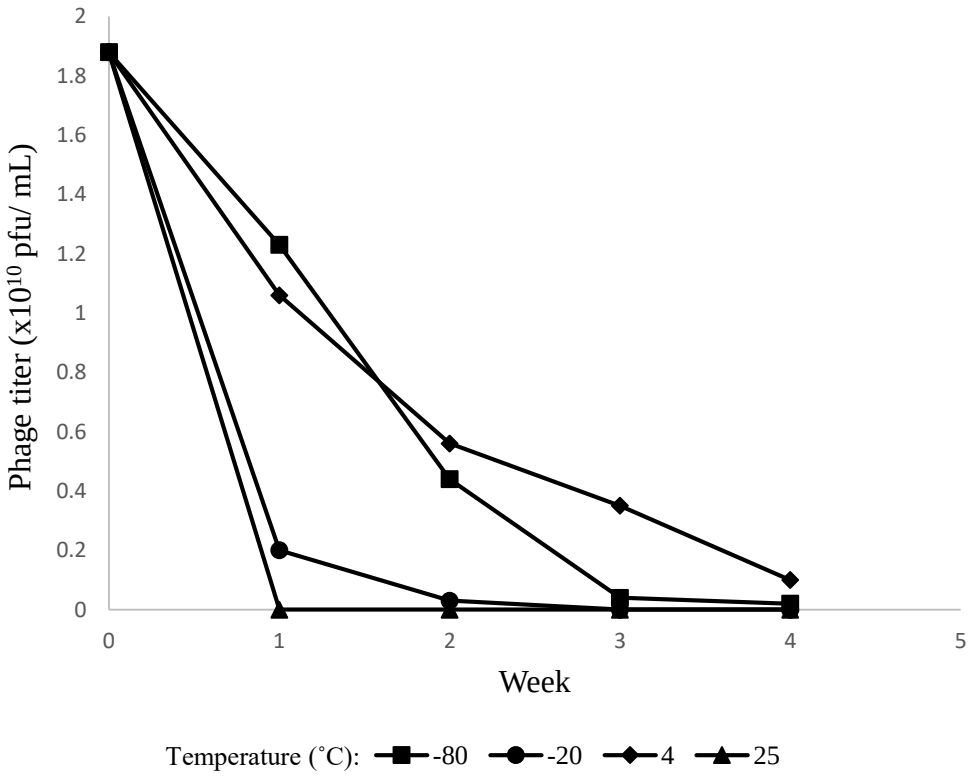

Figure 4.4: Stability of phage LT-B at different storage temperatures.

4.2.7 Host Range

In this study, ten different types of bacterial strains were tested to determine the host range of phage LT-B, which included ETEC, EPEC, *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10, *K. pneumonia*, *S. typhi*, *S. sonnei*, *S. flexneri* and *S. dysenteriae*. *E. coli* BL21 was used as positive. Based on Table 4.3, phage LT-B showed infectivity on *E. coli* strains, such as EPEC, ETEC, *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10 and *E. coli* BL21. Thus, phage LT-B has a broad host range which infects multiple *E. coli* strains (EPEC, ETEC, *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10 and *E. coli* BL21).

Table 4.3: Infectivity of phage LT-B against ten types of bacteria.

Bacterial Hosts	**Infectivity**
Enteropathogenic *E. coli* (EPEC)	+
Enterotoxigenic *E. coli* (ETEC)	+
E. coli JM109 cells	+
E. coli TOP10 cells	+
E. coli TG1 cells	+
Shigella sonnei	-
Shigella flexneri	-
Shigella dysenteriae	-
Salmonella typhi	-
Klebsiella pneumonia	-
E. coli BL21 (Positive control)	+

(+) presence of phage infectivity
(-) absence of phage infectivity

4.3 Partial Genomic Characterization of Bacteriophage

4.3.1 Extraction of Phage Nucleic Acid

The readings of extracted nucleic acid at A260/A280 and A260/A230 for purity were 1.643 and 2.188, respectively. The A260/A280 value was slightly lower than the expected value, which should be around 1.800. This might be due to slight contamination by protein, phenol or other contaminants. On the other hand, the A260/A230 value was in the range of 1.800-2.200, indicating that the isolation technique used was suitable for the sample (Desjardins and Conklin, 2010). The concentration of the extracted nucleic acid obtained was 262 µg/ mL.

4.3.2 Effect of DNAse and RNAse Digestion on Phage Nucleic Acid

The extracted nucleic acid was subjected to DNAse and RNAse digestion. Based on the agarose gel image in Figure 4.5, DNAse-digested nucleic acid of phage LT-B showed no band (Lane 2). On the other hand, RNAse-digested nucleic acid of phage LT-B showed a band with high intensity (Lane 3), which indicated that the nucleic acid was not digested by RNAse. Thus, phage LT-B is a DNA phage. The genome size of phage LT-B was larger than 10 kbp as shown in Lane 3 and 4 (Figure 4.6).

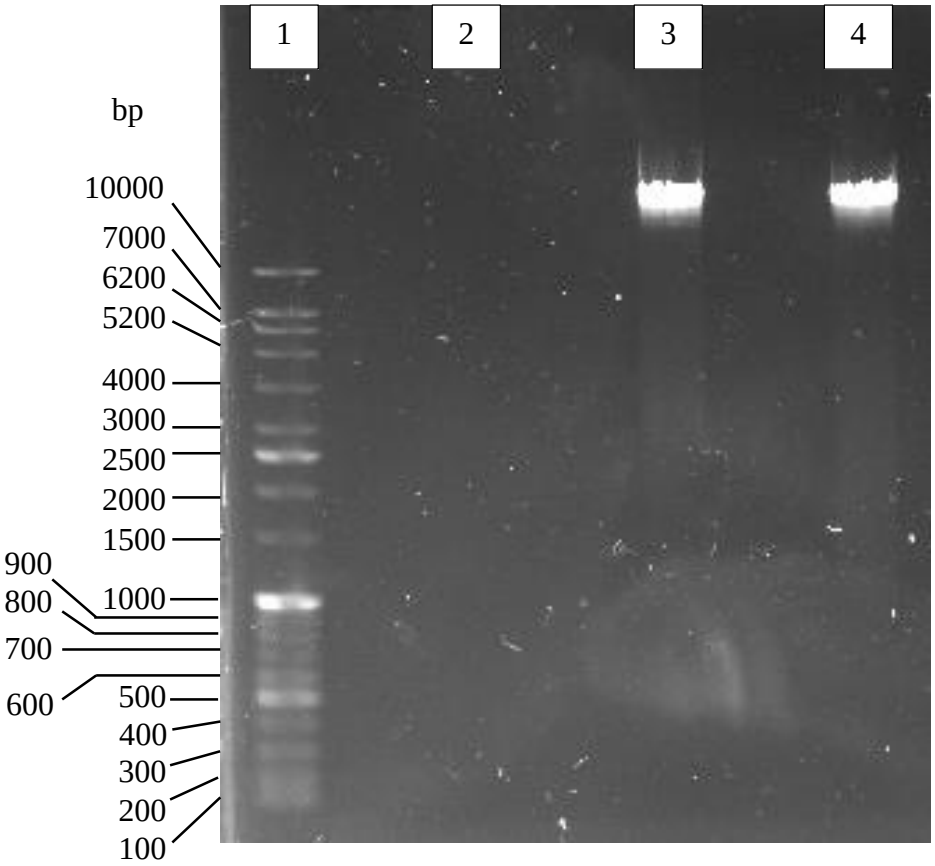

Figure 4.5: Agarose gel electrophoresis image of extracted nucleic acid of phage LT-B after DNAse and RNAse digestion. Lane 1: 1 kb DNA ladder; Lane 2: DNAse-digested phage nucleic acid; Lane 3: RNAse-digested phage nucleic acid; Lane 4: Undigested phage nucleic acid (negative control).

4.3.3 Effect of Restriction Enzyme Digestion on Phage Nucleic Acid

The phage LT-B nucleic acid was subjected to restriction enzyme digestion to identify the possible restriction sites on phage LT-B genome. The restriction enzymes used were *Bam*HI, *Eco*RI, *Eco*RV, *Sac*I and *Xba*I. Figure 4.6 shows the agarose gel image of digested phage LT-B nucleic acid using five different restriction enzymes. The digested product showed that phage LT-B genome was not digested by any of the restriction enzymes as observed in Lane 2 to Lane 6, with only a single band observed with its size larger than 10 kbp. The bands

from Lane 2 to Lane 6 were similar to the undigested phage nucleic acid in Lane 12. Lane 7 to Lane 11 were the positive control with the restriction digestion of pBR322 and pUC19 plasmids by the restriction enzymes used in phage nucleic acid characterization. Cleavage of the plasmids were observed which the band was at the lower position of the gel, indicating smaller DNA strand that migrates faster. The results from positive control showed that the five restriction enzymes were functional up to the date of the experiment.

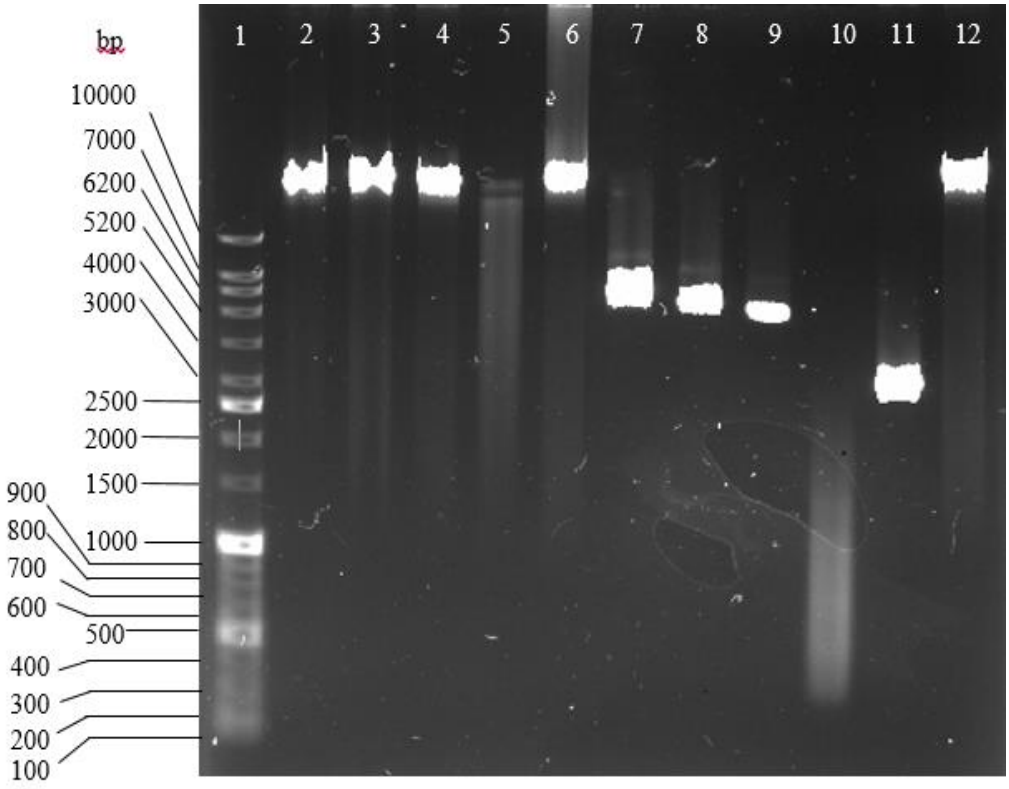

Figure 4.6: Agarose gel electrophoresis image of extracted DNA of phage LT-B after restriction enzymes digestion by five different enzymes. Lane 1: 1 kb DNA ladder; Lane 2: *Bam*HI enzyme with phage DNA; Lane 3: *Eco*RI enzyme with phage DNA; Lane 4: *Eco*RV enzyme with phage DNA; Lane 5: *Sac*I enzyme with phage DNA; Lane 6: *Xba*I enzyme with phage DNA; Lane 7: *Bam*HI enzyme with pBR322 plasmid; Lane 8: *Eco*RI enzyme with pBR322 plasmid; Lane 9: *Eco*RV enzyme with pBR322 plasmid; Lane 10: *Sac*I enzyme with pUC19 plasmid; Lane 11: *Xba*I enzyme with pUC19 plasmid; Lane 12: Undigested phage DNA (negative control).

CHAPTER 5

DISCUSSION

5.1 Physiological Characteristics of Phage

Physiological characteristics of bacteriophage LT-B determines the pathogenicity towards the host bacteria and its infection process. Conformational changes of phage components may be directed under different environmental conditions, which can negatively affect the phage activity when the growth conditions are undesirable (Sillankorva et al., 2004; Orlova, 2012). Therefore, it is important to study about the physiological characteristics of phage LT-B as the information obtained may be useful for different applications. In this study, phage LT-B was investigated based on one step growth curve, adsorption rate, temperature, pH, organic solvent, stability and host range.

5.1.1 One Step Growth Curve

In this study, a one step growth curve was plotted to investigate the infection cycle of phage LT-B with MOI value of 0.00007. Throughout this period, phage LT-B started the processes of attachment, entry into host cells, replication, transcription, translation and assembly of progeny phages. The curve showed the phage titer at different time intervals and the burst size of phage was estimated based on the actual number of phage progeny released from an

infected host cell (Choi et al., 2010). Based on Figure 4.1, the whole process of phage LT-B infection were observed in 50 min. The eclipse phase of phage LT-B took about 30 min to reach release phase. During this eclipse phase, several processes such as phage attachment, uptake of phage nucleic acid, synthesis of phage proteins and nucleic acid, and assembly of progeny phages took place (Hyman and Abedon, 2009).

After the 30th min of phage infection, a rapid surge of phage titer was observed for 20 min, which indicates the release phase of phage LT-B infection. Progeny phages must be released from the host cell to infect other bacterial host cells for replication process. In order for a cell lysis to be successful, phage LT-B must overcome the physical barrier of the bacterial host, including the peptidoglycan layer and cell membrane (Young et al., 2000).

Based on previous findings, different phages showed different growth characteristics. In this study, phage LT-B took 50 min to complete an infection cycle and yielded a burst size of 22 phage particles per bacterial host cell in 20 min. In another study, the infection cycle for a hot spring phage TSP4 isolated from Tengchong hot spring in China was longer compared to phage LT-B, which was about 120 min. Its eclipse period lasted 60 min, which was 2 times longer than the eclipse period of phage LT-B (30 min). However, the burst size of phage TSP4 was 200 phage particles per bacterial host cell in 10 min, which was significantly larger than that observed in phage LT-B (Lin et al., 2010). Apart from that, phage φOH3, isolated from Obama hot spring, Nagasaki, Japan was

reported to have eclipse and release phases of 60 and 40 min, respectively, with a burst size of 109 phage particles per bacterial host cell (Nagayoshi et al., 2016).

For phage that infects *E. coli*, phage øZE1 showed an eclipse phase of 35 min and release phase of 75-80 min, yielding a burst size of 75-80 phage particles per bacterial host cell (Askora et al., 2015). According to Choi et al. (2010), the burst size of phage infecting a single strain of *E. coli* was shown to range from 20 to 1000 phage particles per bacterial host cell. Therefore, the burst size of phage LT-B in this study was 22 phages per bacterial host cell, which is within the estimated range for *E. coli*-infecting phage.

Burst size of a phage varies based on several factors. The sizes of the bacterial host and phage are the two main factors that determine the burst size (Parada et al., 2006; Choi et al., 2010). Bacteria that are larger in size possess higher surface area and more phage receptors are found on the bacterial cell surface. Thus, more phages can be adsorbed onto the cell to infect the host cell. Furthermore, larger bacteria are capable to include more protein synthesis machinery with their high cell volume. Therefore, this can cause higher number of progeny phages to be replicated in a certain duration that leads to a larger burst size. Moreover, the size of the phage is also a contributing factor to its burst size. Larger phages result in smaller burst size due to limited cell volume of the bacterial host that accommodates the progeny phages (Weinbauer and Peduzzi, 1994; Weinbauer and Hofle, 1998). These two factors, the sizes of bacterial host and the phage correlates with each other. In this study, *E. coli* was used as the host for phage

LT-B which is about 2 μm in size (Reshes et al., 2008). Based on these findings, it is possible that the size of phage LT-B may be larger relative to the bacterial cell volume.

Multiplicity of infection (MOI) is also a contributing factor to the burst size of a phage. Low MOI enables one bacterial host to be infected by a single phage, hence co-infection or superinfection is less likely to occur. Co-infection is the infection when the bacterial host cell is infected by a phage, followed by subsequent second infection by another phage. This condition can increase the number of progeny phages synthesized in the host cell. Therefore, this may increase the burst size. For superinfection, re-infection of a host cell with a homologous phage may cause longer eclipse phase (lysis inhibition), which allows more time for the synthesis of progeny phages and increase in burst size (Parada et al., 2006). The MOI used in this study was low to prevent co-infection or superinfection. Thus, it might contributed to the low burst size of phage LT-B.

5.1.2 Adsorption Rate

The adsorption rate of phage LT-B was obtained with MOI value of 0.002. Within 2 min of incubation, the adsorption rate increased drastically to 91.33%, followed by a gradual increase of adsorption rate to 95.76% at the 20th min of infection. Based on a study by Nagayoshi et al. (2016), thermophilic phage φOH3 showed a high adsorption rate of 90.0%. On the other hand, a study conducted by Lau et al. (2012) showed a higher adsorption rate of *E. coli*-

infecting phage ØEC1, which was 98.9% within 2 min of incubation. At the 8th min, the adsorption rate increased to 99.9% and remained constant throughout the experiment. In the current study, adsorption rate of phage LT-B was similar to the adsorption rate of phage φOH3 and ØEC1, which was higher than 90.0% after 2 min of infection.

In a previous study by Mercanti et al. (2015), the MOI value is strongly associated with the adsorption rate of the phage. At a higher MOI value, the adsorption rate decreases drastically. This is likely due to reduced available attachment sites for the phage on the bacterial surface. Therefore, the adsorption rate of phages is usually studied at MOI value lower than 0.1. High MOI value might also lead to "lysis from without", whereby the bacterial host cell undergo cell lysis without phage replication in the host cell due to high amount of phage adsorption on the cell surface (Sritha et al., 2017). Therefore, lower MOI was used for this study to reduce the risk of "lysis from without".

Other than the MOI value that affects the adsorption rate, the physiological state of the bacterial host cells (temperature and pH) also plays an important role in determining the adsorption rate of the phage. A study on *Lactobacillus paracasei* phages i*Lp*84 and i*Lp*1308 showed that high temperature could affect the adsorption rate by changing the conformation of the heat-sensitive phage receptors. With the conformational changes of the receptors, phages could be no longer attached to the bacterial surface (Mercanti et al., 2015; Fister et al., 2016). Since the incubation of phage LT-B was at 37˚C instead of 50˚C (temperature

of hot spring where phage LT-B was isolated), the alteration of phage LT-B adsorption rate by the temperature is less likely to occur.

Apart from that, pH also plays an important role in determining the adsorption rate. In a previous study, *Lactobacillus paracasei* phages i*Lp*84 and i*Lp*1308 showed a decreased in adsorption rate at pH 4 and pH 9. This might be due to the alteration of pH in the environment that causes ionization of the phage receptors, which subsequently decrease in the adsorption ability of the phages (Lau et al., 2012; Mercanti et al., 2015). In another study, phage ØEC1 that infects *E. coli* failed to attach to the bacterial surface at pH 3 but showed the highest adsorption rate at pH 5 to pH 11 (Lau et al., 2012). However, in this study, only one pH was used to study the adsorption rate of phage LT-B, which was at pH 7. Therefore, comparison of adsorption rate at different pH could not be made but the effect of different pH on phage infectivity was discussed in Section 5.1.4.

Other than the physiological state of the bacterial host cell, adhesion molecules of phage and bacterial host cell are crucial for a successful adsorption. A study conducted by Shao and Wang (2008) concluded that the side tail fiber of phage λ determined the adsorption rate of the phage. This was done by introducing frameshift mutation in the side tail fiber (*stf*) gene, which resulted in low adsorption rate compared with the non-mutated *stf* gene. In addition, phage receptors on bacterial cell surface could be blocked by other substances that serve as phage resistance mechanism. Phage T5, one of the *E. coli*-infecting

phage, uses lipoprotein to reduce the chances of superinfection and inactivation of progeny phages by binding to its own receptor. However, *E. coli* uses this lipoprotein to inhibit the phage adsorption onto the phage receptor (Labrie et al., 2010). Based on these findings, it is possible that phage LT-B may have higher amount of adsorption molecules and this may not affect the phage binding process during the adsorption process.

5.1.3 Effect of Temperature on Phage Infectivity

In this study, phage LT-B showed complete absence of infectivity at 70˚C from 20 min onwards. The current study showed that the thermal inactivation temperature of phage LT-B is higher compared to the temperature of its origin which was at 50˚C. This is in accordance to the findings of another study, whereby phage TSP4 was inactivated at a higher temperature compared to the temperature of its origin, which is 65˚C (Lin et al., 2010). Similarly, hot spring phage φOH3 showed a complete absence of infectivity at 100˚C, which was also higher than its original temperature (70˚C) (Nagayoshi et al., 2016). On the other hand, phage øZE1 that infected *E. coli* was reported as a heat-stable phage with the results showed its viability after incubation at 70˚C for 10 min (Askora et al., 2015). Thus, the inactivation temperature of phage reported so far is usually higher than the temperature of its origin, which was also observed in this study.

Variability of the inactivation temperature of each phage is highly dependable on the constitution of phage capsid. Phage capsid is mostly made up by major capsid proteins with polymerization to combine all the proteins. Capsid is highly

important in nucleic acid packaging that is useful in infection and replication of the phage. The stability of capsid under different temperatures may represent the overall resistance to unfavourable environment conditions. Phages that are heat-stable may survive longer in the harsh environment while searching for a new host, which is a great benefit for the phages (White et al., 2012). Apart from that, a previous study showed that the heating of phage led to aggregation of phage tails, detachment of phage head from its tail and production of empty capsid. This observations further confirmed that high temperature results in changes of structural conformation of phage particles, including the disulfide bonds between capsid proteins that stabilize the structure (Caldeira and Peabody, 2007; Mojica and Brussaard, 2014).

However, the phage capsid may be able to reconstitute after the destruction by high temperature. Based on a study conducted by Ahmadi et al. (2017), *Listeria* phages P100 was completely inactivated at 71˚C and able to reconstitute the structure of the phage particles at lower temperature. The reconstitution of the phage might be due to the retained structure of portal complex even at higher temperature. Therefore, each phage comes with structural differences that differ them from their sensitivity of capsid proteins towards different temperatures.

5.1.4 Effect of pH on Phage Infectivity

Phage LT-B stability was investigated under different pH values ranging from pH 3 to pH 11. Based on Figure 4.3, phage LT-B showed tolerance to all the tested pH values, with its optimal pH value at pH 7. This is in line with phage T7 and hot spring phage TSP4, which also showed the highest stability at pH 7 (Lin et al., 2010; Jończyk et al., 2011). A study from Lau et al. (2012) demonstrated the optimum pH of phage ØEC1 that infects *E. coli* also ranged from pH 6 to pH 9.

Alteration of pH in external environment provides changes to the phage stability. Extreme pH increases the hydrogen ion and hydroxyl ion concentrations in the environment which contribute to the phage inactivation. Those ions are highly reactive free radicals that present in the environment and tend to oxidize substances such as capsid proteins. Subsequently, it would lead to deletion, denaturation or conformational changes of the capsid proteins that are responsible for attachment to host bacteria (Feng et al., 2003). According to another study by Nap et al. (2014), the acidity and alkalinity of the environment can also cause changes in the charge state of the capsid, such as from net negative to net positive charge. The normal net charges on the phage capsid is determined by the distribution of the amino acids in each phage and it is crucial in electrostatic interaction between capsid and external environment. On the other hand, low pH was proven to increase phage aggregation ability due to high hydrogen ion concentration, such as phage MS2. The aggregation process can

cause inaccuracy in phage titer and the aggregates can be eliminated easily during adsorption (Langlet et al., 2007).

Other than the optimal pH of phage LT-B, it was found that phage LT-B was more resistant to acidic pH compared to alkaline pH with lower reduction of phage titer at acidic pH. Other discovered phage such as phage MS2 also showed higher survival rate at acidic pH compared to alkaline pH, in which the inactivation observed at pH 5 was lower compared to pH 9 (Feng et al., 2003). Based on a study by Yu et al. (2006), resistance towards acidic pH environment was observed in tectivirus P78-76, which survived at pH 2 for 24 h.

Reduction of the phage titer of phage LT-B at alkaline pH was presented, thus it was more sensitive to alkaline pH. On the contrary, some phages are more stable in alkaline pH environment. Phage Qβ was more resistant to alkaline pH than acidic pH, as it showed higher inactivation rate at pH 5 compared to pH 9 (Feng et al., 2003). A common phage, phage T7, was also more resistant to alkaline pH compared to phage T3 (Jończyk et al., 2011). Furthermore, hot spring phage TSP4 showed a significant reduction of survival rate at pH 3 to 24% remaining phages (Lin et al., 2010). Another hot spring phage φOH3 also showed higher stability at pH 9 with 32.7% of remaining phage particles compared to 4% at pH 3 (Nagayoshi et al., 2016).

5.1.5 Effect of Chloroform on Phage Infectivity

Chloroform is widely use in laboratory for molecular biology work. It is also useful in precipitating phage as mentioned in a study by Bourdin et al. (2014). Therefore, it is important to study the effect of chloroform on phage infectivity to make sure that the precipitation of phage using chloroform does not affect the viability of the phage sample.

The stability of phage LT-B was investigated using chloroform in different concentrations, ranging from 2% (v/v) to 10% (v/v). In this study, phage LT-B was resistant to chloroform treatment up to 10% (v/v) chloroform. This finding is in accordance to the findings of phages V3 and V7 that infect *E. coli* O157:H7, which were resistant to 10% (v/v) chloroform after 1 h incubation. On the contrary, another phage in the similar study, phage V8 was sensitive to chloroform treatment, that resulted in reduction of phage titer (Akindolire and Ateba, 2019). Apart from that, brucellaphage BpL1 was reported to be completely inactivated in 10% (v/v) chloroform within 15 min of incubation at 37˚C (Gupta and Saxena, 2017). On the other hand, *E. coli* phage øZE1 was not affected by chloroform treatment and showed complete resistance towards chloroform (Askora et al., 2015). In another study, a thermophilic phage TSP4 was also reported to be resistant to chloroform concentration up to 25% (v/v) after 5 min of incubation (Lin et al., 2010). Similarly, phage KP34 that infects *Klebsiella pneumonia* was also reported to be resistant to chloroform even after 2 h and 24 h of incubation (Drulis-Kawa et al., 2011).

The stability of phage to chloroform is heavily contributed by the lipid composition. For phages that are composed of lipid, chloroform is able to loosen the lipid membrane structure rich in cholesterol (liquid-ordered phase), instead of lipid membrane with low in cholesterol (liquid-disordered phase). It is believed that chloroform molecule inserts itself into the lipid bilayer and interferes with the chain packing and acyl chains located within the lipids (Turkyilmaz et al., 2009; Reigada, 2013). Examples of lipid membrane-containing phages are *Corticoviridae*, *Tectiviridae*, *Cystoviridae* and *Plasmaviridae*. Their lipids are mainly derived from their host cell cytoplasm, however its composition varies from host cell lipid composition (Poranen et al., 2015). As phage LT-B was found resistant to chloroform, it may postulate the composition of the phage structure which may not contain any lipid membrane to be damaged by chloroform.

5.1.6 Stability of Phage

Storage of phage samples is essential to maintain the initial characteristics and the amount of the phages for future application such as novel drug research, genetic engineering and biotechnological processes. Phages are usually stored without any host cell in the sample, in which the storage conditions could be varied from phages to phages (Fortier and Moineau, 2009; Iqbal et al., 2018). Thus, the optimal storage temperature of phage LT-B was evaluated in this study.

Phage LT-B was found to be the most stable with minimal drop in titer at 4˚C storage for 28 days, followed by -80˚C, -20˚C and 25˚C. The results are in agreement with tailed bacteriophages, such as phage JHP, RLP, RSP, SaPL and IttPL, which reported to have the lowest reduction rate of titer after one year of storage at 4˚C (Iqbal et al., 2018). Phage MS2 was found to be more stable at 4˚C storage than at -80˚C and -20˚C (Olson et al., 2004). Significant reduction of phage titer observed in phage LT-B stored at -20˚C for 14 days might be due to the formation of large ice crystal that led to virion damage (Olson et al., 2004). Furthermore, phage LT-B was the least stable at 25˚C in which its viability was completely lost after 7 days of storage. However, phage CP-51 targeting *Bacillus cereus* showed a higher viability when stored at room temperature than at low temperature (Jończyk et al., 2011).

Based on the study by Ackermann et al. (2004), phages with contractile phages or without lipid membrane are more stable compared to other phages across variety of storage temperatures. Low temperature storage of phage with fragile tails exerts negative impact on them. Low temperature causes contraction and loosening of the phage tail sheath that eventually comes off from the phage particle. This statement is supported by another study that showed both contractile-tailed phages and phages without lipids can be stored for up to 5 to 10 years at 4˚C (Iqbal et al., 2018).

Some phages are more resistant to different storage temperatures, such as phage KP34 for *K. pneumoniae* is only sensitive to storage temperature at 25˚C with a

significant drop in titer (Drulis-Kawa et al., 2011). A study by Emedi (2015) showed drastically reduction in phage titer of phage GV1 at 4˚C compared to the titer of phage stored at -80˚C in the presence of 20% glucose. In addition, phage CP-51, which is a *Myoviridae*, has an optimum storage temperature at 0˚C instead of 4˚C (Jończyk et al., 2011).

5.1.7 Host Range

Each phage requires host cell to replicate, some may be present with narrow host range and some with a broad host range. Adsorption of phage onto the bacteria is the key process to kick start the infection process. It heavily relies on the interactions between phage binding proteins and bacterial cell surface receptor, which eventually results in the specificity of a phage in infecting the host. A successful adsorption of phage to a host cell facilitates the DNA ejection into the host cell. Subsequently, replication process can be carried out and forming progeny phages to infect other host cells (Bertozzi Silva et al., 2016).

Various receptors can be found on the surface of bacteria, ranging from peptide to polysaccharide moieties. Gram-positive bacteria have cell surface components such as peptidoglycans and teichoic acid that served as the important receptors for the phage adsorption. For Gram-negative bacteria, lipopolysaccharides is the main role in phage adsorption, which consist of lipid A, core polysaccharides and O-polysaccharides. Moreover, flagella, pili and capsules or slime layer can also be the target for the phage (Bertozzi Silva et al., 2016).

Despite this, the failure of a phage to infect a host occurs when the receptors are inaccessible or not complement to the phage binding proteins. Furthermore, mutations on the structure of receptors and absence of the phage receptors on the bacterial cell surface can also cause phage resistance. This situation is commonly termed as bacteriophage resistance of the host bacteria, which may be initiated from the selective pressure exerted by the phage. When the host bacteria become resistant towards phage infection, the bacterial lineages can be maintained. However, phages can also evolve to counteract the resistance, causing the bacteria to become susceptible to phage infection again. There are three mechanisms to resist phage adsorption, which are blockage of phage receptors, capsule or slime layer synthesis and inhibition of receptors by competitive inhibitors (Labrie et al., 2010; Bertozzi Silva et al., 2016).

Based on this study, phage LT-B was found to be infectious only towards *E. coli* strains, which included EPEC, ETEC, *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10 and *E. coli* BL21. Since it was not infecting only *E. coli* BL21, the positive control. Phage LT-B is considered a phage with broad host range as defined by Ross et al. (2016) as it infects different strains within a bacterial species. The finding is in contrast to another study by Nagayoshi et al. (2016) on hot spring phage φOH3. It was reported that the phage was highly specific towards thermophile *Thermus thermophilus* HB8 among other *Thermus* spp., *Meiothermus rube*r strain 21 and *Geobacillus kaustophilus* NBRC 102445. In comparison with thermophilic phage, both phage BVW1 and GVE1 have specific host range, which are *Bacillus sp*. W13 and *Geobacillus sp*. E26323 respectively (Liu et al., 2006). In a previous study by Askora et al. (2015)

showed that *E. coli* phage ØZE1 could infect four strains of *E. coli* among eleven tested *E. coli* strains. In this study, phage LT-B was also shown to infect all six tested *E. coli* strains, which both phage LT-B and ØZE1 can be classified as broad host range phage.

Broad host range was also observed in *Staphylococcus aureus* phages ΦSA012 and ΦSA039. Both phages were able to infect multiple strains of *S. aureus*, with phage ΦSA012's ability to infect and lyse all *S. aureus* strains including MRSA (Iwano et al., 2018). In this study, phage LT-B showed similarity with phages ΦSA012 and ΦSA039, in which they can infect multiple strains in one bacterial species. Apart from that, phage with broad host range may also infect multiple bacterial species. In another study by Ghasemian et al. (2017), phage gBSN-MGB13 showed a wide host range across a multiple bacterial species, which included *E. coli* O157, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa*.

5.2 Partial Genomic Characterization of Phage

5.2.1 Effect of DNAse A and RNAse Digestion on Phage Nucleic Acid

In order to have an effective infection, the amount of genome packaged within the phage capsid have to be optimized via evolutionary pressure whether to lose or gain nucleic acid. Genome size of phage ranges from 3405 bp to 500 kbp. Study on phage genome is relatively important to understand the diversity of the phage population, origin, and the involvement of evolution that creates the population pattern (Hatfull and Hendrix, 2011).

One of the concern in phage genome study is the genome diversity of the phage. The genome architectures are said to be in mosaic form, which is formed by horizontal gene transfer (HGT) between different phages or phage and host. Thus, two phages that are closely related in terms of genome similarity can be used in phylogenetic study on their relationship and their evolutionary histories (Mavrich and Hatfull, 2018). Genome similarities can be compared using metagenomic libraries to further confirm the occurrence of evolution. Numerous genes in the phage that expressed restriction transcriptase, recombinase, integrase, and transposase propose that horizontal gene transfer do exists in hot spring phage genomes (Schoenfeld et al., 2008). Furthermore, similarity of genome sizes between multiple phages can occur and may lead to similar phage proteins produced. A study by Scholl et al. (2002) investigated on the closeness between phage SP6 and phages K1-5, K5 and K1E. All the four phages consist of genome size of approximately 40 kb, which are similar in terms of phage morphologies and specific RNA polymerases. All the above characteristics suggested that these phages may have a common ancestor from where they derived from via horizontal gene transfer.

In this study, it was clearly shown that phage LT-B is a DNA phage. There are few other thermophilic hot spring phages, which contain DNA genome, such as *Myoviridae* phages ϕTMA, ϕYS40 and D6E, *Thermus* phages φIN93 and TSP4, and *Filamentous* hot spring phage φOH3 (Matsushita and Yanase, 2009; Lin et al., 2010; Wang and Zhang, 2010; Tamakoshi et al., 2011; Nagayoshi et al., 2016). In contrast, highly thermostable phage PP7 from hot spring that infects *Pseudomonas* spp. consists of RNA genome (Caldeira and Peabody, 2007).

Therefore, hot spring phages could either have DNA or RNA genome packaged in their capsids.

The estimated genome size of LT-B phage is larger than 10 kbp. This result is in line with thermophilic phage D6E, whereby its genome size is 49 kbp (Wang and Zhang, 2010). Similar findings were also reported for other hot spring phages, ϕTMA and ϕYS40, which consist of DNA genome with sizes of 151 kbp and 152 kbp, respectively (Tamakoshi et al., 2011). In another study, hot spring *Thermus* phages φIN93 has a DNA genome size of 20 kbp (Matsushita and Yanase, 2009). On the contrary, there are hot spring phages that have smaller genome size compared to the genome size of phage LT-B. For example, phage TSP4 and φOH3 consist of DNA genome with the sizes of 80 kb and 5688 kb, respectively (Lin et al., 2010; Nagayoshi et al., 2016).

5.2.2 Effect of Restriction Enzyme Digestion on Phage Nucleic Acid

Restriction enzyme digestion on DNA is widely used in DNA manipulation such as in molecular cloning. The mechanism includes the "cutting" process of the enzyme on double-stranded DNA at a specific DNA sequence (restriction site). Then, these digested DNA fragments can be constructed into restriction map that demonstrates the position of each restriction enzyme cleavage site in a DNA molecule (Hepfer and Turchi, 1989).

In this study, the genome of phage LT-B was found to be resistant to digestions by all of the treated restriction enzymes, which included *Bam*HI, *Eco*RI, *Eco*RV, *Sac*I and *Xba*I. On the contrary, various studies have shown that the genome obtained from hot spring phage and thermophilic phage contain restriction sites for common used restriction enzymes such as *Bam*HI, *Eco*RI, *Eco*RV and *Hind*III (Liu et al., 2006; Lin et al., 2010; Wang and Zhang, 2010). Apart from that, phage genome may contain other unique restriction sites that are rarely used in cloning. In a previous study, *Stenotrophomonas maltophilia* phage ΦSMA5's DNA genome was subjected to 50 types of restriction enzymes. The genome only contains restriction sites for *Ava*I, *Hae*II, *Hae*III, and *Hha*I, which are seldom used in cloning (Chang et al., 2005).

One of the reasons that phage LT-B DNA resistance to restriction enzyme digestion is the action of the phage to overcome the restriction-modification system from bacterial host. Bacterial restriction enzymes are released by the host to cleave the phage genome upon DNA injection by the phage. An enzyme called DNA methyltransferase (MTase) is important in protecting host genome from cleavage by restriction enzymes since methylated DNA will not be cleaved by the restriction enzymes. Some of the phages carry out passive mechanisms to evade the restriction-modification system by allowing the methylation of the phage genome by MTase before the action of restriction enzymes. The methylated state of the phage genome then remains stable in the genome for every replication process. Another passive mechanism is to introduce point mutation to the phage genome, causing absence of the restriction recognition sites. In contrast, active mechanism to combat the restriction-modification

system involves the activation of host cell modification enzymes. One of the good example is phage λ which synthesizes antirestriction protein Ral to increase the MTase activity of *Eco*KI (Enikeeva et al., 2010; Samson et al., 2013; Shabbir et al., 2016).

It is proven that phage with broad host range facilitates phage genome evolution. Phages that infect multiple bacterial species receive more pressures from the host's restriction-modification system in every host. This condition exerts a selective pressure to the phage and promotes evolution of the genome. Therefore, the restriction sites on the phage genome may be altered and inhibited restriction enzymes from binding to the genome. For example, phage PP722 genome was not susceptible to the cleavage by *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III and *Sau*3AI similar to that observed in the current study. It is believed that its broad host range is advantageous for successful phage infection by avoiding digestion by restriction enzymes (Lute et al., 2004; Hamdi et al., 2017).

Apart from that, phage LT-B may contain single-stranded DNA which may lead to this outcome. As the restriction enzymes used in this study are Type II restriction endonucleases, hence they are only able to cleave double-stranded DNA at specific cleavage sites (Pingoud and Jeltsch, 2001). Further investigations on the restriction enzyme digestion using restriction enzymes that cleave single-stranded DNA should be carried out to characterize the phage genome. Examples of the restriction enzymes that cleave single-stranded DNA are *Ava*II, *Hae*II, *Dde*I, *Alu*I, *Sau*3AI, *Acc*II, *TthHB*8I and *Hap*II, which could

be used in further characterization of phage LT-B genome (Nishigaki et al., 1985).

5.3 Limitations of Study

The plateau phase of phage LT-B in the one step growth curve could not be determined. Besides that, the exact genome size of the phage LT-B could not be confirmed.

5.4 Future Studies

Cesium chloride ultracentrifugation may be used to further purify the phage LT-B in order to visualize the morphology under TEM. Apart from that, the extracted phage LT-B DNA can be sequenced to obtain a complete set of DNA sequences. This information obtained based on its morphology and genome sequence could be useful in the classification of phage LT-B. Besides that, type III restriction enzymes can be used to determine whether the phage DNA is in single-stranded form.

CHAPTER 6

CONCLUSION

In the present study, bacteriophage LT-B that was isolated from Lubuk Timah hot spring was characterized based on its physiological and partial genomic characteristics. One step growth curve was carried out using MOI of 0.00007, while the adsorption rate was determined using MOI of 0.002. One step growth curve of phage LT-B was obtained within 50 min post infection with a burst size of approximately 22 phages per bacterial host cell. This study also demonstrated that phage LT-B reached an adsorption rate of 91.33% within 2 min of post incubation. Furthermore, the phage infectivity was maintained even after heat treatment at 60˚C and 65˚C for 1 h. When the phage was heat-treated at 70˚C, the viability of the phage was still maintained for the first 10 min. However, when the heat treatment was prolonged to 20 min, the viability of the phage was completely lost. In terms of pH, phage LT-B produced the highest phage titer at pH 7 in comparison to the titer obtained at acidic and alkaline pH. Furthermore, it was also stable under chloroform treatment with concentration ranging from 2% to 10% (v/v). Apart from that, the most suitable storage temperature for phage LT-B was at 4˚C with highest phage titer remained after 28 days of storage, compared to -80˚C, -20˚C and 25˚C. In addition, this study showed that the host range of phage LT-B included EPEC, ETEC, *E. coli* TG1, *E. coli* JM109, *E. coli* TOP10 and *E. coli* BL21 among all the bacteria tested in this study. Phage LT-

B was infectious to multiple strains of *E. coli* species, which is considered as broad host range.

Genomic characterization of phage LT-B revealed that the phage is a DNA phage with a genome size larger than 10 kbp. Apart from that, none of the tested restriction enzymes such as *Bam*HI, *Eco*RI, *Eco*RV, *Sac*I and *Xba*I, were able to cleave the phage genome. Thus, it showed that phage LT-B genome does not contain the restriction sites for any of the above restriction enzymes.

REFERENCES

Abedon, S.T., Herschler, T.D. and Stopar, D., 2001a. Bacteriophage latent-period evolution as a response to bacterial availability. *Applied and Environmental Microbiology*, 67(9), pp. 4233 – 4241.

Abedon, S.T., Hyman, P. and Thomas, C., 2001b. Experimental examination of bacteriophage latent-period evolution as a response to bacterial availability. *Applied and Environmental Microbiology*, 69(12), pp. 7499 – 7506.

Ackermann, H., 2005. Bacteriophage classification. In: Kutter, E., and Sulakvelidze, A. (eds.). *Bacteriophages: Biology and Applications*. Florida: CRC Press, pp. 68 – 86.

Ackermann, H., Tremblay, D. and Moineau, S., 2004. Long-term bacteriophage preservation. *WFCC Newsletter*, 38, pp. 35 – 40.

Ahmadi, H., Radford, D., Kropinski, A. M., Lim, L.T. and Balamurugan, S., 2017. Thermal-stability and reconstitution ability of *Listeria* phages P100 and A511. *Frontiers in Microbiology*, 8, pp. 1 – 11.

Akindolire, M. A. and Ateba, C. N., 2019. Isolation and characterisation of bacteriophages with lytic activity against virulent *Escherichia coli* O157:H7: Potential bio-control agents. *Preprints*.

Askora, A., Merwad, A., Gharieb, R.M. and Maysa, A.I.A., 2015. A lytic bacteriophage as a biocontrol for some enteropathogenic and enterohemorrhagic *Escherichia coli* strains of zoonotic risk in Egypt. *Revue De Medecine Veterinaire,* 166(3–4), pp. 76 – 83.

Baptista, C., Santos, M. A. and São-José, C., 2008. Phage SPP1 reversible adsorption to *Bacillus subtilis* cell wall teichoic acids accelerates virus recognition of membrane receptor YueB. *Journal of Bacteriology*, 190(14), pp. 4989 – 4996.

Beal, H., n.d.. *Microbial life in extremely hot environments* [Online]. Available at: https://serc.carleton.edu/microbelife/extreme/extremeheat/index.html [Accessed: 14 March 2019].

Bertozzi Silva, J., Storms, Z. and Sauvageau, D., 2016. Host receptors for bacteriophage adsorption. *FEMS Microbiology Letters*, 363(4), pp. 1 – 11.

Biotech Khan, 2014. *One-step multiplication curve for bacteriophages* [Online]. Available at: https://biotechkhan.wordpress.com/2014/07/01/one-step-multiplication-curve-for-bacteriophages/ [Accessed: 14 March 2019].

Bourdin, G. et al., 2014. Amplification and purification of T4-Like *Escherichia coli* phages for phage therapy: From laboratory to pilot scale. *Applied and Environmental Microbiology*, 80(4), pp. 1469 – 1476.

Bradley, D.E., 1967. Ultrastructure of bacteriophage and bacteriocins. *Bacteriological Reviews*, 31(4), pp. 230 – 314.

Breitbart, M., Wegley, L., Leeds, S., Rohwer, F. and Schoenfeld, T., 2004. Phage community dynamics in hot springs. *Applied and Environmental Microbiology*, 70(3), pp. 1633 – 1640.

Caldeira, J.C. and Peabody, D.S., 2007. Stability and assembly *in vitro* of bacteriophage PP7 virus-like particles. *Journal of Nanobiotechnology*, 5(10), pp. 1 – 10.

Chaby, R. and Girard, R., 1980. Adsorption and endo-glycosidase activity of phage φ1(40) on *Salmonella johannesbury* O polysaccharide. *Virology*, 105, pp. 136 – 147.

Chang, H. et al., 2005. Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage SMA5. *Applied and Environmental Microbiology*, 71(3), pp. 1387 – 1393.

Choi, C., Kuatsjah, E., Wu, E. and Yuan, S., 2010. The effect of cell size on the burst size of T4 bacteriophage infections of *Escherichia coli* B23. *Journal of Experimental Microbiology and Immunology*, 14, pp. 85 – 91.

Choi, Y., Shin, H., Lee, J.H. and Ryua, S., 2013. Identification and characterization of a novel flagellum-dependent *Salmonella*-infecting bacteriophage, iEPS5. *Applied and Environmental Microbiology*, 79(16), pp. 4829 – 4837.

Click, E. M. and Webster, R. E., 1997. Filamentous phage infection: Required interactions with the TolA protein. *Journal of Bacteriology*, 179(20), pp. 6464 – 6471.

Desjardins, P. and Conklin, D., 2010, NanoDrop microvolume quantitation of nucleic acids. *Journal of Visualized Experiments*, 45, pp. 1 – 4.

Drulis-Kawa, Z. et al., 2011. Isolation and characterisation of KP34-a novel φkMV-like bacteriophage for *Klebsiella pneumonia*. *Applied Microbiology and Biotechnology*, 90, pp. 1333 – 1345.

Emedi, B.T., 2015. *Isolation and preliminary characterization of bacteriophages of thermophilic Bacillus and Geobacillus species*. Master Degree thesis, University of the Western Cape, Republic of South Africa.

Encyclopaedia Britannica, n.d.. *Hot spring: Geology* [Online]. Available at: https://www.britannica.com/science/hot-spring (Accessed: 29 October 2018).

Enikeeva, F.N., Severinov, K.V. and Gelfand, M.S., 2010. Restriction-modification systems and bacteriophage invasion: who wins?. *Journal of Theoretical Biology*, 266(4), pp. 550 – 559.

Erez, Z. et al., 2017. Communication between viruses guides lysis-lysogeny decisions. *Nature*, 541(7638), pp. 488 – 493.

Feng, Y.Y., Ong, S.L., Hu, J.Y., Tan, X.L. and Ng, W.J., 2003. Effects of pH and temperature on the survival of coliphages MS2 and Qβ. *Journal of Industrial Microbiology and Biotechnology*, 30, pp. 549 – 552.

Fister, S. et al., 2016. Influence of environmental factors on phage-bacteria interaction and on the efficacy and infectivity of phage P100. *Frontiers in Microbiology*, 7(1152), pp. 1 – 13.

Fortier, L.C. and Moineau, S., 2009. Phage production and maintenance of stocks, including expected stock lifetimes. In: Clokie, M.R. and Kropinski, A.M. (eds.). *Bacteriophages. Methods in Molecular Biology*. New York: Humana Press, pp. 203 – 219.

Ghasemian, A., Bavand, M. and Moradpour, Z., 2017. A broad-host range coliphage against a clinically isolated *E. coli* O157: Isolation and characterization. *Journal of Applied Pharmaceutical Science*, 7(3), pp. 123 – 128.

Goh, K.M. et al., 2011. A comparison of conventional and miniprimer PCR to elucidate bacteria diversity in Malaysia Ulu Slim hot spring using 16S rDNA clone library. *Romanian Biotechnological Letters*, 16(3), pp. 6273 – 6281.

Gupta, V. and Saxena, H.M., 2017. Isolation and characterization of BpL1, a broad acting lytic bacteriophage against *Brucella*. *International Journal of Current Microbiology and Applied Sciences*, 6(11), pp. 2486 – 2496.

Guttman, B., Raya, R. and Kutter, E., 2005. Basic phage biology. In: Kutter, E., and Sulakvelidze, A. (eds.). *Bacteriophages: Biology and Applications*. Florida: CRC Press, pp. 29 – 63.

Hamdi, S. et al., 2017. Characterization of two polyvalent phages infecting *Enterobacteriaceae*. *Scientific Reports*, 7, pp. 1 – 12.

Harada, L.K. et al., 2018. Biotechnological applications of bacteriophages : State of the art. *Microbiological Research*, 212(213), pp. 38 – 58.

Hatfull, G.F. and Hendrix, R.W., 2011. Bacteriophage and their genomes. *Current Opinion in Virology*, 1(4), pp. 298 – 303.

Henry, M. and Debarbieux, L., 2012. Tools from viruses: Bacteriophage successes and beyond. *Virology*, 434(2012), pp. 151 – 161.

Hepfer, C.E. and Turchi, S.L., 1989. An introduction to restriction mapping of DNA. *Biochemical Education*, 17(1), pp. 48 – 50.

Hoa, N.X., Tang, F., Bai, Q., Zhang, W. and Lu, C., 2014. Isolation and characterization of two T4-like bacteriophages against pathogenic *Escherichia coli* of piglet. *African Journal of Microbiology Research*, 8(41), pp. 3604 – 3611.

Howard-Varona, C., Hargreaves, K.R., Abedon, S.T. and Sullivan, M.B., 2017. Lysogeny in nature: Mechanisms, impact and ecology of temperate phages. *International Society for Microbial Ecology*, 11, pp. 1511 – 1520.

Hyman, P. and Abedon, S.T., 2009. Chapter 18 - Practical methods for determining phage growth parameters. In: Clokie, M.R. and Kropinski, A.M. (eds.). *Bacteriophages. Methods in Molecular Biology^{TM}, vol 501.* New York: Humana Press, pp. 175 – 202.

Hyman, P. and Abedon, S.T. 2010. Chapter 7 - Bacteriophage host range and bacterial resistance. *Advances in Applied Microbiology*, 70, pp. 217 – 248.

Iqbal, A.A., Muhammad, A., Rabia, T., Zaigham, A. and Shafiq, U.R., 2018. Storage of bacteriophages at 4℃ leads to no loss in their titer after one year. *Pakistan Journal of Zoology*, 50(6), pp. 2395 – 2398.

Iwano, H. et al., 2018. Bacteriophage ΦSA012 has a broad host range against *Staphylococcus aureus* and effective lytic capacity in a mouse mastitis model. *Biology*, 7(8), pp. 1 – 13.

Jamal, M. et al., 2017. Isolation and characterization of a bacteriophage and its utilization against multi-drug resistant *Pseudomonas aeruginosa*-2995. *Life Sciences*, 190, pp. 21 – 28.

Jończyk, E., Kłak, M., Miedzybrodzki, R. and Górski, A., 2011. The influence of external factors on bacteriophages-review. *Folia Microbiologica*, 56(3), pp. 191 – 200.

Kim, M., Kim, S., Park, B. and Ryu, S., 2014. Core lipopolysaccharide-specific phage SSU5 as an auxiliary component of a phage cocktail for *Salmonella* biocontrol. *Applied and Environmental Microbiology*, 80(3), pp. 1026 – 1034.

Kokare, C.R., 2008. *Pharmaceutical microbiology: principles and applications*, 6th ed. Pune: Nirali Prakashan.

Koskella, B. and Meaden, S., 2013. Understanding bacteriophage specificity in natural microbial communities. *Viruses*, 5, pp. 806 – 823.

Kovacs, E.W. et al., 2007. Dual-surface-modified bacteriophage MS2 as an ideal scaffold for a viral capsid-based drug delivery system. *Bioconjugate Chemistry*, 18(4), pp. 1140 – 1147.

Kutter, E., Raya, R. and Carlson, K., 2005. Molecular mechanisms of phage infection. In: Kutter, E. and Sulakvelidze, A. (eds.). *Bacteriophages: Biology and Applications*. Florida: CRC Press, pp. 165 – 222.

Kutter, E. et al., 2010. Phage therapy in clinical practice : Treatment of human infections. *Current Pharmaceutical Biotechnology*, 11(1), pp. 69 – 86.

Labrie, S.J., Samson, J.E. and Moineau, S., 2010. Bacteriophage resistance mechanisms. *Nature Reviews Microbiology*, 8, pp. 317 – 327.

Langlet, J., Gaboriaud, F. and Gantzer, C., 2007. Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. *Journal of Applied Microbiology*, 103, pp. 1632 – 1638.

Lau, G.L., Sieo, C.C., Tan, W.S. and Ho, Y.W., 2012. Characteristics of a phage effective for colibacillosis control in poultry. *Journal of the Science of Food and Agriculture*, 92(13), pp. 2657 – 2663.

Lavigne, R., Molineux, I.J. and Kropinski, A.M., 2012. Order Caudovirales. In: King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (eds.). *Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses*. London: Elsevier Academic Press, pp. 39 – 45.

Lin, D.M., Koskella, B. and Lin, H.C., 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, 83(3), p. 162.

Lin, L. et al., 2010. Isolation and characterization of an extremely long tail *Thermus* bacteriophage from Tengchong hot springs in China. *Journal of Basic Microbiology*, 50, pp. 452 – 456.

Lin, L. et al., 2011. Isolation and characterization of a new bacteriophage MMP17 from *Meiothermus. Extremophiles*, 15(2), pp. 253 – 258.

Liu, B., Wu, S., Song, Q., Zhang, X. and Xie, L., 2006. Two novel bacteriophages of thermophilic bacteria isolated from deep-sea hydrothermal fields. *Current Microbiology*, 53, pp. 163 – 166.

Lute, S. et al., 2004. Characterization of coliphage PR772 and evaluation of its use for virus filter performance testing. *Applied and Environmental Microbiology*, 70(8), pp. 4864 – 4871.

Majewska, J. et al., 2015. Oral application of T4 phage induces weak antibody production in the gut and in the blood. *Viruses*, 7, pp. 4783 – 4799.

Mann, N. H., 2005. The third age of phage. *PLoS Biology*, 3(5), pp. 0753 – 0755.

Marcó, M.B., Reinheimer, J. and Quiberoni, A., 2015. Phage adsorption and lytic propagation in *Lactobacillus plantarum*: Could host cell starvation affect them?. *BMC Microbiology*, 15(273), pp. 1 – 7.

Matsushita, I. and Yanase, H., 2009. The genomic structure of *Thermus* bacteriophage φiN93. *Journal of Biochemistry*, 146(6), pp. 775 – 785.

Mavrich, T.N. and Hatfull, G.F., 2018. Bacteriophage evolution differs by host, lifestyle and genome. *Nature Microbiology*, 2, pp. 1 – 22.

Mendez, J. et al., 2002. Conservation of phage reference materials and water samples containing bacteriophages of enteric bacteria. *Journal of Virological Methods*, 106, pp. 215 – 224.

Mercanti, D.J., Ackermann, H.W. and Quiberoni, A., 2015. Characterization of two temperate *Lactobacillus paracasei* bacteriophages: Morphology, kinetics and adsorption. *Intervirology*, 58, pp. 49 – 56.

Moghimian, P., Srot, V., Pichon, B.P., Facey, S.J. and Aken, P.A.V., 2016. Stability of M13 Phage in organic solvents. *Journal of Biomaterials and Nanobiotechnology*, 7, pp. 72 – 77.

Mojica, K.D.A. and Brussaard, C.P.D., 2014. Factors affecting virus dynamics and microbial host-virus interactions in marine environments. *FEMS Microbiology Ecology*, 89(3), pp. 495 – 515.

Nagayoshi, Y. et al., 2016. Physiological properties and genome structure of the hyperthermophilic filamentous phage φOH3 which infects *Thermus thermophilus* HB8. *Frontiers in Microbiology*, 7, pp. 1 – 11.

Nap, R.J., Božič, A.L., Szleifer, I. and Podgornik, R., 2014. The role of solution conditions in the bacteriophage pp7 capsid charge regulation. *Biophysical Journal*, 107, pp. 1970 – 1979.

Nishigaki, K., Kaneko, Y., Wakuda, H., Husimi, Y. and Tanaka, T., 1985. Type II restriction endonucleases cleave single-stranded DNAs in general. *Nucleic Acids Research*, 13(16), pp. 5747 – 5760.

Norashirene, M.J., Umi Sarah, H., Siti Khairiyah, M.H. and Nurdiana, S. et al., 2013. Biochemical characterization and 16S rDNA sequencing of lipolytic thermophiles from Selayang Hot Spring, Malaysia. *International Conference on Agricultutral and Natural Resources Engineering*. Elsevier B.V., 5(2013), pp. 258 – 264.

Novik, G., Ladutska, A. and Rakhuba, D., 2017. Bacteriophage taxonomy and classification. In: Méndez-Vilas, A. (eds.). *Antimicrobial Research: Novel Bioknowledge and Educational Programs.* Spain: Formatex Research Center, pp. 251 – 259.

Olofsson, L., Ankarloo, J., Andersson, P.O. and Nicholls, I.A., 2001. Filamentous bacteriophage stability in non-aqueous media. *Chemistry and Biology*, 8(7), pp. 661 – 671.

Olson, M.R., Axler, R.P. and Hicks, R.E., 2004. Effects of freezing and storage temperature on MS2 viability. *Journal of Virological Methods*, 122(2), pp. 147 – 152.

Orlova, E.V., 2012. *Bacteriophages and Their Structural Organisation*. United Kingdom: Intech Open Access Publisher.

Parada, V., Herndl, G.J. and Weinbauer, M.G., 2006. Viral burst size of heterotrophic prokaryotes in aquatic systems. *Journal of the Marine Biological Association of the United Kingdom*, 86, pp. 613 – 621.

Pelczar, M.J., Chan, E.C.S. and Krieg, N.R., 2010. *Microbiology: An Application Based Approach*. New Delhi: Tata McGraw Hill Education Private Limited.

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

Poranen, M.M., Bamford, D.H. and Oksanen, H.M., 2015. Membrane-containing bacteriophages. *Encyclopedia of Life Sciences*, pp. 1 – 11.

Prangishvili, D., Stedman, K. and Zillig, W., 2001 Viruses of the extremely thermophilic archaeon *Sulfolobus*, *Trends in Microbiology*, 9, pp. 289 – 296.

Rakhuba, D.V., Kolomiets, E.I., Szwajcer Dey, E. and Novik, G.I., 2010. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish Journal of Microbiology*, 59(3), pp. 145 – 155.

Reigada, R., 2013. Atomistic study of lipid membranes containing chloroform: looking for a lipid-mediated mechanism of anesthesia. *PLoS ONE*, 8(1), pp. 1 – 10.

Reshes, G., Vanounou, S., Fishov, I. and Feingold, M., 2008. Cell shape dynamics in *Escherichia coli*. *Biophysical Journal*, 94, pp. 251 – 264.

Rigoldi, F., Donini, S., Redaelli, A., Parisini, E. and Gautieri, A., 2018. Review: Engineering of thermostable enzymes for industrial applications. *APL Bioengineering*, 2(1), pp. 1 – 17.

Ross, A., Ward, S. and Hyman, P., 2016. More is better : Selecting for broad host range bacteriophages. *Frontiers in Microbiology*, 7(1352), pp. 1 – 6.

Sakaki, Y. and Oshima, T., 1975. Isolation and characterization of a bacteriophage infectious to an extreme thermophile, *Thermus thermophilus* HB8. *Journal of Virology*, 15(6), pp. 1449 – 1453.

Samson, J.E., Magadán, A.H., Sabri, M. and Moineau, S., 2013. Revenge of the phages: Defeating bacterial defences. *Nature Reviews Microbiology*, 11, pp. 675 – 687.

Schoenfeld, T. et al., 2008. Assembly of viral metagenomes from Yellowstone hot springs. *Applied and Environmental Microbiology*, 74(13), pp. 4164 – 4174.

Scholl, D., Adhya, S. and Merrill, C.R., 2002. Bacteriophage SP6 is closely related to phages K1-5, K5, and K1E but encodes a tail protein very similar to that of the distantly related P22. *Journal of Bacteriology*, 184(10), pp. 2833 – 2836.

Shabbir, M.A.B. et al., 2016. Bacteria vs. bacteriophages: Parallel evolution of immune arsenals. *Frontiers in Microbiology*, 7(1292), pp. 1 – 8.

Shao, Y. and Wang, I.N., 2008. Bacteriophage adsorption rate and optimal lysis time. *Genetics Society of America*, 180(1), pp. 471–482.

Sillankorva, S., Oliveira, R., Vieira, M.J., Sutherland, I. and Azeredo, J., 2004. *Pseudomonas fluorescens* infection by bacteriophage ΦS1: The influence of temperature, host growth phase and media. *Federation of European Microbiological Societies Microbiology Letters 241*, pp. 13 – 20.

Silva, Y.J. et al., 2013. Influence of environmental variables in the efficiency of phage therapy in aquaculture. *Microbial Biotechnology*, 7(5), pp. 401 – 413.

Skurray, R.A., Hancock, R.E.W. and Reeves, P., 1974. Con- mutants: Class of mutants in *Escherichia coli* K 12 lacking a major cell wall protein and defective in conjugation and adsorption of a bacteriophage. *Journal of Bacteriology*, 119(3), pp. 726 – 735.

Sørensen, M.C.H. et al., 2011. Bacteriophage F336 recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *Journal of Bacteriology*, 193(23), pp. 6742 – 6749.

Sritha, K.S., Shemymol, K.A. and Sarita, G.B., 2017. Infectivity criteria for use of ϕSEP2 and ϕSEP3 as bio-control agents. *Journal of Bacteriology & Mycology: Open Access*, 5(4), pp. 322 – 326.

Stedman, K.M., 2012. Family Guttaviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (eds.). *Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses*. London: Elsevier Academic Press, pp. 191 – 192.

Summers, W.C., 2005. Bacteriophage research: early history. In: Kutter, E., and Sulakvelidze, A. (eds.). *Bacteriophages: Biology and Applications.* Florida: CRC Press, pp. 5 – 23.

Tamakoshi, M. et al., 2011. Genomic and proteomic characterization of the large *Myoviridae* bacteriophage ϕTMA of the extreme thermophile *Thermus thermophiles*. *Bacteriophage*, 1(3), pp. 152 – 164.

Tey, B.T. et al., 2009. Production of fusion M13 phage bearing the di-sulphide constrained peptide sequence C-WSFFSNI-C that interacts with hepatitis B core antigen. *African Journal of Biotechnology*, 8(2), pp. 268 – 273.

Thorne, C.B. and Holt, S.C., 1974. Cold lability of *Bacillus cereus* bacteriophage CP 51. *Journal of Virology*, 14(4), pp. 1008 – 1012.

Turkyilmaz, S., Chen, W., Mitomo, H. and Regen, S.L., 2009. Loosening and reorganization of fluid phospholipid bilayers by chloroform. *Journal of the American Chemical Society*, 131(14), pp. 5068 – 5069.

Uchiyama, J. et al., 2013. Characterization of *Helicobacter pylori* bacteriophage KHP30. *Applied and Environmental Microbiology*, 79(10), pp. 3176 – 3184.

Ventola, C.L., 2015. The antibiotic resistance crisis part 1: Causes and threats. *Pharmacy and Therapeutics*, 40(4), pp. 277 – 283.

Wang, Y. and Zhang, X., 2010. Genome analysis of deep-sea thermophilic phage D6E. *Applied and Environmental Microbiology*, 76(23), pp. 7861 – 7866.

Weinbauer, M.G. and Hofle, M.G., 1998. Size-specific mortality of lake bacterioplankton by natural virus communities. *Aquatic Microbial Ecology*, 15, pp. 103 – 113.

Weinbauer, M.G. and Peduzzi, P., 1994. Frequency, size and distribution of bacteriophages in different marine bacterial morphotypes. *Marine Ecology Progress Series*, 108, pp. 11 – 20.

White, H.E. et al., 2012. Capsid structure and its stability at the late stages of bacteriophage SPP1 assembly. *Journal of Virology*, 86(12), pp. 6768 – 6777.

Young, R., Wang, I.N. and Roof, W.D., 2000. Phages will out: Strategies of host cell lysis. *Trends in Microbiology*, 8(3), pp. 120 – 128.

Yu, F. and Mizushima, S., 1982. Roles of lipopolysaccharide and outer membrane protein OMPc of *Escherichia coli* K-12 in the receptor function for bacteriophage T4. *Journal of Bacteriology*, 151(2), pp. 718 – 722.

Yu, M.X., Slater, M.R. and Ackermann, H.W., 2006. Isolation and characterization of *Thermus* bacteriophages. *Archives of Virology*, 151, pp. 663 – 679.

APPENDIX A

Reagents, Chemicals and Equipment

Table A: The list of reagents and chemicals used throughout this study.

Reagents/ Chemicals	Manufacturers, Country
1 Kb DNA ladder	Vivantis Technologies Sdn. Bhd., Malaysia
10X Buffer Tango (with BSA)	Thermo Scientific, USA
Acetic acid glacial	QRec, Malaysia
Agarose powder	Fisher Scientific, USA
Chloroform	GENE Chemical
DNAse A	Vivantis Technologies Sdm. Bhd., Malaysia
Ethyl alcohol absolute (99.8%)	ChemSoln, Malaysia
Ethylenediaminetetraacetic acid (EDTA)	QReC, Malaysia
Glycerol	Systerm, Malaysia
Hydrochloric acid	VWR Prolabo, Singapore
Luria Bertani agar	Laboratories Conda, Spain
Luria Bertani broth	Laboratories Conda, Spain
Magnesium chloride	Sigma Aldrich, USA
Novel juice	Gene DireX Inc., USA
Phenol/chloroform/isoamyl alcohol (1 phase)	Amresco, USA

Table A: Continued

Reagents/ Chemicals	**Manufacturers, Company**
Polyethylene glycol 8000 (PEG 8000)	Bio Basic Canada Inc., Canada
Proteinase K	Fermentas
Restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Xba*I)	BIORON, Germany
Restriction enzyme (*Sac*I)	Thermo Scientific, USA
RNAse A	Bio Basic Inc., Malaysia
Sodium dodecyl sulphate (SDS)	Bio Basic Canada Inc., Canada
Sodium acetate	R&M Chemicals, Malaysia
Sodium chloride	Merck, USA
Tris	Bio Basic Inc., Malaysia

APPENDIX B

Table B: The list of instruments, apparatus and laboratory wares used throughout this study.

Instruments/ Apparatus/ Laboratory wares	**Manufacturers, Company**
Autoclave machine	HIRAYAMA, Japan
Acrodisc 0.45 µm syringe filter	Pall Corporation, USA
Centrifuge tubes (15 mL and 50 mL)	Nest Biotechnology Co., Ltd., China
Gel electrophoresis set	Major Science, Taiwan
High speed centrifuge	Sigma 2-16PK Sartorius, Germany
Hot plate stirrer	Laboratory and Medical Supplies, Japan
Incubator	Memmert, Germany
Laminar flow cabinet	Esco Micro Pte. Ltd., Singapore
MP-300 V power supply	Medigene Sdn. Bhd., Malaysia
Media bottles	KIMAX, Germany
Microcentrifuge tubes (200 µL and 1.5 mL)	Nest Biotechnology Co., Ltd., China
Microwave oven	Sharp, Japan
Nano-spectrophotometer	Implen, USA
Petri dishes	Nest Biotechnology Co., Ltd., China
pH meter	Eutech Instruments, Singapore
Shaking incubator	Yihder Co., Ltd., Taiwan
Spectrophotometer and cuvettes	Biochrom, USA

Table B: Continued:

Instruments/ Apparatus/ Laboratory wares	**Manufacturers, Company**
Syringe (1 mL/ cc and 5 mL/ cc)	Terumo, Belgium
Table-top microcentrifuge machine	Thermo Scientific, USA
UV transilluminator	Bio-Rad, USA
Vortex mixer	Stuart, UK
Water bath	Memmert, Germany
Weighing balance	KERN & Sohn GmbH, Germany

APPENDIX C

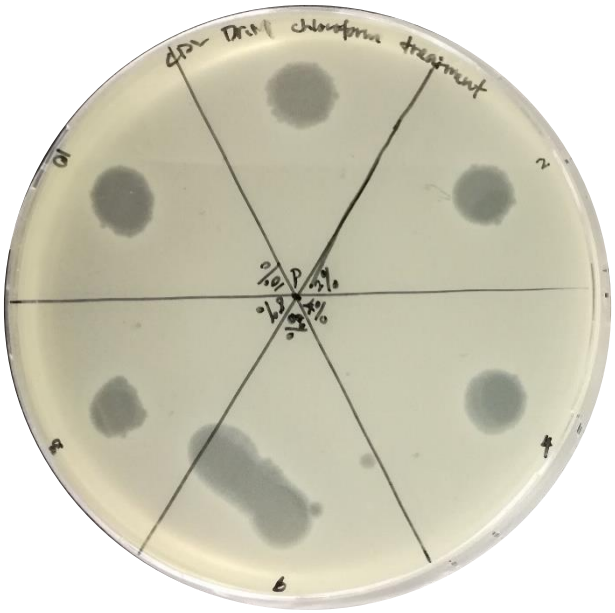

Figure A: Effect of chloroform on phage LT-B infectivity at 2% to 10% (v/v) concentrations.

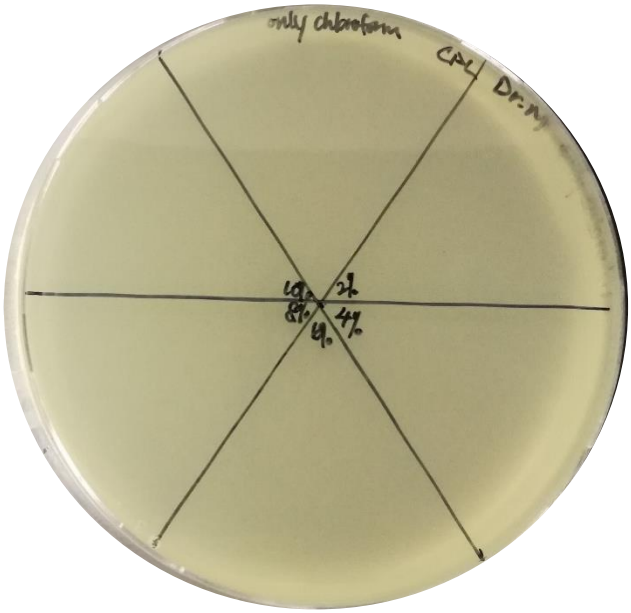

Figure B: Effect of chloroform on *E. coli* BL21 lawn at 2% to 10% (v/v) concentrations (negative control).