EXTRACTION OF ENDOLYSIN FROM BACTERIOPHAGE KW01-A ISOLATED FROM KUALA WOH HOT SPRING, PERAK.

By

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ABSTRACT

EXTRACTION OF ENDOLYSIN FROM BACTERIOPHAGE KW01-A ISOLATED FROM KUALA WOH HOT SPRING, PERAK.

WONG SEE WEI

Due to the global increase of multidrug resistance bacteria, more attention has been drawn towards the potential use of bacteriophage endolysins as the antimicrobial agents. Bacteriophages release their viral progeny by producing endolysin that degrades the peptidoglycan cell wall, which results in cell lysis. In this study, the extraction of endolysin from crude phage lysate of a thermophilic phage, KW01-A was optimized using different ammonium sulfate saturation. In addition, both the phage and its extracted endolysin were characterized and studied based on their deactivation temperature, pH stability, and host susceptibility. The KW01-A phage isolate was grown against *Escherichia coli* BL21 (DE3) and precipitated by using polyethylene glycol (PEG). Then, the endolysin were extracted from the crude phage lysate with 0-20%, 20-40%, 40-60% and 60-80% ammonium sulfate precipitation. Next,

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and spot lytic assays were performed to determine the presence of endolysin. KW01-A phage was deactivated when heated at 75^{o}C for 10 min to reveal the lytic activity of endolysin in the precipitated protein samples. The endolysin activity was detected in the precipitated protein of 20-40% ammonium sulfate saturation. In this study, it was shown that the precipitated endolysin required outer membrane permeabilizers (OMPs) to permeabilize intact Gram-negative bacteria in order for the endolysin to exert its lytic activity. When the precipitated endolysin was heat-treated, the enzyme could not tolerate high temperature at 80^{o}C and above. Apart from that, both the KW01-A phage and its endolysin have broad pH stability, ranging from pH 4.0 to 10.0. In addition, the KW01-A phage was only specific to *E. coli* BL21 (DE3) whereas its endolysin was able to lyse other *E. coli* strains. Therefore, based on the results obtained from this study, further investigation on the phage endolysin is definitely necessary to explore its potential as antibacterial agents.

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Finally, last but not least, I would like to thank my family and all my friends for their constant helps, encouragements and support throughout this project.

DECLARATION

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

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WONG SEE WEI

APPROVAL SHEET

This project report entitled "**EXTRACTION OF ENDOLYSIN ISOLATED FROM BACTERIOPHAGE KW01-A FROM KUALA WOH HOT SPRINGS, PERAK**" was prepared by WONG SEE WEI 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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PERMISSION SHEET

It is hereby certified that **WONG SEE WEI** (ID No: **14ADB02397**) has completed this final year project entitled "**EXTRACTION OF ENDOLYSIN ISOLATED FROM BACTERIOPHAGE KW01-A FROM KUALA WOH HOT SPRINGS, PERAK**" under the supervision of Dr. Michelle Ng Yeen Tan (Supervisor) from the Department of Biomedical Science, Faculty of Science.

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

Yours truly,

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(WONG SEE WEI)

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LIST OF ABBREVIATIONS

x*g*	Times gravity (acceleration due to gravity)
A. baumannii	*Acinetobacter baumannii*
APS	Ammonium Persulfate
B. anthracis	*Bacillus anthracis*
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
CBD	Cell wall binding domain
DNA	Deoxyribonucleic acid
E. faecium	*Enterococcus faecium*
E. coli	*Escherichia coli*
EAD	Enzymatically active domain
EDTA	Ethylenediaminetetraacetic acid
EPEC	Enteropathogenic *Escherichia coli*
EPS	Extracellular polymeric subatance
ETEC	Enterotoxigenic *Escherichia coli*
HCl	Hydrochloric acid
ICTV	International Committee for Taxonomy of Viruses

kDa	Kilodaltons
kPa	Kilopascal
K. pneumoniae	*Klebsiella pneumoniae*
LB	Luria Bertani
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharide
M	Molar
mA	Milliampere
MDR	Multidrug resistant
mM	Millimolar
MRSA	Methicillin resistant *Staphylococcus aereus*
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
OD_{600}	Optical density measure at wavelength 600 nm
OMP	Outer membrane permeabilizer
P. aeruginosa	*Psuedomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
pfu/mL	Plaque forming units per millilitre
RNA	ribonucleotide
S. aeurus	*Staphylococcus aureus*
S. dysenteriae	*Shigella dysenteriae*
S. enterica	*Samonella enteric*
S. flexneri	*Shigella flexneri*
S. pneumoniae	*Streptococcus pneumoniae*
S. typhi	*Samonella typhi*
SDS	Sodium dodecyl sulphate
sp.	Species
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

According to the Health Ministry of Malaysia (2016), antimicrobial resistance issues have been rising at an alarming rate. Bacterial resistance to antibiotics has become a serious problem and challenge to the healthcare sector. As a result, this weakens the ability of antibiotics to effectively control and kill bacteria (Kannan, 2016). The emergence of multidrug resistance of bacteria can be life-threatening for infected patients. In order to overcome these problems, one of the alternative ways of treatment is by using bacteriophage therapy.

Bacteriophages are the most abundant organisms in the world (Deresinski, 2009). Due to their unique host specificity, the bacteriophage can be potentially used as antimicrobial agents (Clokie, et al., 2011). Many kinds of research have reported the success of phages to specifically kill various human pathogens, especially against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. According to Matsuzaki, et al. (2003), the protective effect of bacteriophage ϕMR11 has been accessed against *S. aureus* including

methicillin-resistant bacteria infection mice. In addition, by using a novel murine model, Waters, et al. (2017) have demonstrated that *P. aeruginosa* phage PELP20 could effectively kill *P. aeruginosa* within a biofilm-associated cystic fibrosis lung-like environment. Besides that, there was a clinical trial phase I and II of a therapeutic bacteriophage preparation (Biophage-PA) against *P. aeruginosa* infection such as chronic otitis have been successfully completed (Wright, et al., 2009).

Despite the optimistic outlook on the application of phage therapy in human, there are still doubts about its ability to become the alternative to antibiotic treatment. The restriction of internationally recognized human clinical trials and the phage resistance issues denied its ability as the antibacterial agent (Roach and Donovan, 2015). Other challenges that restrict their use of antimicrobial include sequestration of the phage by the spleen and liver, antibodies against the phage and poor accessibility of phage to the infected tissue. Researchers are now focusing on the studies of the phage-encoded bacteriolytic enzyme as the alternative antimicrobial agent. One of the bacteriolytic enzymes which are known as endolysin has been shown to have enormous potentials and advantages as an effective antibacterial in killing various bacterial cells (Fenton, et al, 2010). The potent ability of phage-encoded endolysin to cause rapid lysis of bacterial host when applied externally makes it a potential alternative as an antibacterial agent (Schmelcher,

Donovan and Loessner, 2012).

In general, purified endolysins have been shown to have antimicrobial potential against pathogens such as *S. pneumoniae*, *Bacillus anthracis*, *Bacillus subtilis*, *S. aureus*, *Clostridium perfringens*, *Enterococcus faecalis* and *Listeria monocytogenes* (Lim, et al., 2014). These studies showed that lysins could work more effectively against Gram-positive bacteria than the Gram-negative bacteria. The lytic activity is restricted by the presence of outer membrane of Gram-negative bacteria that acts as a protective barrier. The Gram-negative bacteria require pre-treatment with outer membrane permeabilizers (OMPs) such as ethylenediaminetetraacetic acid (EDTA) and Triton X-100 (Fischetti, 2005). However, some studies have been reported that the lysins were able to kill the Gram-negative bacteria without pre-treatment with OMPs. This was evident in a research conducted by Guo, et al. (2017), whereby the purified endolysins such as LysPA26 was able to kill not only *P. aeruginosa* but also other Gram-negative bacteria when applied externally.

Therefore, the objectives of this study were to optimize the extraction of endolysin from the phage KW01-A using ammonium sulfate precipitation method. Secondly, the lytic activity of the extracted endolysin against different hosts in comparison to the phage was carried out. Finally, the characterization of the extracted endolysin was performed based on its pH and thermal stability.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteriophage

2.1.1 History of Bacteriophage

An unidentified substance with marked antibacterial activity in the waters of Ganges and Yamuna rivers in India has been reported by Ernest Hankin, a British bacteriologist in 1896. This unidentified substance has marked antibacterial activity against cholera. Then, bacteriophages were first discovered by Frederick William Twort in 1915. The name "bacteriophage" or "bacteria eater" was coined by Felix d'Herelle. He has conducted many types of research on bacteriophage and introduced the therapeutic use of phage. He demonstrated anti-Shiga microbe activity of the phage as a treatment for shigellosis (Sulakvelidze, Alavidze, and Morris, 2001).

Phage therapy was only available in the 1920s and 1930s. However, this therapy was limited to serum therapy against selected pathogens such as pneumococci and diphtheria. Due to the emerging antibiotic chemotherapy, the phage research has been largely abandoned in the western countries (Wittebole, De Roock, and Opal, 2014).

2.1.2 Nature of Bacteriophages

Bacteriophage is a virus that specifically infects bacterial hosts. There are three basic components of a bacteriophage, which are the head, genetic materials, and phage tail. In the "head", protein capsid is important to give protection to the nucleic acids, which can either be in double-stranded DNA, single-stranded DNA or RNA (Mayer, 2016). The structures of the viral capsid have been studied using the electron microscopy and X-ray diffraction. The capsid can be in icosahedral, helical or head-tail forms (Heritage, Evans and Kilington, 1996). Phage tail, which consists of a baseplate with tail fibers and a long tube with contractile sheath allows the viral genome to be injected into the bacterial host (Aksyuk, et al., 2009).

International Committee on Taxonomy of Viruses (ICTV) has been used to classify phages based on their morphology and nucleic acid composition.

There are 14 different families of phage, which have been organized according to this approach (Elbreki, et al., 2014). Based on the morphological groups of phages, they can be classified into filamentous phages, icosahedral phages without tails, tailed phages, and even lipid-containing envelope phages (Comeau, et al., 2012). Among the all the phage studied, double-stranded DNA tailed phage or *Caudovirus* is the most abundant, which account for 96%. Under the order *Caudovirus*, there are three families, which are *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Orlova, 2012). Figure 2.1 illustrates the overview of the 14 phage families.

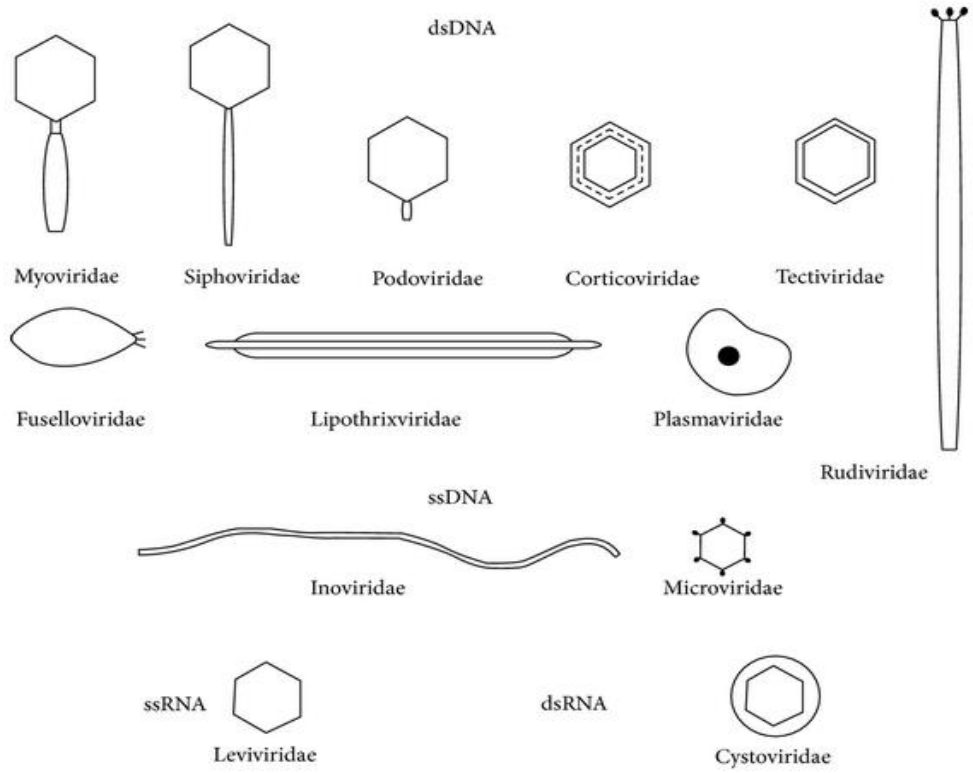

Figure 2.1: Overview of the morphology of 14 phage families. (Adapted from Elbreki, et al., 2014)

2.1.3 Life Cycle of Bacteriophage

As an obligate intracellular parasite, the host energy and protein biosynthetic machinery were highly relied upon by bacteriophages to complete their life cycles. To infect and replicate, they undergo either lytic or lysogenic life cycles depending on their types. Lytic or virulent phage will undergo a lytic cycle whereas lysogenic or temperate phage will undergo a lysogenic cycle (Elbreki, et al., 2014).

The life cycle of phages must begin with adsorption to their specific bacterial hosts regardless of which cycle. By random collision, phages have the first contact with the bacterial hosts and provide a specific interaction with the receptors on the bacterial cells (Elbreki et al., 2014). After the attachment is stable and irreversible, phages will create holes to allow the injection of nucleic acids into the host cells. Successful penetration of the phages is important for the survival and continuation of phage life cycle (Rakhuba, et al., 2010).

In lytic cycle, after the viral genome has entered the host cells, the phage will harvest and exploit the host's metabolism to synthesize its own genes to produce nucleic acids and proteins. These viral particles will then assemble and package (Nicastro, et al., 2016). At the same time, phage's late proteins such as holin and endolysin will be produced to create holes to allow the

release of progeny viruses. The bacterial cells will then burst and died. As a result, the released progeny viruses will infect other surrounding bacterial cells.

Instead of directly killing the host cells, lysogenic phage integrates its viral genome into the bacterial chromosome that can disrupt gene expression of the host. At this stage, the integrated viral genome is known as a prophage, while the bacterial host that contains viral genome is called as lysogen. In addition, the lysogenic conversion may occur and the phenotype of the bacterial cells will be altered by expressing the gene products with special functions in the bacterial cells that are not part of a usual course of infection (Clokie, et al., 2011). The phage can remain dormant in the host without killing the host. According to Erez, et al. (2017), the lysogenic phage can control the lytic-lysogenic decision using a small molecule communication system, which is known as "arbitrium" system. There are many factors or conditions that can trigger the phage to enter either the lytic or lysogenic stage. Based on the "arbitrium" system, it can be concluded that when a single phage encounters a large colony of bacteria, they will favor lytic cycle. On the other hand, if the host numbers are limited, the phage will enter the lysogenic cycle. Figure 2.2 summarizes the life cycle of a bacteriophage.

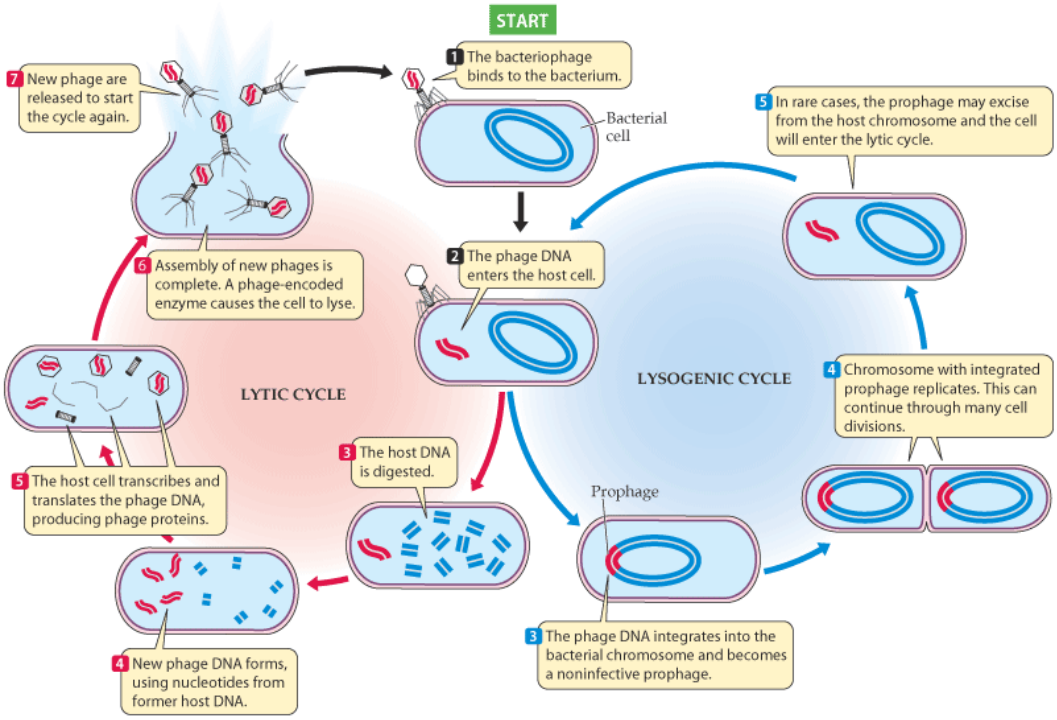

Figure 2.2: The life cycle of lytic and lysogenic bacteriophage. (Adapted from Aman and Ciobanu, 2011)

2.1.4 Applications of Bacteriophage

Due to the increasing awareness of global antimicrobial resistance, researchers are urged to investigate for alternative approaches or treatments to overcome this global issue. One of the alternative treatments for bacterial infections is phage therapy. Bacteriophage targets specific bacterial species and kills them by lysis mechanism, which made them a potential antibacterial agent (Schooley, et al., 2017). A few studies showed that bacteriophage can be used to kill multidrug-resistant bacteria. One study has demonstrated that co-therapy using lytic phage and linezolid has effectively eliminated Methicillin-Resistant

Staphylococcus aureus (MRSA) that caused diabetic foot infections (Chhibber, Kaur and Kaur, 2013). In addition, Biswas, et al. (2002) has reported that phage ENB6 was effective in treating mice that were infected with vancomycin-resistant *Enterococcus faecium* (VRE). Therefore, these findings showed that bacteriophage can be considered as the alternative therapeutic method against multidrug-resistant bacterial infections.

Colonization of bacteria on indwelling medical devices such as catheters can result in the formation of biofilm. However, there was lack of anti-infective strategies to eliminate biofilm. Some of the findings found that bacteriophages can penetrate the biofilms and eventually kill the bacteria. The presence of bacteriophage-borne polysaccharide depolymerize on the external surface of capsid enables it to degrade the extracellular polymeric substance (EPS) of the biofilm and allows the phage to access the bacteria embedded within the EPS matrix (Hughes, Sutherland and Jones, 1998). The ability of a phage cocktail containing phages Pm5460 and Pm5461 to prevent catheters' colonization by *Proteus* was reported (Melo, et al., 2016). Besides that, by using a cocktail of *Clostridium difficle* phages, *C. difficle* was killed and the biofilm formation could be prevented (Nale, et al., 2016). Therefore, bacteriophage can be utilized in the treatment of devices-associated biofilm bacterial infection.

Besides the conventional phage therapy by using lytic phages to treat the infections, phage display technique can be used for identification of monoclonal antibodies (mAbs) against pathogens, as well as for vaccine development (Criscuolo, 2017). The mAb can be produced by insertion of the desired coding sequence of antibody variable regions into the nucleotides of viruses that encode for the coat protein. Then, the antibodies will be expressed together with the phage DNA as the fusion protein on the phage surface (Carmen and Jermutus, 2002). This allows the development of mAbs targeted against cancer or inflammatory diseases. One of the examples of mAb that was successfully applied is palivizumab, which was used in the treatment of respiratory syncytial virus (RSV) infection (Huang, Bishop-Hurley, and Cooper, 2012). Thus, the phage display techniques can be a valuable tool to improve the diagnostic method and develop a potential treatment against different kinds of diseases.

In addition, phage has also been applied as a biocontrol agent in the food industry. The biofilm formation on food has become a challenging problem in the food industry. One of the most promising strategies to eradicate biofilms is the use of bacteriophage. For example, the number of adherence foodborne pathogen, particularly *E. coli* O157:H7 on the surface was significantly reduced after the treatment with bacteriophage BPECO (Sadekuzzaman, et al, 2017). Due to their specificity on the host, these bacteriophages are harmless to

humans, animals, and plants. In 2013, Food and Drug Administration (FDA) has categorized some bacteriophage as Generally Recognized as Safe (GRAS) for the use as an antimicrobial ingredient to control bacterial growth in foods. One of the commercially available phage products that were approved by FDA is ListShield^{TM} (FDA, 2013). ListShield^{TM} specifically targets foodborne pathogen such as *L. monocytogenes* on the ready-to-eat meat and poultry products (Strydom and Witthuhn, 2015). These bacteriophages have been approved to be used as direct food additives due to their minimal risk to human health (Sharma, 2013).

On the other hand, bacterial diseases in plants have become a challenging problem in the agriculture field as the current management strategies, especially the chemical controls are limited. Biological control such as bacteriophage has been used for combating plant disease. Álvarez and Biosca (2017) reported that bacterial wilt disease, which is caused by *Ralstonia solanacearum sp.* can be effectively killed by the phages φRSL1 and PE204. Unlike the chemical pesticides, phages will not persist in the soil and will not cause any pollution to the environment (Buttimer, et al., 2017). These are the reasons why phages are chosen as the biological control agents for plant disease. On the other hand, bacteriophages can also be used to improve the intestinal health in pigs and poultry. Kim, et al. (2014) have reported that bacteriophage was more effective than probiotics to promote the growth of

pigs. Another group of researchers has shown that the pathogenic bacteria in the piglets' intestines were significantly reduced after the bacteriophages were supplemented in piglets' creep feed (Einstein-Curtis, 2016). Therefore, these showed that bacteriophages can be beneficial in the agriculture industry.

2.2 Advantages and Disadvantages of Bacteriophage Therapy

High specificity of bacteriophage is the main advantage for phage therapy which helps to minimize the risk of secondary infections. In contrast, the chemical antibiotics, which have broader spectrum activities target both pathogens and normal flora of the patients. This will eventually result in secondary infections or superinfections. Besides that, phage specifically targets on the site of infection while antibiotics tend to circulate throughout the body, targeting not only the site of infection but also the normal tissues (Golkar, Bagasra and Pace, 2014). A study has demonstrated that only minimal side effect was observed on the gastrointestinal tract in mice resulted from oral administration of *E. coli* phage (Chibani-Chennoufi, et al., 2004). Besides that, Denou, et al. (2009) also demonstrated that orally applied phage cocktail against *E. coli* that cause diarrhea had no negative impacts on gut flora. Therefore, these studies demonstrated that phage therapy is a relatively safe treatment to use without affecting human's normal flora.

As bacteriophage consists of nucleic acids and proteins only, it is considered to be inherently less toxic to patients compared to antibiotics. The safety of patients can be assured. However, lysis of phage which is then followed by the release of bacterial contents and toxins may trigger an immunological response (Nilsson, 2014). In order to overcome this problem, phage preparations for therapy must be free from any toxins or substances that can cause allergy. Few studies have shown that phages have been engineered as nonreplicative or lysis-deficient mutants. For instance, lysogenic phage M13 has been engineered to encode for lethal proteins in two ways without causing lysis to the host cells to minimize the release of endotoxin (Hagens and Bläsi, 2003). Lastly, phages exhibit self-replicating properties, which means that they can replicate at the site of infection as long as the bacterial host is present. Therefore, complete eradication of the bacteria can be achieved without the need to apply them repeatedly (Loc-Carrillo and Abedon, 2011).

Despite the numerous advantages of phage therapy, there are also some limitations in this application. Phages will evoke an immune response when administrated intravenously as the phage antigens will be recognized as foreign antigens for the immune system, especially in immunocompromised patients (Wittebole, De Roock, and Opal, 2014). Merrils, et al. (1996) demonstrated that phage therapy was not effective because of the clearance of phage particles by reticuloendothelial system of patients, which eventually reduced

the number of phages to a level that was not sufficient to kill the bacterial cells. Besides that, repeated exposures of the same strain of phage to the body will activate the adaptive immune response, which resulted in antibody production. These antibodies will neutralize and clear the particular phages, which eventually decrease their efficacy in killing their target pathogens (Henein, 2012).

Next, the increasing growth of phage-insensitive bacterial strains has limited the application of phage to treat bacterial infections. Some bacteria have gained the ability to subvert the antiviral mechanisms in order to survive in most environments. There are a few antiviral mechanisms used by bacterial cells as reported by Labrie, Samson, and Moineau (2010). For examples, to prevent the entry of viral genome into bacterial cells, some bacterial cells will cleave the unmethylated phage DNA by the restriction enzyme. This mechanism is known as restriction-modification systems. In addition, bacteriophage can become the vector for transferring antibiotic-resistant genes to bacterial cells through transduction (Balcazar, 2014). In this process, phages may transfer the virulence genes, such as toxins and antibiotics to bacterial cells, which will transform a non-pathogenic bacterial cell into a virulent pathogen (Brabban, Hite and Callaway, 2005). This may contribute to the development of even more resistant bacteria. Therefore, the use of phage therapy should be reconsidered due to the risk of resistance development.

Due to the various disadvantages and limitations of phage therapy and lack of internationally recognized human clinical trials currently, phage therapy for human use has not been approved in most of the countries. Therefore, many researchers have shifted their focus to bacteriostatic and bacteriolytic proteins secreted by phage, for example, endolysin as an antibacterial agent.

2.3 Endolysin

2.3.1 Nature of Endolysin

According to Young (1992), newly formed phage particles are released outside the cell with the help of a set of enzymes after completion of replication cycle inside the infected bacterial cells. These enzymes- endolysins, along with holins are encoded by all double-stranded DNA phages, which together form a holin-lysin system (Jarábková, Tišáková, and Godány, 2015). Holins act as the protein clock of the bacteriophage infection, which will oligomerize and form holes on the host cell cytoplasmic membrane. This provides a route for the endolysins to attack and lyse the host cells.

Endolysin, which is a peptidoglycan hydrolase can be purified and act on the peptidoglycan externally to cause the “lysis from without” (Nelson, et al., 2012). This characteristic makes the endolysin as an effective antimicrobial agent against various bacterial cells.

2.3.2 Structure and Classification of Endolysin

Phage endolysin is composed of different functional domains or modules. Loessner (2015) proposed that endolysins are usually comprised of N-terminal catalytic domain and a C-terminal cell wall binding domain. The structure of endolysin from Gram-positive organisms was reported to vary from Gram-negative endolysin structure. Endolysins with a modular structure usually belongs to the bacteriophage against Gram-positive organisms (Diaz, Lopezand Garcia, 1990), whereas phage endolysin associated with Gram-negative host cells consists of a single-module, globular protein (Briers, et al., 2009).

Endolysins encoded by phages against Gram-positive host cells which have the modular structure are generally between 25 to 40 kDa in size. There are two functional domains of the endolysin, termed as cell wall binding domains (CBDs) which responsible for the endolysin's catalytic activity and enzymatically active domains (EADs) which functions for substrate recognition. The CBDs are typically located at C-terminus while EADs are situated at the N-terminus (Schmelcher, Donovan and Loessner, 2012). For example, the endolysin derived from the pneumococcal phage Cp-1 has a modular structure (Hermoso, et al 2003). In contrast, the Gram-negative host which has an outer membrane in the periplasmic space prevents direct contact and damage by endolysin. Therefore, this explains why most of the lysins from

phage infecting Gram-negative hosts are small, single domain globular proteins with a mass size of 15 to 20 kDa, without specific CBD modules (Nelson, et al., 2012).

Their classifications are very much depending on their enzymatic activity against specific bond on the peptidoglycan layer of Gram-negative bacteria. They can be classified into at least five different groups, which are (I) N-acetyl-β-d-muramidases or lysozymes; (II) lytic transglycosylases; (III) N-acetyl-β-d-glucosaminidases or glycosidases; (IV) N-acetylmuramoyl-l-alanine amidases and (V) endopeptidases (Barrera-Rivas, et al., 2017). The N-acetylglucosaminil-β-1,4-N-acetylmuramine bonds were cleaved by both the N-acetyl-β-d-muramidases and lytic transglycosylases whereas the other glycosidic bond in the sugar strand was hydrolyzed by N-acetyl-β-d-glucosaminidases. On the other hand, amidases catalyze the hydrolysis of the amide bond, which connects glycan to the amino acids whereas the endopeptidases cut peptide bonds on amino acids chains linked to the glycan moiety (Schmelcher, Donovanand Loessner, 2012). Figure 2.3 shows the structure of bacterial peptidoglycan and cleavage sites by endolysins.

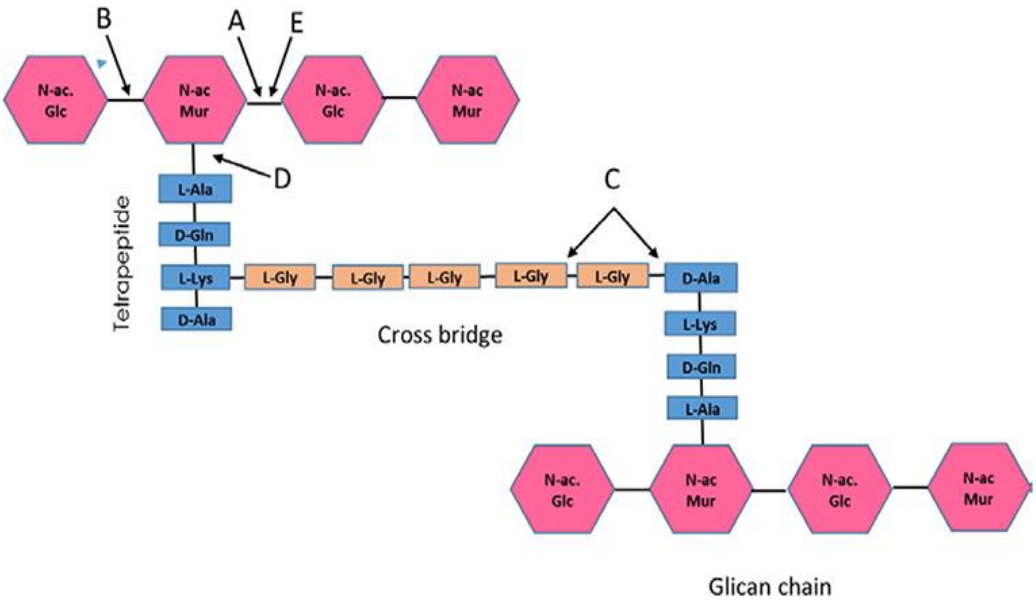

Figure 2.3: Structure of bacterial peptidoglycan and cleavage sites by endolysins. (A) N-acetyl-muramidase (B) N-acetylglucosaminidase (C) Endopeptidase (D) N-acetylmuramoyl-L-alaninamidase (E) Lytic transglycosylases (Adapted from Barrera-Rivas, et al., 2017)

2.4 Characteristics of Endolysin

2.4.1 Endolysin Specificity

Similar to bacteriophage, the bacteriophage-encoded endolysins also target specifically to certain bacterial hosts. Generally, endolysins are species-specific or strain-specific (Pastagia, et al, 2013). According to Na, Kong, and Kyu (2016), LysPBC4, an endolysin isolated from a *Bacillus cereus*-specific phage PBC4 was only able to kill most of the *B.cereus* strains but showed no effect on other bacteria such as *B. subtilis* or *Listeria*, implying that the endolysin has a specific and narrow host spectrum of lytic activity

towards the *B. cereus* group. However, some endolysins have also been observed for their broad lytic activity against different strains of Gram-positive pathogens. For instance, an enterococcal phage lysin, PlyV12 has been identified and shown to not only kill the enterococci but also other types of Gram-positive bacteria such as *B. anthracis*, *Streptococcus pyogenes* and *S. pneumoniae* (Young, et al., 2004).

Limited findings were reported on the success of endolysin to lyse Gram-negative bacteria as the outer membrane prevents the access of endolysins. Some outer membrane permeabilizers such as EDTA, citric acid, and polycationic agents have been used to allow the penetration of antibiotics for the treatment of bacterial infections (Vaara, 1992). Similarly, the outer membrane permeabilizers can be applied to Gram-negative bacteria to allow the access of externally applied endolysin. One of the studies demonstrated that different permeabilizing agents have been used to permeabilize *P. aeruginosa* to allow the entry of endolysin EL188. As a result, endolysin EL188 was able to lyse the bacterial and treated the infection effectively (Briers, Walmagh and Lavigne, 2011).

In addition, there are also some endolysins that are able to act on both Gram-positive and Gram-negative bacteria. Dong, et al. (2015) has reported *Stenotrophomonas maltophilia* endolysin P28 has a broad antibacterial activity

against both Gram-positive and Gram-negative bacterial cells. Moreover, it could effectively lyse the bacterial hosts without the treatment of outer membrane permeabilizers (OMPs). This is further supported by another study, in which endolysin SPN9CC has been shown to exogenously killed intact Gram-negative bacteria without the presence of OMP (Lim, et al., 2014). Likewise, *Bacillus amyloliquefaciens* phage endolysin could retain its antibacterial activity against *P. aeruginosa* even in the absence of OMP (Morita, et al., 2001). In short, some endolysin against Gram-negative bacteria could exert its lytic activity without being obstructed by the presence of the outer membrane in the Gram-negative host.

2.4.2 Endolysin Immunogenicity

When administrated systematically or mucosally, endolysin can elicit the immune response as it is a protein similar to bacteriophage. Few studies have explored the possibility of endolysin elimination by the immune system. For example, positive IgG antibodies test that is specific to the phage lysin was detected in the rabbits immunized with several doses of the pneumococcal phage lysin Cpl-1 (Loeffler, Djurkovicand Fischetti, 2003). Furthermore, similar results were also reported with *B. anthracis* and *S. pyogenes* endolysins (Fischetti, 2005).

However, it was found that the lytic activity of the endolysin was slowed down but not inactivated in an *in vitro* test (Loeffler, Djurkovicand Fischetti, 2003). This finding was further supported by an *in vivo* study demonstrated by Jado, et al. (2003). The antibodies that formed against two pneumococcal endolysins, Cpl-1 and Pal showed the similar result as observed in the *in vitro* study, whereby the endolysins were still remained active in killing their hosts. In addition, no other adverse effects or signs of anaphylaxis were observed. Therefore, these results suggested that the antibodies raised were not neutralizing antibodies, thus it would not inactivate the lytic activity of endolysins (Lobocka and Szybalski, 2012). Some of the studies hypothesized that the strong binding affinity of some CBDs are greater than the affinity of endolysin-specific antibodies. As a result, the endolysins can escape from being neutralized by the antibodies (Loessner, et al., 2002). In short, these endolysin-specific antibodies did not inhibit the lytic activity of endolysins.

2.4.3 Endolysin Resistance

To date, no specific endolysin resistance has been reported so far. Endolysins bind to the highly conserved and immutable targets in the cell wall due to the coevolution of bacteriophage together with the bacterial host. This could be the reason why endolysin resistance is a rare event. Besides that, endolysins,

which have two active domains, are predicted to be more difficult to develop resistance because the host cells will need to mutate and modify several target bonds to resist the lytic activity of the endolysin (Fischetti, 2005). Moreover, external application of endolysin also prevents the bacterial host to develop resistance since most of the classical resistance mechanisms develop from the inside of the cells (Donovan, 2009).

According to Totté, van Doorn and Pasmans (2017), patients treated with topical endolysin Staphefekt SA.100 did not develop resistance during long-term daily therapy. Another study showed that three lytic fusion enzymes targeting *S. aureus* have successfully served as an effective treatment for *S. aureus* infection without any development of endolysin resistance (Becker, 2016). In a study conducted by Loeffler, Nelson and Fischetti (2001), cells of *S. pneumonia*, which were repeatedly exposed to a purified pneumococcal endolysin Pal either *in vitro* or *in vivo*, showed no Pal-resistant pneumococci. Therefore, these findings clearly showed that unlike antibiotics, endolysin did not develop resistance even with prolonged exposure.

2.5 Applications of Endolysin

Endolysin has been widely investigated to be used as an alternative way to treat bacterial infections, especially those caused by multidrug-resistant bacteria. For example, the spread of MRSA which causes chronic and recurrent *S. aureus*-related dermatoses has become increasingly serious. To overcome this problem, a topical recombinant phage endolysin Staphefekt SA.100 was produced. Both methicillin-sensitive and methicillin-resistant *S. aureus* were successfully eliminated by using this product without developing resistance (Totté, van Doorn and Pasmans, 2017). Another engineered endolysin, Artilysin®s was reported to show high bactericidal activity against Gram-negative bacteria, including *P. aeruginosa* and *A. baumannii*. In short, endolysin has been proven for its ability to kill bacteria effectively and could become the alternative antimicrobial agents in the future. Besides that, endolysins have also been applied to eliminate biofilm on medical devices such as catheters. Guo, et al. (2017) has demonstrated that LysPA26 was able to effectively kill *P. aeruginosa* in biofilms. Similarly, endolysin LysH5 (Gutierrez, et al., 2014) and recombinant φ11 endolysin (Sass and Bierbaum, 2007) have shown the similar positive result in *S. aureus* biofilm elimination. These studies indicated that the endolysin has the potential to act as the agent that prevents biofilm formation on medical devices.

In addition, endolysin has extended its application in the food for food safety and preservation. According to Zhang, et al. (2012), the Listeria bacteriophage endolysin LysZ5 has high and specific lytic activity against *L. monocytogens* in soy milk at refrigeration temperature. Another endolysin, such as endolysin LysH5 was able to kill *S.aeurus* growing in pasteurized milk (Obeso, et al., 2008). Besides acting as biocontrol agents in food, endolysin-derived CBD was used to detect specific foodborne pathogens. The function of CBD in specific host recognition and binding allows it to become a good candidate for rapid detection of specific foodborne pathogens through specific binding. For example, fluorescent-labeled CBD was developed to detect various Gram-positive food-borne pathogens including *L. monocytogenes, S. aureus* and *B. cereus* (Bai, et al., 2016). Therefore, endolysin not only can be used as food antimicrobial but also in rapid detection of the foodborne pathogen.

The recombinant technology was widely used to study the characteristics of endolysin. Obeso, et al. (2008) has reported that the recombinant endolysin has significantly resembled most of the endolysin encoded by the phages. Researchers can engineer the modular structure of the endolysin to alter the bacteriolytic activity of the endolysin. For example, a recombinant endolysin, LysBPS13 was shown to exhibit high lytic activity under various conditions, which include a wide range of temperatures and ionic strengths. It was also stable in the presence of detergents, such as Triton X-100 which supports its

potential to act as the detergent additive or disinfectant. In addition, chimeric endolysin was developed by genetically fusing multiple catalytic domains and cell wall binding domains. Chimeric endolysin could be a more effective than the parental enzyme. This was evident in a study by Mao, et al. (2013) in which chimeric Ply187 endolysin showed higher lytic activity against *S. aureus* compared to its parental endolysin. Therefore, a recombinant endolysin or chimeolysin can be designed to have an improved lytic activity compared to its parental endolysin.

On the other hand, endolysin can synergize with other antimicrobial agents to improve the lytic activity. For example, the activity of endolysin LysH5 was enhanced when combined with nisin, a bacteriocin which is currently used as biopreservative in food. Nisin improved the lytic activity of LysH5 up to 8-fold and successfully cleared the *S.aeurus* in the contaminated milk (García, et al., 2010). Other studies also showed the synergistic effect of endolysin and antibiotics such as colistin (Thummeepak, et al., 2016). Synergistic effects were also found in the combination of phage SAL200 with SOC anti-staphylococcal antibiotics such as nafcillin and vancomycin in killing *S. aureus* (Kim, et al., 2017). In short, the combination of endolysins and antibiotics produced the synergistic effect for effective killing of some bacterial strains.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacteriophage

KW01-A phage sample isolated from Kuala Woh hot spring, Tapah, Perak and bacterial strain of *E. coli* BL21 (DE3) were used throughout this study. Other bacteria such as *Salmonella* sp., *Shigella* sp. and other *E. coli* strains were obtained from Dr. Tan Gim Cheong.

3.2 Reagents, Chemicals and Equipment

The reagents and chemicals used throughout this study with their respective manufacturers are listed in Appendix A and Appendix B.

3.3 Preparation of Buffers and Media

Refer to Appendix C.

3.4 Growing and Amplification of Bacteriophage

3.4.1 Obtaining Single Bacterial Colony

The *E. coli* glycerol stock was streaked on Lunia Bertani (LB) agar plate. The plate was incubated at 37ºC for 16-18 h. After 16-18 h of incubation, distinct single colonies were observed.

3.4.2 Preparation of Overnight Culture and Log Phase Bacterial Culture

The overnight bacterial culture was prepared and incubated in a shaking incubator at 37ºC with constant agitation at 200 rpm for 16-18 h by inoculating a single bacterial colony into LB broth,.

The log phase bacterial culture was prepared by adding overnight bacterial culture into fresh LB broth at a 1:20 ratio. The culture was then incubated at 37ºC with a constant agitation at 200 rpm until the OD_{600} of the bacterial culture reached 0.4-0.6.

3.4.3 Amplification of Bacteriophage from Single Plaque

A single plaque was picked from a plate and resuspended in 1 mL of TBS buffer in a sterile 1.5 mL microcentrifuge tube. The resuspended plaque was

dialyzed at 4°C for 4-5 h. Meanwhile, a log phase bacterial culture was prepared as described in Section 3.3.6. Next, LB top agar was melted in a microwave oven and placed in a water bath at 55°C.

After the bacterial culture reached OD_{600} = 0.4-0.6, 200 µL of log phase bacterial culture and 500 µL of the dialyzed single-plaque were added into 3 mL of top agar and immediately mixed by using a vortex mixer. Then, the mixture was quickly poured onto the LB agar plate and swirled gently to cover the entire base agar evenly. Two plates were prepared by using all 1 mL dialyzed single-plaque. A negative control plate was prepared by adding 4 mL top agar to 200 µL log phase host cells and 500 µL TBS. The plates were incubated at 37°C for 16-18 h.

3.4.4 Small Scale of Bacteriophage Amplification

To dialyze the phage, 5 mL of TBS was transferred to the plates the next day. The dialyzed plates were stored at 4°C for 6 h or overnight. Meanwhile, two tubes containing 40 mL of log phase of bacterial culture were prepared as described in Section 3.4.2. After the OD_{600} of the bacterial culture reached 0.4-0.6, TBS containing diffused phages were transferred into 1.5 mL tubes and centrifuged at 17,000 x*g* for 5 min. The supernatant was transferred to the tubes containing the log phase growth host cells and incubated at 37°C with a

constant agitation at 200 rpm until the OD_{600} of the bacterial culture dropped to 0.1-0.2. The lysate was centrifuged at 8,600 x*g* for 15 min and the supernatant was stored at 4°C.

3.4.5 Large Scale of Bacteriophage Amplification

Five hundred milliliters of log phase growing host was prepared as described in the Section 3.4.2. After the OD_{600} of the culture reached at 0.4-0.6, the lysate was added to the log phase growing host and incubated until total lysis was observed. After that, the culture was centrifuged at 8,600 x*g* for 15 min to remove cell debris. The supernatant was used for subsequent precipitation of bacteriophage whereas the pellet was discarded.

3.5 Precipitation of Bacteriophage

3.5.1 Preparation of 20% (w/v) PEG 8000 containing 2.5 M NaCl

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

3.5.2 Precipitation of Bacteriophage with 20% (w/v) PEG 8000

To precipitate the bacteriophage, 20% (w/v) PEG 8000 was added to the supernatant obtained from Section 3.3.5 at a ratio of 1:4. The mixture was then stirred for 2 h at 4^{o}C. Next, the mixture was centrifuged at 12,000 x*g* for 20 min at 4^{o}C. The pellet was collected and resuspended in TBS.

3.5.3 Enumeration of Bacteriophage

To determine the titer of bacteriophage, the plaque assay was performed. Firstly, 10 mL of log phase *E. coli* was prepared as described in Section 3.3.2. Next, serial dilutions of phage sample were carried out up to a dilution factor of 10^{-15}. Dilution factors of 10^{-10}, 10^{-12} and 10^{-15} were chosen to carry out plaque assay. Ten microlitres of diluted phage sample were added into 200 µL of log phase bacterial culture and incubated at 37^{o}C for 20 min to allow adsorption of phage onto the bacterial cells. Meanwhile, top agar was melted in a microwave oven and equilibrated at 55^{o}C in a water bath. After 20 min, 3 mL of top agar was added to the culture and immediately mixed by using a vortex mixer. Then, the mixture was quickly poured onto the LB agar plate and swirled gently to cover the entire base agar evenly. Once the top agar has solidified, the plates were incubated at 37^{o}C for 16-18 h. The number of plaques was calculated the next day and the phage titer was determined based on the following equation below:

$$\frac{\text{pfu}}{\text{mL}} = \frac{\text{Number of plaques x Dilution factor}}{\text{Volume plated}}$$

3.6 Extraction of Endolysin

3.6.1 Preparation of Crude Phage Lysate

Five hundred milliliters of log phase *E. coli* culture was prepared as described in Section 3.3.2. Next, 100 µL of amplified phage sample was added to log phase bacterial culture. The culture was incubated at 37ºC with a constant agitation at 200 rpm until total lysis occurred. Then, the culture was centrifuged at 12,000 x*g* for 15 min to remove cell debris. The supernatant was kept for ammonium sulfate precipitation and the pellet was discarded.

3.6.2 Ammonium Sulfate Precipitation

The proteins from the crude phage lysate were precipitated using 0-20%; 20-40%; 40-60% and 60-80% ammonium sulfate saturation. The amount of solid ammonium sulfate required for each saturation percentage was calculated using an online ammonium sulfate calculator (accessed at http://www.encorbio.com/protocols/AM-SO4.htm). The solid ammonium sulfate was added to the lysate slowly with stirring on ice until the ammonium sulfate was fully dissolved. Then, the mixture was stirred for another 1 h at 4ºC.

After that, the mixture was centrifuged at 12,000 x*g* for 20 min at 4ºC. The supernatant was kept for 20-40% ammonium sulfate precipitation whereas the pellet was resuspended in an appropriate volume of TBS buffer solution and stored at 4ºC. Same steps were carried out for the remaining ammonium sulfate saturation. After the completion of ammonium sulfate precipitation, all the protein samples were subjected to dialysis as described in Section 3.5.3 to remove excess ammonium sulfate in the samples.

3.6.3 Dialysis

Dialysis tubing (10,000 MWCO) was cut into appropriate sizes with a pair of sterile scissors. One end of dialysis tubing was first moistened with TBS and clamped tightly. The precipitated protein samples were then transferred into the dialysis tubing. Then, the other end of dialysis tubing was clamped tightly. Next, the dialysis tubing was placed into 500 mL TBS solution and dialyzed at 4ºC with gentle stirring. After 6 h of dialysis, the TBS was replaced with new TBS and dialyzed overnight at 4ºC. The next day, the dialyzed samples were removed from the tubing and SDS-PAGE was conducted to analyze the precipitated proteins.

3.6.4 Ultrafiltration of Precipitated Protein Samples

Ultrafiltration was carried out by using Microsep Advance Centrifugal Devices with 10k Omega Membrane to further concentrate the precipitated protein samples,. Five milliliters of precipitated protein sample was first transferred to the sample reservoir. Then, the sample was centrifuged at 5, 000 x*g* for 15 min. After centrifugation, the sample in the sample reservoir was collected and stored at 4^{o}C whereas the sample in the filtrate receiver was discarded.

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

3.7.1 Preparation of 4X Resolving Gel Buffer Solution (pH 8.8)

Resolving gel buffer solution (1.5 M Tris-base, 0.4% SDS) was prepared by adding 18.171 g of Tris base and 4 mL of 10% SDS solution into approximately 60 mL of sterile deionized water and mixed thoroughly. The pH of the solution was then adjusted to 8.8 by using 1 M HCl. After the desired pH was achieved, the solution was topped up to 100 mL with sterile deionized water. Lastly, the solution was filtered through 0.45 μm syringe filter and kept at 4^{o}C until use.

3.7.2 Preparation of 4X Stacking Gel Buffer Solution (pH 6.8)

Stacking gel buffer solution (0.5 M Tris-base, 0.4% SDS) was prepared by adding 6.057 g of Tris base and 4 mL of 10% SDS solution into approximately 60 mL of sterile deionized water and mixed thoroughly. The pH of the solution was then adjusted to 6.8 by using 1 M HCl. After the desired pH was achieved, the solution was topped up to 100 mL with sterile deionized water. Lastly, the solution was filtered through 0.45 μm syringe filter and kept at 4^{o}C until use.

3.7.3 Preparation of 10X and 1X Running Buffer Solution

To prepare 500 mL of 10X running buffer (0.025 M Tris-base, 0.1% SDS), 15 g of Tris base and 72 g of glycine was added into 500 mL of sterile deionized water and mixed thoroughly. In order to run SDS-PAGE, 10X running buffer was diluted to 1X running buffer by mixing 100 mL of 10X running buffer with 900 mL of sterile deionized water. Lastly 10 mL of 10% SDS solution was added into 1X running buffer solution. The final concentration of SDS solution in 1X running buffer was 1%.

3.7.4 Preparation of Staining and Destaining Solution

Two hundred milliliters of staining solution (0.1% Coomassie Brilliant Blue R-250, 1% acetic acid, 40% methanol) was prepared by adding 100 mg of Coomassie Brilliant Blue R-250 into 1 mL of acetic acid. The solution was then mixed with 40 mL of methanol and 59 mL of deionized water. To prepare 100 mL of destaining solution (10% acetic acid, 40% methanol), 10 mL of acetic acid was mixed with 40 mL of methanol and 50 mL of deionized water.

3.7.5 Casting of SDS-PAGE Gel

The casting frame was first set up on the casting stand. Resolving gel solution and stacking gel solution were prepared according to the tables in Appendix D. Next, the resolving gel solution was loaded into the gap between the glass plates. After that, the 70% ethanol was added immediately to overlay the resolving gel in order to eliminate bubbles and level the gel surface. The gel was allowed to solidify at room temperature. Once the resolving gel has solidified, the ethanol was removed completely by using a filter paper. The stacking gel solution was then loaded into the gap between the glass plates and the comb was carefully inserted. The gel was allowed to solidify at room temperature. After the stacking gel has solidified, the whole gel was transferred into a gel tank and 1X running buffer was added into the tank.

3.7.6 SDS-PAGE Analysis

First, 5 µL of 4X sample loading dye was mixed with 15 µL of protein sample. The sample was boiled at 100°C for 10 min. Then, 3 µL of protein marker and 8 µL of each protein sample were loaded into their respective wells. After that, electrophoresis was conducted at 16 mA/gel. The electrophoresis was stopped once the loading dye ran off. The gel was removed from the plates and stained for 10 min, followed by destaining stop until no blue background was observed.

3.8 Spot Lytic Assay

3.8.1 Spot Lytic Assay for Non-EDTA-treated Bacterial Cells

Spot lytic assay was carried out to determine which ammonium sulfate saturation precipitated protein sample contained the endolysin. Firstly, log phase *E. coli* was prepared as described in Section 3.4.2. After that, 1 mL of log phase bacterial culture was centrifuged at 10,000 x*g* for 1 min. The supernatant was then discarded whereas the pellet was resuspended in TBS. Next, 200 µL of non-EDTA-treated bacteria was mixed with 3 mL of top agar and poured onto the LB agar evenly. Five microlitres of each precipitated protein sample were spotted onto the agar. At the same time, 5 µL of amplified phage sample and TBS were also spotted to act as positive and negative

controls, respectively. The plates were allowed to air dry and then incubated at 37^{o}C for 16-18 h.

3.8.2 Spot Lytic Assay for EDTA-treated Bacterial Cells

Similar steps were repeated for bacterial culture with EDTA treatment. Log phase *E. coli* was grown as described in Section 3.3.2. After that, centrifugation was conducted at 10,000 x*g* for 1 min. The supernatant was then discarded whereas the pellet was washed with 200 µL of Tris buffer solution containing 0.1 M EDTA. The pellet was later incubated at 37^{o}C with a constant agitation at 200 rpm for 20 min. To remove ETDA, centrifugation was carried out at 10,000 x*g* for 1 min and the pellet was resuspended in TBS buffer. Subsequently, 200 µL of EDTA-treated bacterial cells was mixed with 3 mL of top agar and poured onto the LB agar evenly. Five microlitres of each precipitated protein sample were spotted onto the agar, together with 25 µg/mL of lysozyme (positive control) and TBS (negative control) were spotted onto the agar. The plates were allowed to air dry and then incubated at 37^{o}C for 16-18 h.

3.8.3 Heat Treatment of Bacteriophage Sample

To determine the deactivation temperature, heat treatment was carried out on PEG-precipitated KW01-A phage. Ten microlitres of PEG-precipitated phage were aliquoted separately into five microcentrifuge tubes. Next, all the microcentrifuge tubes were heated at 60ºC, 65ºC, 70ºC, 75ºC, and 80ºC for 5 and 10 min separately in a water bath. The non-EDTA-treated bacterial cells were spread onto the LB agar evenly. Subsequently, 5 μL of each heat-treated phage sample was spotted onto the agar and the plates were allowed to air dry before incubation at 37ºC for 16-18 h.

3.8.4 Heat Treatment for Precipitated Protein Samples

Heat treatment was carried out on the precipitated protein samples to deactivate co-precipitated phages that were present in the samples. All precipitated protein samples were heated for 60ºC, 70ºC, 75ºC and 80ºC for 5 and 10 min separately. Firstly, 20 μL of 0-20% saturation precipitated protein sample was transferred separately into eight different microcentrifuge tubes. Next, all the microcentrifuge tubes were heat-treated at the different temperatures for 5 and 10 min in the water bath. Both the non-ETDA-treated and EDTA-treated bacterial cells were spread onto the LB agar evenly. This was followed by spotting of 5 μL of each heat-treated 0-20% saturation

precipitated protein samples onto the agar and the plates were allowed to air dry. Similar steps were conducted for the remaining precipitated protein samples. All the plates were incubated at 37ºC for 16-18 h.

3.8.5 pH Treatment for Bacteriophage Sample

To determine the phage pH stability, 10 µL of PEG-precipitated phages were aliquoted separately into five microcentrifuge tubes. Each of the microcentrifuge tubes was added LB broth at different pHs (pH 2, pH 4, pH 6, pH 8 and pH 10). The tubes were incubated at 37ºC for 30 min. The non-ETDA-treated bacterial cells were spread onto the LB agar evenly. Lastly, 5 µL of each pH treated phage sample was spotted onto the agar and the plates were allowed to air dry before incubation at 37ºC for 16-18 h.

3.8.6 Determination of pH Stability of the Precipitated Endolysin

pH treatment was carried out on precipitated protein sample containing endolysin to determine the deactivation pH of the enzyme. The precipitated protein sample containing the endolysin was treated with different pH (pH 2, pH 4, pH 6, pH 8 and pH 10). Firstly, 10 µL of the precipitated protein sample was transferred separately into five different microcentrifuge tubes. Next, all

the microcentrifuge tubes were added with LB broth at different pH and incubated at 37°C for 30 min. Both the non-ETDA-treated and EDTA-treated bacterial cells were spread onto the LB agar evenly. Lastly, 5 μL of each treated precipitated protein sample was spotted onto the agar. The plates were then allowed to air dry and incubated at 37°C for 16-18 h.

3.8.7 Host Range Test for PEG-Precipitated Phage and Endolysin

Spot lytic test was carried out to determine the lytic activity of the precipitated endolysin against different Gram-negative bacteria. The precipitated protein sample containing endolysin was first heat treated to remove the co-precipitated phage. The bacterial hosts tested in this study were *Salmonella typhi*, *S. enteric*, *Shigella flexneri*, *S. dysenteriae*, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), and *E. coli* TOP10 cells. Then, log phase bacterial hosts were prepared. Both the non-ETDA-treated and EDTA-treated bacterial cells were spread onto the LB agar evenly. Next, 5 μL of heat treated precipitated protein sample containing endolysin was spotted onto both agar plates whereas the PEG-precipitated KW01-A phage was spotted onto the non-EDTA-treated cells. Lysozyme with a concentration of 0.025 μg/mL was spotted as the positive control of EDTA-treated cells. The plates were then allowed to air dry and incubated at 37°C for 16-18 h.

CHAPTER 4

RESULTS

4.1 Extraction of Endolysin by Ammonium Sulfate Precipitation

The precipitated protein profile can be determined based on the SDS-PAGE gel. Based on the SDS-PAGE gel of the precipitated protein samples as shown in Figure 4.1, several bands were observed for all the precipitated protein samples. The precipitated protein using 0-20% saturation precipitated protein in lane 1 and 20-40% saturation in lane 2, showed several very faint bands as compared to the remaining precipitated protein samples. It was obvious that more protein was precipitated with 40-60% and 60-80% ammonium sulfate saturation, as shown in lane 3 and lane 4, respectively. However, the percentage of ammonium sulfate saturation that was able to precipitate the endolysin was not able to be determined based on the SDS-PAGE. This is because the molecular weight of the endolysin is still unknown. Therefore, in order to test the presence of endolysin, spot lytic assay was conducted.

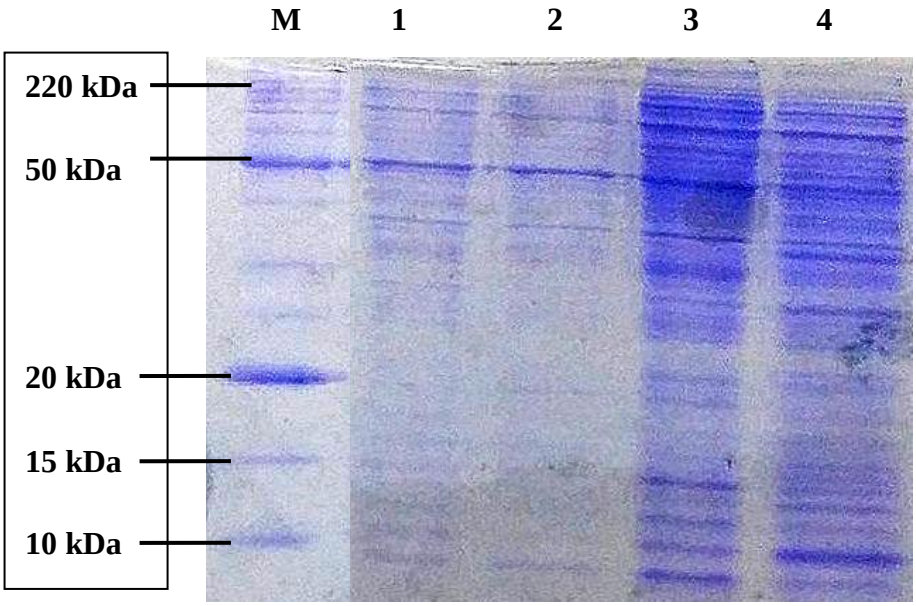

Figure 4.1: SDS-PAGE analysis of precipitated protein samples. Lane M: Protein marker; Lane 1: 0-20% ammonium sulfate saturation precipitated protein sample; Lane 2: 20-40% ammonium sulfate saturation precipitated protein sample; Lane 3: 40-60% ammonium sulfate saturation precipitated protein sample; Lane 4: 60-80% ammonium sulfate saturation precipitated protein sample.

4.2 Spot Lytic Assay

4.2.1 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment

In order to determine which precipitated protein samples containing the phage endolysin, spot lytic assay was conducted. Figure 4.2 demonstrates the results of spot lytic assay for both ETDA-treated and non-EDTA-treated bacterial cells. Clear zones were observed on all the regions spotted with precipitated protein samples against EDTA-treated bacterial cells (Figure 4.2A). This indicated that all the precipitated protein samples have the ability to kill the

bacterial cells. However, when they were compared to the lysis pattern produced by lysozyme, the lysis zones caused by the precipitated protein samples were similar to the lysis pattern caused by KW01-A phage. In addition, the precipitated protein samples also formed clear zones on non-EDTA treated cells (Figure 4.2B). Thus, this can be concluded that the lysis was due to the presence of co-precipitated bacteriophages in the precipitated protein samples, As a result, the lysis activity of the endolysin could not be identified in all the precipitated protein samples.

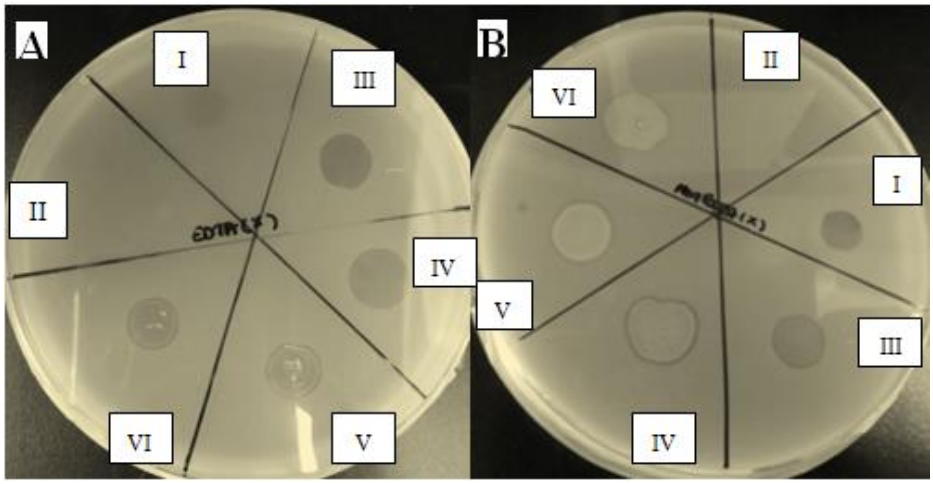

Figure 4.2: Spot lytic assay for non-EDTA-treated and EDTA-treated bacterial cells. The plates were incubated at 37ºC for 16-18 h. (A) EDTA-treated bacterial lawn spotted with 5 µL of (I) 0.025 µg/mL lysozyme as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B) Non-EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) PEG precipitated KW01-A phage as a positive control.

4.2.2 Determination of Deactivation Temperature for KW01-A Phage

The PEG precipitated KW01-A phage was heat-treated to determine the deactivation temperature of phage. The results of heat-treated PEG precipitated KW01-A phage at different temperatures for 5 min are shown in Figure 4.3A. There were clear zones observed for phage samples heated at 60^{o}C and 65^{o}C, which indicated that the phage was still active and able to lyse the bacterial cells at these particular temperatures. There were no clear zones observed but only a few plaques were formed for phage samples that were heat-treated at 70^{o}C and 75^{o}C. This could be due to the weak lytic activity of the phage sample. However, no clear zone was observed on the bacterial lawn for the phage sample that was heat-treated at 80^{o}C. This indicated that no lysis occurred because phage was successfully deactivated at this particular temperature. Next, the KW01-A phage sample was heated for 10 min at different temperatures (Figure 4.3B). The phage samples that were heated at 60^{o}C and 65^{o}C produced clear zones. Few plaques were observed on the spot with phage sample heated at 70^{o}C. However, phage samples heated at 75^{o}C and 80^{o}C did not produce any clear zones, which meant that no lysis has occurred. Based on the results obtained, KW01-A phage was successfully found to be deactivated at 75^{o}C after heat treatment for 10 min.

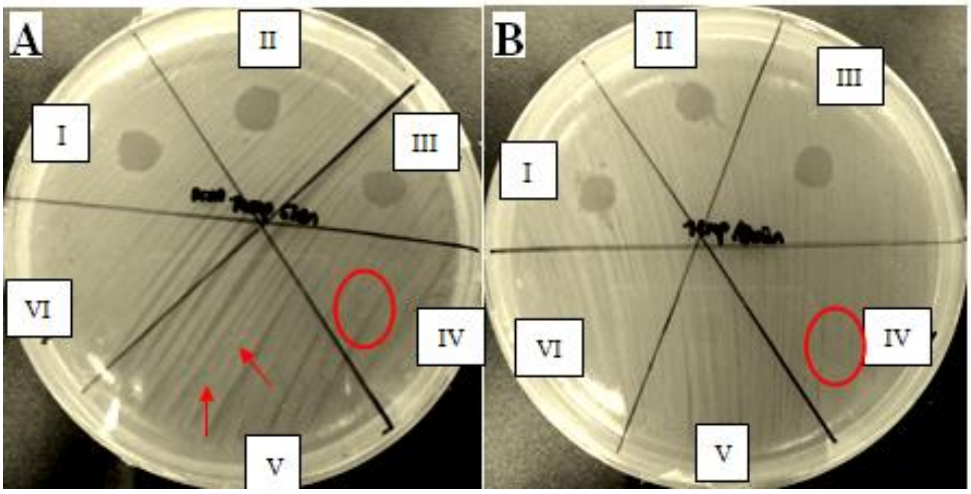

Figure 4.3: Heat treatment of PEG precipitated KW01-A phage. These plates are non EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control and KW01-A phage sample heated at (II) 60^{o}C; (III) 65^{o}C; (IV) 70^{o}C; (V) 75^{o}C; (VI) 80^{o}C for (A) 5 min and (B) 10 min. The plates were incubated at 37^{o}C for 16-18 h.

4.2.3 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using Heat-treated Protein Samples

After the co-precipitated bacteriophages have been deactivated by heat treatment, spot lytic assay was conducted again to determine the presence of the endolysin in the precipitated protein samples. All the precipitated protein samples were subjected to heat treatment at different temperatures (60^{o}C, 70^{o}C, 75^{o}C and, 80^{o}C) for 5 and 10 min. The heat-treated protein samples were tested against bacterial cells with and without EDTA-treatment.

Based on Figure 4.4 (A & B), Figure 4.5 (A & B) and Figure 4.6 (A & B), all the precipitated protein samples heat-treated at 60^{o}C, 70^{o}C and, 75^{o}C for 5 min showed presence of clear zones on both EDTA-treated and non-EDTA-treated cells. These lysis patterns produced by the precipitated protein samples were similar to the lysis patterns produced by KW01-A phage instead of lysozyme. Hence, the lysis was due to the presence of co-precipitated KW01-A phage and this showed that heating at 60^{o}C, 70^{o}C, and 75^{o}C for 5 min was insufficient to deactivate all the phages. After all the precipitated protein samples were heat-treated at 80^{o}C for 5 min, no lysis was observed on both EDTA-treated and non-EDTA-treated bacterial cells as shown in Figure 4.7 (A & B). This showed that the co-precipitated phage in the sample has been deactivated at this temperature.

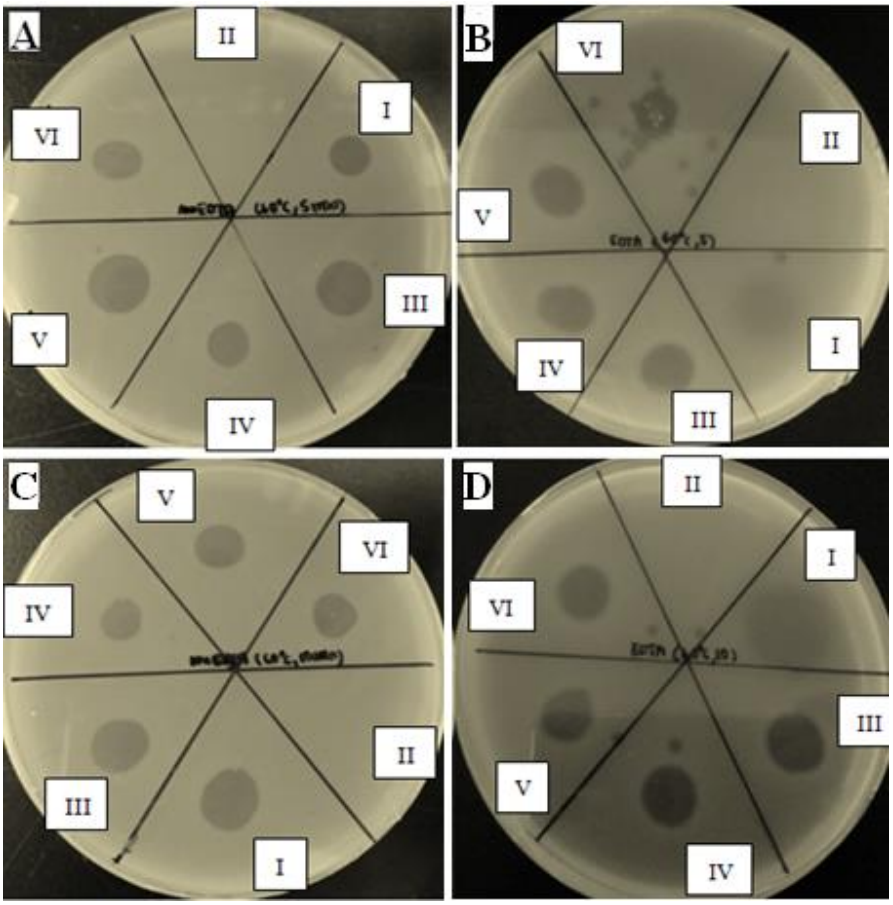

Figure 4.4: Spot lytic assay for non-EDTA and EDTA-treated bacterial cells using precipitated protein samples that underwent heat treatment at 60ºC. These precipitated protein samples were heat-treated for 5 (A & B) and 10 min (C & D). (A & C) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B & D) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A & C) except (I) 0.025 µg/mL lysozyme as a positive control. The plates were incubated at 37ºC for 16-18h.

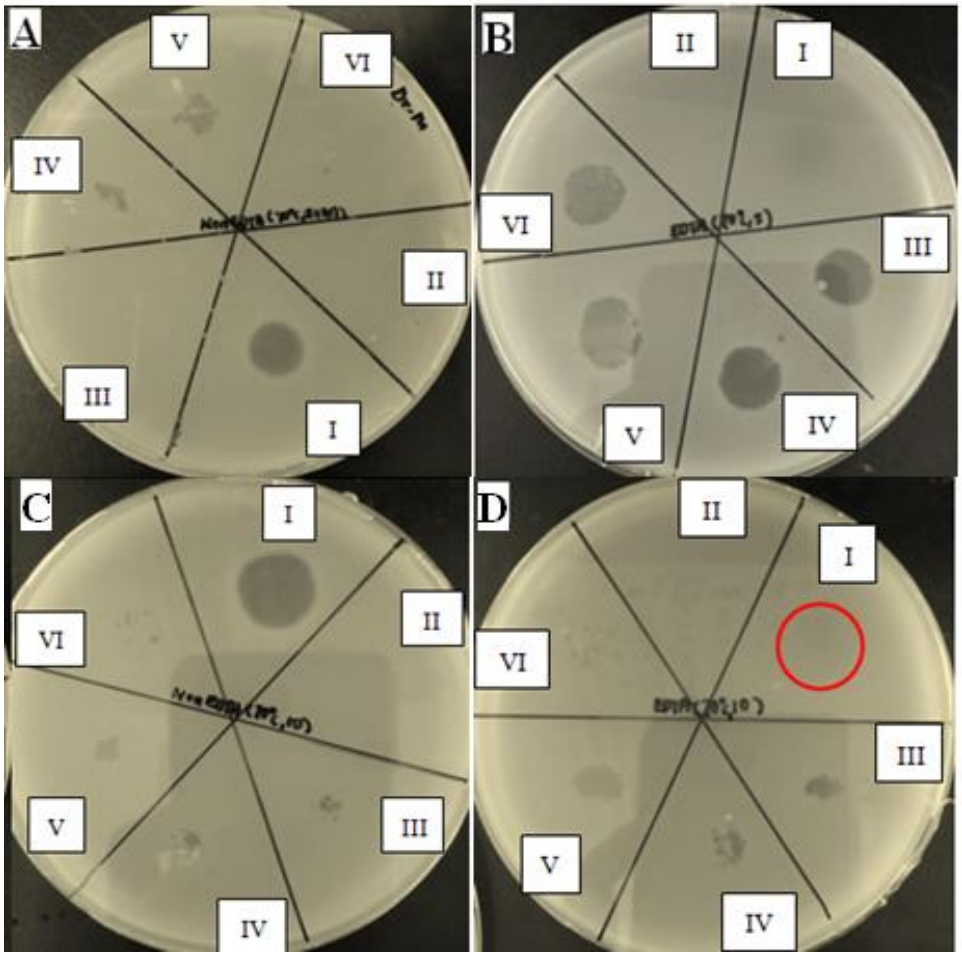

Figure 4.5: Spot lytic assay for non-EDTA-treated bacterial cells using precipitated protein samples that were heat-treated at 70ºC. These precipitated protein samples were heat-treated for 5 (A & B) and 10 min (C & D). (A & C) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B & D) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A & C) except (I) 0.025 µg/mL lysozyme as a positive control. The plates were incubated at 37ºC for 16-18h.

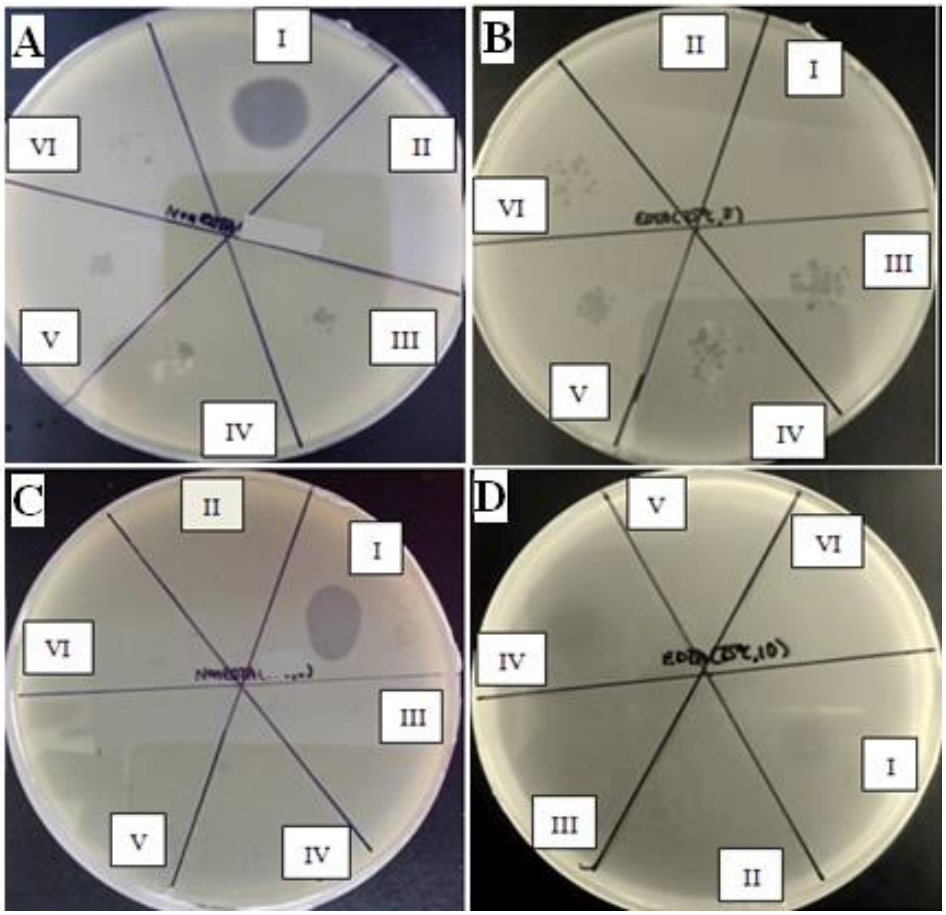

Figure 4.6: Spot lytic assay for non-EDTA-treated bacterial cells using precipitated protein samples that were heat-treated at 75°C. These precipitated protein samples were heat-treated for 5 (A & B) and 10 min (C & D). (A & C) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B & D) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A & C) except (I) 0.025 µg/mL lysozyme as a positive control. The plates were incubated at 37°C for 16-18h.

After the heating duration increased to 10 min, heat treatment between 60°C to 70°C was still unable to deactivate the phage. Lysis of all the precipitated protein samples was still observed on both the EDTA and non-EDTA-treated

bacterial cells as shown in Figure 4.4 (C & D) and Figure 4.5 (C & D). However, after the precipitated protein sample heat-treated at 75^{o}C for 10 min, there were no clear zones produced on the areas spotted with precipitated protein sample on the non-EDTA-treated bacterial cells as shown in Figure 4.6C. This showed that all the co-precipitated phages in the precipitated protein samples have been successfully deactivated. On the other hand, Figure 4.6D shows the lysis of the EDTA-treated bacterial cells, which were spotted with 20-40% saturation precipitated protein. The lysis pattern produced was similar to the lysis pattern produced by lysozyme. Besides that, the co-precipitated phages were also deactivated as shown on non-EDTA-treated bacterial cells in Figure 4.6C. Therefore, this lysis could be caused by the precipitated endolysin in 20-40% ammonium sulfate saturation.

Based on Figure 4.7, the areas spotted with precipitated protein samples that were heated at 80^{o}C for 5 and 10 min on the non-EDTA-treated bacterial cells could not produce any clear zones. This indicated that the co-precipitated phages lost their lytic activities after heat treatment at 80^{o}C. However, the absence of lysis occurred on the EDTA-treated plate could be suggested that the endolysin was denatured when the protein sample was heated for more than 5 min at 80^{o}C.

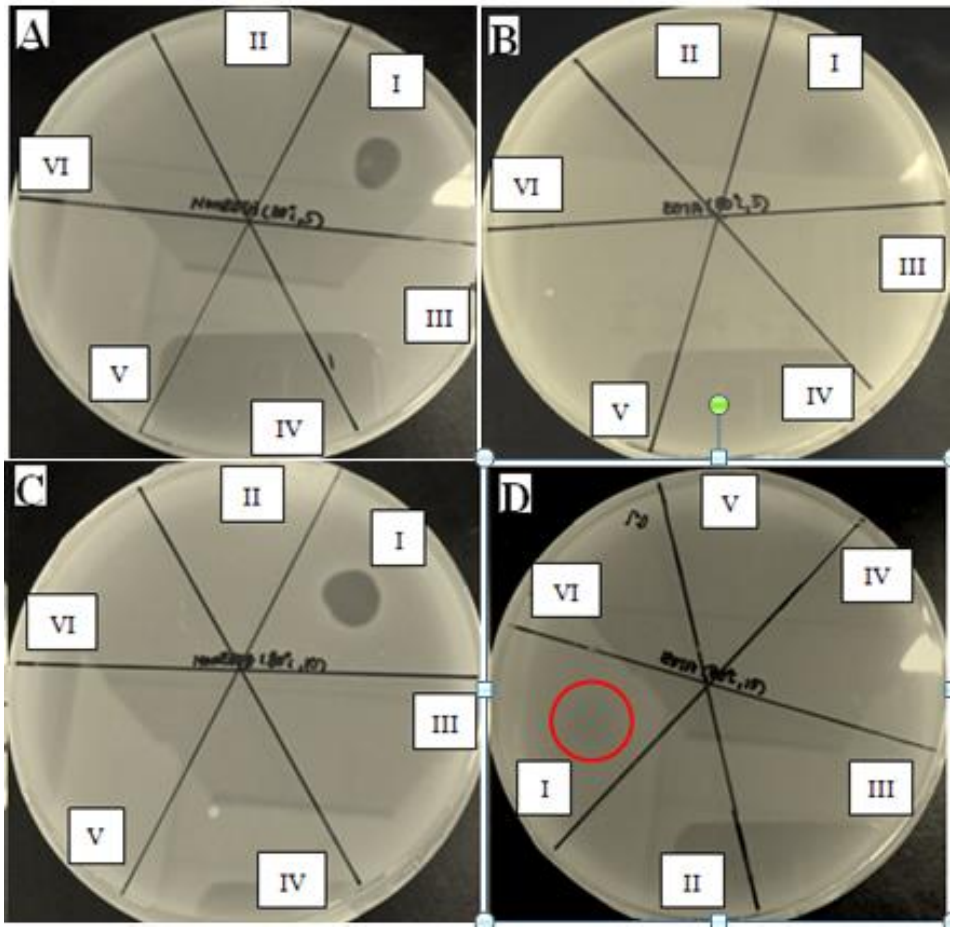

Figure 4.7: Spot lytic assay for non-EDTA-treated bacterial cells using precipitated protein samples that were heat-treated at 80ºC. These precipitated protein samples were heat-treated for 5 (A & B) and 10 min (C & D). (A & C) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control; (II) TBS buffer as negative control; (III) 0-20% ammonium sulfate saturation precipitated protein; (IV) 20-40% ammonium sulfate saturation precipitated protein; (V) 40-60% ammonium sulfate saturation precipitated protein; (VI) 60-80% ammonium sulfate saturation precipitated protein. (B & D) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A & C) except (I) 0.025 µg/mL lysozyme as a positive control. The plates were incubated at 37ºC for 16-18h.

4.2.4 Determination of Deactivation pH of KW01-A Phage

To determine the deactivation pH of KW01-A phage, the phages were incubated in different pH LB broth (pH 2.0, 4.0, 6.0, 8.0 and 10.0) and the spot lytic assay was performed. Figure 4.12 shows the results of spot lytic assay for non-EDTA-treated bacterial cells by using KW01-A phages treated in different pHs.

At pH 4.0 to 10.0, clear zones were observed on the areas spotted with phages treated with different pH. This demonstrated that the phages were stable in a wide pH range between pH 4.0 and pH 10.0. However, only a few plaques were observed on the area spotted with KW01-A phage treated at pH 2.0. In short, the lytic activity of phage would decrease or deactivate at pH 2.0 while retained its infectivity at pH 4.0 to 10.0.

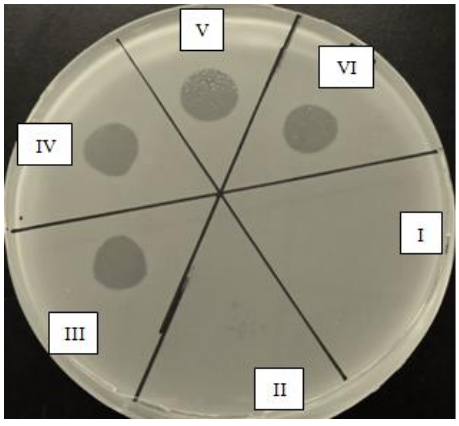

Figure 4.8: pH treatment of PEG-precipitated KW01-A phage. These plates are non-EDTA-treated bacterial lawn spotted with 5 µL of (I) TBS buffer as negative control and KW01-A phage sample treated with different pH LB broth: (II) pH 2.0; (III) pH 4.0; (IV) pH 6.0; (V) pH 8.0; (VI) pH 10.0.The plate was incubated at 37ºC for 16-18 h.

4.2.5 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using pH-treated Protein Samples containing Endolysin

Based on the results obtained in Section 4.2.3, the phage endolysin was precipitated using 20-40% ammonium sulfate saturation. Therefore, this precipitated protein sample was used to determine the endolysin's deactivation pH. Figure 4.9A and 4.9B show the results of spot lytic assay for non-EDTA-treated and EDTA-treated bacterial cells using pH-treated endolysin.

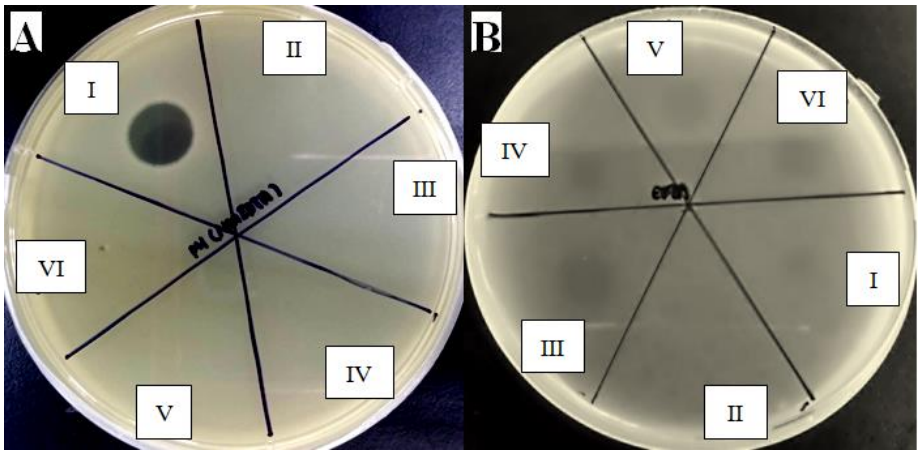

Figure 4.9: pH treatment of 20-40% saturation precipitated protein. (A) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage as positive control and endolysin treated with different pH LB broth: (II) pH 2.0; (III) pH 4.0; (IV) pH 6.0; (V) pH 8.0; (VI) pH 10.0. (B) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) 0.025µg/mL lysozyme as a positive control. These plates were incubated at 37^{o}C for 16-18 h.

Based on Figure 4.9A (pH-treated endolysin on non-EDTA-treated plate), there were no clear zones observed, as this endolysin was unable to exert its lytic

activity on the bacterial cells without EDTA treatment. In contrast, based on Figure 4.9B (pH-treated endolysin on the EDTA-treated plate), there were clear zones observed on areas spotted with endolysin treated with LB broth with different pHs range between 4.0 and 10.0. However, the area spotted with endolysin treated with LB broth at pH 2.0 did not on show any clear zones. This may indicate that endolysin only work well at pH between pH 4.0 and 10.0 and deactivated at pH 2.0 similar to that observed with the KW01-A phage as described in Section 4.2.4.

4.2.6 Comparison of Different Host Susceptibility against Phage KW01-A and Its Endolysin

In this study, the KW01-A phage and its endolysin were tested against different Gram-negative bacterial cells. The bacterial hosts tested in this study were *S. typhi*, *S. enteric*, *S. flexneri*, *S. dysenteriae*, Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) and *E. coli* TOP10 cells.

EDTA -treated and non-EDTA-treated bacterial cells were used to test the lytic activity of both the precipitated endolysin and KW01-A phage. Table 4.1 shows the results of host susceptibility test against the KW01-A phage and its endolysin. Based on the results, the KW01-A phage was unable to lyse any of the bacterial hosts tested on all the non EDTA-treated plates. This showed that

the phage exhibited no lytic activity against other bacterial hosts except *E.coli* BL21 (DE3), which was the original host used for the phage isolation.

Table 4.1: Comparison of different host susceptibility against KW01-A phage and its endolysin.

Bacterial Hosts	KW01-A Phage Infectivity	Endolysin Host Susceptibility	
		EDTA-treated plate	Non-EDTA-treated plate
E. coli TOP10 cells	-	+	-
Enteropathogenic *E. coli* (EPEC)	-	+	-
Enterotoxigenic *E. coli* (ETEC)	-	+	-
Shigella dysenteriae	-	-	-
Shigella flexneri	-	-	-
Salmonella typhi	-	-	-
Salmonella enteric	-	-	-

(+) there was lysis occurred
(-) no lysis occurred

In addition, based on Figure 4.10B (endolysin against EDTA-treated ETEC), Figure 4.11B (endolysin against EDTA-treated EPEC) and Figure 4.12B

(endolysin against EDTA-treated *E. coli* TOP10 cells), clear zones were observed on the areas spotted with precipitated endolysin. This indicated that the endolysin could work effectively against other *E. coli* strains. However, it was unable to cause lysis on *Salmonella* sp. and *Shigella* sp. as shown in Table 4.1.

In short, KW01-A phage was unable to cause lysis against other bacterial cells whereas the endolysin when applied exogenously was able to kill other EDTA-treated *E. coli* strains but not *Salmonella* sp. and *Shigella* sp when applied exogenously.

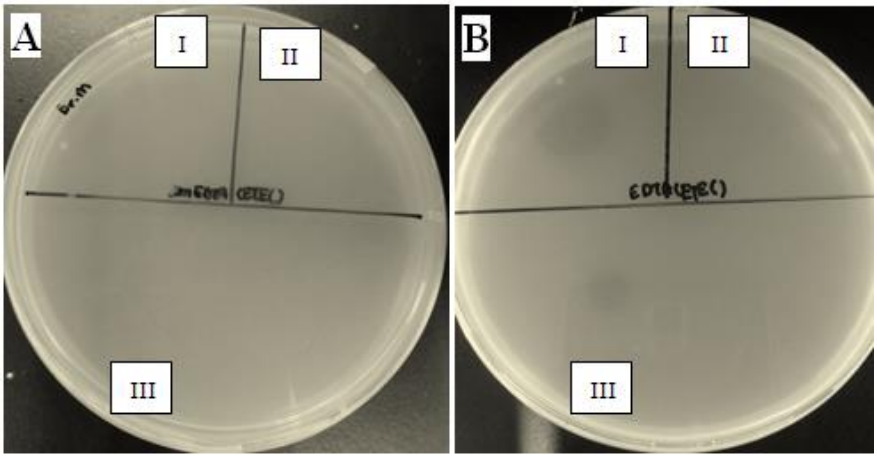

Figure 4.10: Spot lytic assay of non-EDTA-treated and EDTA-treated Enterotoxigenic *E. coli* (ETEC). These plates were incubated at 37^{o}C for 16-18 h. (A) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage; (II) TBS buffer as negative control (III) Endolysin for 20-40% saturation precipitated protein sample. (B) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) 0.025µg/mL lysozyme as a positive control.

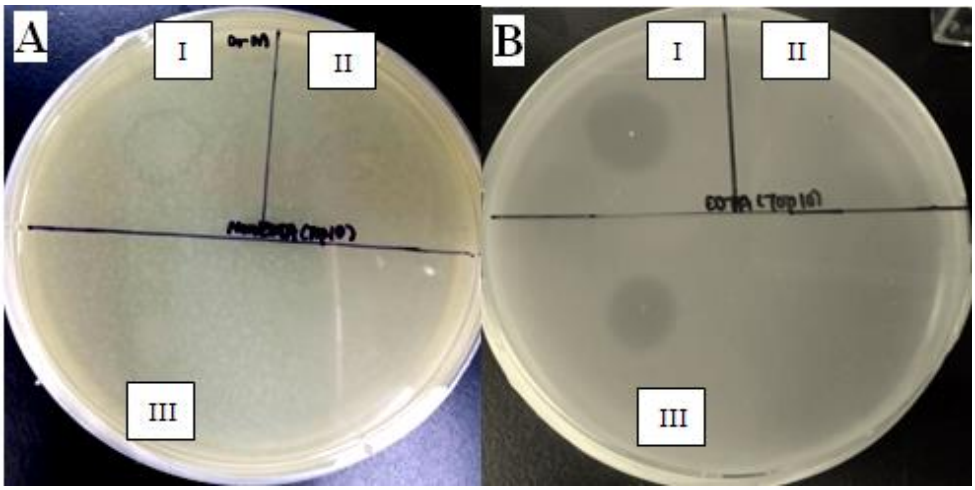

Figure 4.11: Spot lytic assay of non-EDTA-treated and EDTA-treated Enteropathogenic *E. coli* (EPEC). These plates were incubated at 37ºC for 16-18 h. (A) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage; (II) TBS buffer as negative control (III) Endolysin for 20-40% saturation precipitated protein sample. (B) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) 0.025µg/mL lysozyme as a positive control.

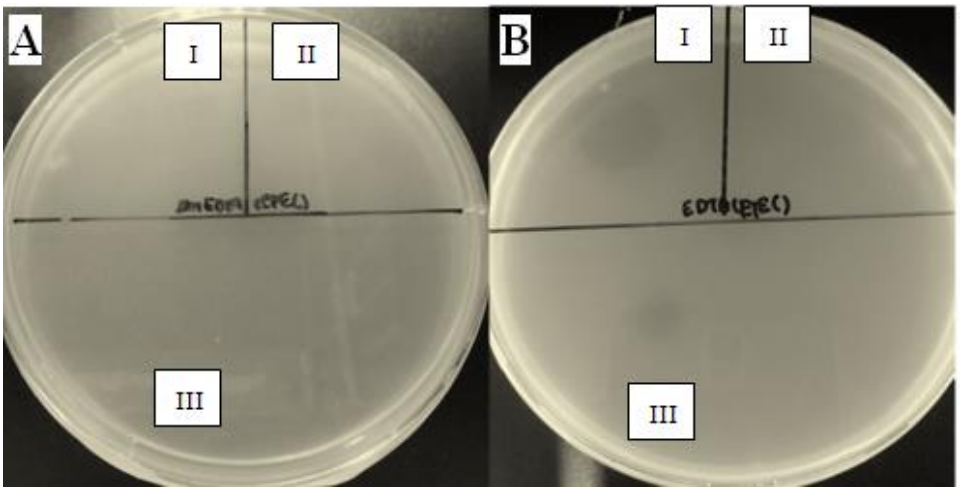

Figure 4.12: Spot lytic assay of non-EDTA-treated and EDTA-treated *E. coli* TOP10 cells. These plates were incubated at 37ºC for 16-18 h. (A) Non-EDTA-treated bacterial lawn spotted with 5 µL of (I) PEG precipitated KW01-A phage; (II) TBS buffer as negative control (III) Endolysin for 20-40% saturation precipitated protein sample. (B) EDTA-treated bacterial lawn spotted with 5 µL of similar samples as (A) except (I) 0.025µg/mL lysozyme as a positive control.

CHAPTER 5

DISCUSSION

5.1 Extraction of Endolysin by Ammonium Sulphate Precipitation

A precipitating agent is required in order to precipitate endolysin from the crude phage lysate. There is a variety of protein precipitation techniques that can be utilized as precipitating agents. One of the most commonly used precipitating agents is ammonium sulfate. The main principle of this method is the salting-out effect, whereby the ionic strength of an aqueous salt solution is altered (Duong-Ly and Gabelli, 2014). When an increasing salt concentration is added to the solution, the water on the surface of protein will be withdrawn, thus resulting in protein aggregation, which will eventually precipitate the protein from the solution (Ryan, 2011). According to Wingfield (2001), due to its non-denaturing properties, ammonium sulfate is suitable to use in precipitation of biological samples without affecting their activities. Thus, ammonium sulfate precipitation was used in this study for the extraction of endolysin as it would not affect its enzymatic activity.

Several studies have reported the use of ammonium sulfate to precipitate endolysin from the lysate. Shen, et al. (2012) have utilized 30% ammonium

sulfate to precipitate the endolysin from *A. baumannii* (XDRAB) phage lysate. Besides that, double precipitation with ammonium sulfate was carried out by another group of researchers to precipitate the endolysins from *S. aureus* phages (Mishra, et al., 2014). Apart from that, Gupta and Prasad (2011) have also employed ammonium sulfate method to concentrate P-27/HP lysin (a lysin from phage infecting *S. aureus*). There are also other studies, which employed different methods to isolate phage endolysin. For example, Chai, Tao and Mou (2014) used acetone to purify the endolysin from phage infecting *Klebsiella*. However, most of the studies preferred to use ammonium sulfate method as it does not denature the proteins.

In this study, extraction of endolysin was carried out by using four different ammonium sulfate saturations, which were 0-20%, 20-40%, 40-60%, and 60-80%. After precipitation, excess ammonium sulfate in the protein sample was removed by dialysis prior to SDS-PAGE analysis (Thermo Fisher Scientific, 2018). Presence of ammonium sulfate may interfere with subsequent purification step or with its enzyme activity (Dako, et al., 2012). It is also reported that dialysis was able to concentrate the extracted protein by removing the water content (Andrew, Titus and Zumstein, 2001). After that, the precipitated protein samples were subjected to ultrafiltration by using 10k Omega membrane. As supported by Schmelcher, Donovan, and Loessner (2012), the molecular weight of endolysin from phage infecting Gram-negative

bacteria is generally in between 15 and 20 kDa. Therefore, by prediction, the size of the precipitated endolysin should be larger than 10 kDa. Few studies demonstrated that the endolysin against Gram-negative bacteria has a molecular weight larger than 10 kDa. For example, the *E. faecalis* endolysin, Lys 168 and Lys 170 have a molecular weight of 27.6 kDa and 32.6 kDa respectively. Therefore, it can be hypothesized that the extracted endolysin should be larger than 10 kDa. However, further investigation must be done to confirm this hypothesis.

Based on Figure 4.1, the presence of endolysin could not be determined as its molecular weight is still unknown. Therefore, spot lytic assay was carried out to determine the presence of endolysin in the precipitated protein samples.

5.2 Spot Lytic Assay

5.2.1 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment

In this study, spot lytic assay was conducted by using EDTA-treated and non-EDTA-treated bacterial cells. The aim of this study was to investigate which ammonium sulfate saturation was able to precipitate the endolysin. In addition, the ability of the precipitated endolysin to lyse the bacteria with or without the treatment of membrane permeabilizer can also be determined.

In 1921, Alexander Fleming has discovered the ability of lysozyme to kill bacteria. According to Chheda, Keeney, and Goldman (2004), lysozyme is a 15 kDa single chain peptide, which is capable of killing susceptible bacteria by hydrolyzing the β-1,4-glycosidic bonds between the N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan. Peptidoglycan is the main component of bacteria, which is essential to maintain the cell membrane from high internal osmotic pressure (Sillhavy, Kahne and Walker, 2010). Therefore, disruption of peptidoglycan will lyse the bacterial cells due to high osmotic pressure. Similarly, endolysin, a phage-encoded peptidoglycan hydrolase also possess the ability to lyse bacterial host cells (Jarábková, Tišáková, and Godány, 2015). Generally, lysozyme and endolysin are effective antibacterial enzyme against Gram-positive bacteria. However, the Gram-negative outer membrane (OM) has provided a shield for the use of endolysin against Gram-negative bacteria (Salazar and Asenjo, 2007; Jarábková, Tišáková, and Godány, 2015). For this reason, lysozyme was used as the positive control for the bacterial cells treated with EDTA in this study.

The presence of OM shields the cell wall peptidoglycan from the exogenous application of lysozyme as well as endolysin (Upadhayay, Evamand Sansthan, 2014). According to Sillhavy, Kahne, and Walker (2010), lipopolysaccharide (LPS) molecules on the surface play a critical role in the barrier function of

OM. To increase the permeability of OM, permeabilizers can be used to treat the bacterial cells. Outer membrane permeabilizers lead to the loss of permeability barrier functions by either releasing the LPS and other components from OMs or intercalate in the membranes (Alakomi, et al., 2000).

There are many chemical agents that can be used as outer membrane permeabilizers. For instance, EDTA, which chelates divalent cations from their binding sites so that LPS can be released and cause OM disruption, polycations such as polyethyleneimine or polymyxin B nonapeptide (PMBN), which causes OM damage without release of LPS and weak organic acids such as citric acid, which exhibits similar chelating activity as EDTA in addition to acidifying the medium and cause OM disruption (Briers, Walmagh and Lavigne, 2011; Alakomi, et al., 2000). However, it was reported that EDTA is the most suitable agent to use among these membrane permeabilizers due to its strong ability to withdraw the stabilizing divalent cations from the LPS structure, thereby causing a significant liberation of LPS molecules (Briers, Walmagh, and Lavigne, 2011). Other agents such as PMBN are insufficient to cause a destructive action because of its inability to liberate LPS and citric acid. On the other hand, it will cause agglutination of cells, which is most likely due to neutralization of the negative charged OM. Thus, EDTA was selected as the membrane permeabilizer in this study.

As shown in Figure 4.2, lysis was occurred on the bacterial host cells with or without EDTA treatment for all the precipitated protein samples. Since the OM is usually insensitive to exogenous application of endolysin, most probably the precipitated protein samples were not able to lyse non-EDTA treated bacterial cells. Several studies have reported that the endolysin only work effectively against Gram-negative bacteria when combined with membrane permeabilizer such as EDTA. Apart from that, the addition of 0.5 mM EDTA has been reported to significantly increase the efficiency of antibacterial activity of OBPgp279, PVP-SE1gp146 and 201φ2-1gp229 endolysins against *P. fluorescens*, *S. enteric* and *P. chlororaphis* (Walmagh, et al., 2012). In the presence of the OM permeabilizer EDTA, *P. aeruginosa* PAO1 showed sensitivity towards five endolysins (BcepC6gp22, P2gp09, PsP3gp10, K11gp3.5 and KP32gp15) (Walmagh, et al., 2013). According to Son, et al. (2012), LysB4 showed a broad range of antibacterial activity against Gram-negative bacteria such as *E. coli*, *P. aeruginosa* and some *Shigella* sp. when these cells were treated with 0.1 M EDTA. Therefore, the results obtained showed that the lysis was most likely due to the co-precipitated KW01-A phage in the precipitated protein sample.

Similar observations in two previous studies were reported by Gan (2015) and Muhilarasi (2015), whereby co-precipitation of bacteriophage with protein occurred during ammonium sulfate precipitation. This is because

bacteriophage was mainly composed of proteins, especially its head and protein coat (Abedon, 2016; Wittebole, De Rock, and Opal, 2014). Hence, the high salt concentration could lead to the precipitation of phage particles out from the solutions due to electrolyte-nonelectrolyte interactions as the solubility of phage particle would be reduced at a high salt concentration (Czajkowski, Ozymko and Lojkowska, 2016). Furthermore, some studies have successfully utilized ammonium sulfate in precipitating phage particles. For instance, ammonium sulfate precipitation method was employed to purify the phage for *Thiobacillus novellus* (Johnson, et al., 1973). Therefore, these studies showed that the phage was precipitated with the proteins by using ammonium sulfate. As a result, further investigation was carried out in this study to determine the presence of endolysin by deactivating the co-precipitated phage in the sample as discussed in section 5.2.2 and 5.2.3.

As mentioned earlier, ammonium sulfate precipitation was a very suitable method to apply in precipitation of the endolysin from crude lysate because the active site of the enzyme can be maintained and its biological activity can be preserved (Wingfield, 2001). Thus, to remove co-precipitated bacteriophage in the precipitated protein sample, an extra purification step can be performed. For example, Sonstein, Hammel, and Bondi (1971) have demonstrated that the phage particles can be removed by chromatography method. After ammonium sulfate precipitation, gel filtration through Sephadex G-200 was performed to

fractionate staphylococcal phage-associated lysin (PAL) and remove the bacteriophage. A similar study was carried out by Lood, et al. (2014), whereby anion exchange chromatography was performed to remove any impurities and then the eluted fractions were analyzed on SDS-PAGE to determine the purity of PlyPy lysin. This purification step is effective as the phage particle and endolysin will be eluted at different fractions. However, in this study, heat deactivation method was utilized to deactivate the co-precipitated phages in the precipitated protein samples.

5.2.2 Determination of Deactivation Temperature for KW01-A Phage

According to Jończyk, et al. (2011), temperature plays a crucial role in bacteriophage survival, capacity for attachment, and the length of the latent period. A higher temperature can decrease the stability of phage and eventually degrade the phage particles because the phage is composed mainly of proteins (Abedon, 2016). Therefore, in this study, heat deactivation method was employed to deactivate the phage lytic activity.

As shown in Figure 4.3, phage sample, which was heated at 60^{o}C, 65^{o}C, and 70^{o}C for 5 and 10 min were insufficient to deactivate the phage at these particular temperatures and still able to lyse the host cells. As KW01-A phage

was the phage isolated from hot spring with an environmental temperature of 55^{o}C, so theoretically the phage should be able to withstand higher temperature than 55^{o}C. Jończyk, et al. (2011) reported that high thermal resistance is the characteristic of environmental bacteriophages. According to Qiu (2012), the protein capsid of λ phage was melted at approximately 87^{o}C. This could be one of the reasons why the phage was still stable even after heat treatment at 75^{o}C. In addition, *Thermus phage* TSP4, isolated from 65^{o}C hot spring was still able to retain its lytic activity even heat-treated up to 70^{o}C and 80^{o}C after 1 h of incubation (Lin, et al., 2010). Thus, this may explain why heat treatment at these particular temperatures was insufficient to cause KW01-A phage deactivation.

Heat treatment of the phage samples at 75^{o}C and 80^{o}C for 10 min has completely deactivated the phage at these particular temperatures. According to Chandra, et al. (2011), prolonged exposure to high temperature will gradually decrease the phage lytic activity. This is because excessive heat could lead to irreversible damage to the phage envelope. Hence, this study has demonstrated that heat treatment at 75^{o}C for 10 min was sufficient to deactivate all the co-precipitated KW01-A phage in the precipitated protein samples.

Most of the bacteriophages deactivate at the temperature above 60°C. For example, the T4 phage, which can infect *E. coli* BL21 was completely deactivated at 70°C as shown by Taj, et al. (2014). In addition, Colindres, et al. (2011) also discovered that MCR-10 and MCR-11 phages infecting *K. pneumonia* and *P. aeruginosa* were not able to form plaques after incubation at 70°C for more than 1 h. Some of the environmental phages also showed similar outcomes. For example, most of the bacteriophages isolated from the urban sewage did not show lysis after more than 30 min incubation at 62°C and above. Besides, Yamaki, et al. (2014) found that the *Myoviridae* phage FSP1 isolated from river water was deactivated after 1 h incubation at 60°C. These studies showed that the KW01-A phage in this study has higher deactivation temperature compared to some environmental phages.

As compared to environmental phage, bacteriophages isolated from hot spring exhibited higher tolerance to high temperature. For instance, most of the thermophilic *Bacillus* phages were still active at temperatures up to 103°C (Hazem, 2002). According to Shakaki and Oshima (1975), ϕYS40 phage infecting an extreme thermophile, Thermus thermophilus HB8 was found to be thermostable at 95°C. Therefore, based on the deactivation temperature obtained, the KW01-A phage, is also a thermostable thermophilic phage, since it could tolerate temperatures up to 75°C.

5.2.3 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using Heat-treated Protein Samples

After the deactivation temperature for phage KW01-A was determined, the precipitated protein samples were also subjected to heat treatment at 75^{o}C for 5 and 10 min. Apart from that, this study also investigates the possibility of using a lower temperature to deactivate the co-precipitated phages in precipitated protein sample to avoid denaturation of the enzyme.

In this study, the crude phage lysate that was obtained after total lysis was centrifuged at 12, 000 x*g* for 15 min to remove cell debris. The phage titer in precipitated protein samples could be lesser than the phage titer in PEG-precipitated phage sample. According to Winkler, Rüger, and Wackernagel (2012), centrifugation of crude lysate at high speed will sediment some of the phage particles together with other cell debris, thus eventually decreases the phage titer in the precipitated protein samples. Therefore, it was hypothesized that the phage present in the precipitated proteins can be deactivated at a lower temperature.

Rode, et al. (2011) have reported that no infective phage in the ground beef detected after heat treatment at 60^{o}C for 30 min whereas the phages in LB broth were still active even 2 h of incubation. Phage titer in ground beef (approximately 10^{7} pfu/mL) was much lower than the phage titer in broth

(approximately 10^9 pfu/mL). Thus, it demonstrated that phage titer could affect the deactivation temperature of the phage. However, based on the results obtained, lysis was still observed on both EDTA-treated and non-EDTA-treated bacterial cells for all the precipitated protein samples heated at 60ºC and 70ºC. As the PEG-precipitated KW01-A phage was only deactivated at 75ºC for 10 min, this clearly showed that heat treatment at these particular temperatures was insufficient to kill the phage in the precipitated protein samples. Although it is important to understand that bacteriophage titer could affect its lytic activity but there were also many other factors such as salinity, ions, and acidity of the environment that will affect the activity and survival of bacteriophage (Jończyk, et al., 2011; Ly-Chatain, 2014). Therefore, as demonstrated in this study, the phage titer did not affect the deactivation temperature of KW01-A phage.

Furthermore, this study demonstrated that heat treatment at 75ºC for 10 min was sufficient to deactivate all the co-precipitated KW01-A phage in the precipitated protein samples. The lytic activity of endolysin on EDTA-treated bacterial cells was observed as a result of complete phage deactivation. The lysis was only observed on the area spotted with precipitated protein sample at 20-40% ammonium sulfate saturation as shown in Figure 4.6D. Since the phage particles were shown to be deactivated at this particular temperature and heating duration, the lysis was most probably caused by endolysin. Similar

results also obtained by Shen, et al. (2012), whereby the endolysin φkm18p of the *A. baumannii* phage activity was detected in the 30% ammonium sulfate saturation. Besides that, endolysin SAL-1 derived from *S. aureus* was successfully precipitated by using 35% ammonium sulfate saturation (Yoon, et al., 2013). These findings showed that ammonium sulfate within this range of saturation has successfully precipitate endolysin from crude phage lysate similar to that observed in this current study.

Based on the result obtained, it is predicted that the precipitated endolysin is a medium to large size protein. This prediction can be supported by a study by Wingfield (2001), whereby a low molecular weight protein requires higher ammonium sulfate concentration to be precipitated. Shen, et al. (2012) have demonstrated that the endolysin φkm18p, which was precipitated at 30% ammonium sulfate saturation has a protein size of about 185 kDa based on the bacterium overlay assay. This study showed that at lower ammonium sulfate concentration (30%), a large molecular weight protein can be precipitated. Since the endolysin was precipitated at 20-40% ammonium sulfate saturation, therefore, this further confirmed that KW01-A phage endolysin could be a medium to large protein. However, further investigation needs to be done in order to determine the molecular weight of this protein as KW01-A phage is a newly discovered thermophilic phage.

When the precipitated protein samples were heat-treated at 80ºC for 5 min and 10 min, no lysis was observed on both the EDTA-treated and non-EDTA-treated bacterial cells as shown in Figure 4.7. This could due to degradation of endolysin at this temperature. It was sensitive to high temperature as the endolysin is an enzyme. Since the endolysin was derived from a thermophilic phage, it is assumed to have high thermal stability. Few studies have demonstrated that thermophilic phage endolysin has very high thermal stability as compared to other phage endolysin. For example, the endolysin from *Thermus scotoductus* MAT2119 bacteriophage Ph2119 could resist high heat treatment and retained its lytic activity after 6 h incubation at 95ºC (Plotka, et al., 2014). A similar study done by Plotka, et al. (2015) showed that Ts2631 endolysin that infect *Thermus scotoductus* MAT2631 has high thermoresistant because most of its lytic activity was retained after 2 h of incubation at 95ºC.

Besides that, endolysin derived from mesophilic phage could cause lysis after heat treatment at high temperature. According to Guo, et al. (2017), LysPA26 still exert high bactericidal activity against *P. aeruginosa* after heat treatment at 100ºC. Similarly, a recent study showed that AP3gp15 endolysin isolated from phage infecting *Burkholderia cenocepacia* only lost its lytic activity after heat treatment at 80ºC (Maciejewska, et al., 2017). Thus, the extracted KW01-A

phage endolysin can be considered as a heat stable endolysin as it was able to cause cell lysis even after heat treatment at 75^{o}C for 10 min.

5.2.4 Determination of Deactivation pH of KW01-A Phage

Various studies have shown that pH could be a limiting factor for phage activity. According to Langlet, Gaboriaud, and Gantzer (2007), pH enhances aggregation in viral suspension. It was shown that MS2 phage (pI 3.9) was aggregated when pH of the media was near the isoelectric point (pI) of the phage and contributed to the decrease in the PFU counts. In another study by Taj, et al. (2014), pH could indirectly influence the ability of the virus adsorption to other particles or surfaces. In addition, pH could able to change the conformation of the capsid protein (Sobsey and Meschke, 2003). Although there is no detailed mechanism described, it can be concluded that pH could affect the overall lytic activity of the phage.

In this study, the lytic activity of KW01-A phage was also analyzed at different pH. Figure 4.8 shows the results of pH stability of the phage. The phage caused cell lysis at pH 4.0 to 10.0. However, a few plaques were formed at pH 2.0. This indicated that KW01-A phage was stable over a broad pH range (pH 4.0 -10.0) but the infectivity of phage was significantly reduced at pH 2.0.

In general, phage is stable in a broad pH range between pH 5.0 and 9.0 (Jończyk, et al., 2011). For example, T4 bacteriophage was found to be stable at pH ranges from pH 4.0 to 10.0 (Taj, et al., 2014). In addition, *Acinetobacter* phage Bϕ-C62 was also able to retain high stability at pH 4.0-10.0 for a duration up to 10 months (Jeon, et al., 2016). Besides that, *Siphoviridae* phage Z was shown to have an effective lytic activity at pH ranges for 5.0 to 11.0, with a maximum stability at pH 7.0 (Jamal, et al., 2015). Therefore, in general, these studies demonstrated that most of the phages could survive well at a wide pH range between pH 4.0 and 10.0 as observed with the KW01-A phage in this study.

Apart from that, phages may be more sensitive to acidic condition compared to the alkaline condition as observed with KW01-A phage. A similar observation was reported by Nagayoshi, et al. (2016), whereby survivability phages ϕOH3 reduced to 4% after incubation at pH lower than pH 3.0 but 32.7% of phages survived at pH higher than pH 9.0. Yu, et al. (2013) reported that four isolated phages (P3K, P4A, P7A, and P9C) only showed 50-70% survivability at pH 2.0 while more than 80% of them survived at pH 11.0. Another study also demonstrated that phage P100 titer reduced rapidly after incubation at pH $\leq$ 2.0 within 1 h (Fister, et al., 2016). In short, these observations suggest that phage KW01-A incubated in the extreme acidic condition, such as pH 2.0 might affect phage stability, which eventually resulted in low phage titer.

5.2.5 Spot Lytic Assay for Bacterial Culture with and without EDTA Treatment using pH-treated Protein Samples containing Endolysin

Besides testing the phage pH stability, pH stability test was also carried out on the precipitated endolysin. As shown in Figure 4.9, the endolysin showed lysis at a wide pH range (pH 4.0 to 10.0) on the EDTA-treated bacterial cells. However, there was no lysis observed at pH 2.0, which indicated that the endolysin was deactivated at pH 2.0.

Many studies have proposed that endolysin is also stable at a broad range of pH similar to the phage from where the endolysin is extracted from. According to Oliveria, et al. (2012), the phage endolysin has an optimum pH that lies between pH 4.0 and 6.0. Swift, et al. (2016) proposed that *E. faecalis* endolysin VD13 has lytic activity over a broad pH range (pH 4.0-8.0), with a peak activity at pH 5.0. Moreover, *B. anthracis* endolysin PlyPH was the first endolysin described to be active over a wide range of pH (pH 4.0-10.5), with a maximal activity at pH 4.5 to 8.0 (Yoong, et al., 2006). These observations are in accordance with the results obtained in this study, whereby the precipitated endolysin tolerated a wide pH range between pH 4.0 and 10.0. Therefore, both the KW01-A phage and its endolysin could be exhibited a broad pH range stability.

The precipitated endolysin was unable to lyse the bacterial cells after incubation at pH 2.0. This may be due to the aggregation of endolysin at low pH condition. When the pH is near the pI value of the endolysin, hydrophobic patches on the endolysin will be exposed and this will eventually increase the chances of endolysin to contact and aggregate. As a result, the aggregated protein was unable to function properly and therefore cannot lyse the bacterial cells (Roberts, 2014). This is in accordance with a study by Filatova, et al. (2010), whereby the endolysin LysK (pI 8.6) was inactivated drastically although the increase of pH was very small (pH 8.2-8.5). Besides that, there is a possibility that the active sites of the endolysin may be altered that caused structure destabilization when pH was above the stable pH range (Filatova, et al., 2010). Similar to KW01-A phage, the precipitated endolysin could not tolerate extremely low pH as this could also result in protein aggregation.

5.2.6 Comparison of Different Hosts Susceptibility against KW01-A Phage and Its Endolysin

Host susceptibility test was carried out by using precipitated protein sample containing endolysin. Based on the Figure 4.10B, 4.11B and 4.12B, only EDTA-treated *E. coli* strains showed lysis. In contrast, the endolysin was unable to kill EDTA-treated *Salmonella* and *Shigella* sp. Furthermore,

endolysin derived from KW01-A phage crude lysate was incapable to cause lysis on non-EDTA treated bacterial cells which similar to other reported endolysins on Gram-negative bacteria (Schmelcher, Donovan, and Loessner, 2012; Fischetti, 2010). These results were then compared to the phage KW01-A. It was shown that no lysis was observed for all the EDTA and non-EDTA treated bacterial hosts tested in this study. The KW01-A phage was only able to kill its original bacterial host, *E. coli* BL21 (DE3).

According to Turner, et al. (2004), endolysins typically have narrow substrate specificities with limited antimicrobial activity to members of either intra-species or -genus. Loessner, et al. (2002) have demonstrated that cell wall binding domain (CBD) is important for endolysin to target its substrate and responsible for the recognition specificity on the proteins. Thereby, it significantly affects the activity range of the endolysin. Therefore, it was assumed that the CBDs of endolysin that was isolated from phage KW01-A could specifically recognize only the *E. coli* sp. This can be evidenced in a study that reported that the two endolysins, Lys160 and Lys170 from phage infecting *E. faecalis* could only act specifically to *Enterococcus* species, particularly *E. faecalis*, but not to other bacterial hosts (Proença, et al., 2012). Their narrow host specificity is not necessarily a drawback for their application. Endolysin with a narrow host range can be a potential alternative to antibiotics, which reduces the risk of developing multi-drug resistance.

On the other hand, few findings have found that endolysins showed broad lytic activity spectrum against different types of bacterial cells. For example, endolysin PlyV12, which was derived from the *E. faecalis* infecting bacteriophage not only has a substantial lytic effect on multiple *E. faecalis* strains but also against some disease-causing streptococcal and staphylococcus strains (Yoong, et al, 2004). In addition, Dong, et al. (2015) have demonstrated that the endolysin P28 from the *S. maltophilia* phage also exhibited a broad range of lytic activity against Gram-positive and Gram-negative bacteria such as *B. cereus*, *S. aureus*, *K. pneumoniae*, and *S. flexneri*. Thus, these studies showed that endolysins are able to cause lysis on a narrow or broad range of bacteria depending on the targeted bond between the peptidoglycan substrate and specificity of substrate-binding domains of the endolysins.

As compared to endolysin, phage may display a narrow spectrum of host range as observed in this study. Kong and Ryu (2015) found that phage PBC1 showed a very narrow host range by infecting only *B. cereus* strain. In contrast, its endolysin, LysPBC1 showed much broader host specificity against different members of genus *Bacillus*, such as *B. subtilis*, *B. megaterium* and *B. licheniformis*. This study indicated that phage has a limited lytic activity against other strains of bacterial cells when compared to endolysin. The reasons of narrow host specificity for the phage particles may due to bacterial

phage resistance mechanism to bacteria, specificity of phages' binding proteins and ability to adhere and evade the bacterial cells (Ross and Hyman, 2016). According to Rakhuba, et al. (2010), the specificity of phage infection was determined by the success of viral adsorption, which depends on the presence of surface receptors on the bacterial surface. For example, phages that recognize smooth (S)-type lipopolysaccharides (LPS) of Gram-negative bacteria usually exhibit narrow host susceptibility (Rakhuba, et al., 2010). Thus, the spectrum for the lytic activity of the phage particles is always limited to intra-species or -genus. As for the endolysin, CBD is responsible for its recognition specificity and this specificity is usually the same in the entire bacterial genus (Loessner, et al., 2002). Therefore, this explains why the endolysin has a broader lytic spectrum as compared to the KW01-A phage.

5.3 Limitations of Study

The molecular weight of the endolysin was not known, thus it cannot be identified on the SDS-PAGE. In addition, the endolysin extracted from the crude phage lysate was partially purified. Hence, further purification steps are necessary to obtain a purified enzyme for further analysis.

5.4 Future studies

In order to remove the co-precipitated bacteriophage in the precipitated protein samples, techniques such as chromatography method can be employed to purify the extracted endolysin. To further investigate the lytic activity of the phage and endolysin, the colony forming units (CFU) of the bacterial cells can be quantified to determine the effectiveness of their lytic activity. Other properties of the endolysin can be investigated such as the effect of ionic strength and divalent metal ions on their lytic activity. Last but not least, *in silico* analysis, modular organization and three-dimensional structures of the KW01-A phage and its purified endolysin can be further explored.

CHAPTER 6

CONCLUSION

Endolysin has been successfully extracted from the crude phage lysate by using ammonium sulfate precipitation. However, due to the co-precipitation of bacteriophage with endolysin, the lytic activity of endolysin was unable to be determined before heat treatment. In this study, KW01-A phage was found to be deactivated when heat treated at 75^{o}C for 10 min. Similarly, the precipitated protein samples were subjected to heat-treatment at 75^{o}C for 10 min to deactivate the phage. The lytic activity of endolysin was then revealed in the precipitated protein sample of 20-40% ammonium sulfate saturation.

In addition, the KW01-A phage endolysin can only lyse the bacterial cells after pre-treatment with outer membrane permeabilizer. Furthermore, the KW01-A phage endolysin was found to be thermostable as it could withstand heat treatment up to 75^{o}C for 5 min. KW01-A phage and its endolysin have been shown to have a broad pH stability range, between pH 4.0 and 10.0. The KW01-A phage has narrow host susceptibility, as it could only specifically kill

E. coli BL21 whereas its endolysin has a broader spectrum of lytic activity towards different strains of *E. coli*. The endolysin was able to lyse *E. coli* TOP10 cells, Enterotoxigenic *E. coli* (ETEC) and Enteropathogenic *E. coli* (EPEC). However, the endolysin was not able to lyse other Gram-negative bacteria such as *Shigella* sp. and *Salmonella* sp.

In conclusion, with the favorable antibacterial activities against various *E. coli* strains, the endolysin derived from KW01-A phage has high potential to be developed into antibacterial agent against *E. coli* infections. Further investigation should be carried out to explore other potentials of the endolysin in order to engineer more effective endolysins against Gram-negative bacteria.

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APPENDIX A

LIST OF REAGENTS AND CHEMICALS

Reagents/ Chemicals	Manufacturers, Country
0.1% Coomassie Blue R-250	Bio Basic Canada Inc., Canada
Acetic acid glacial	QRec, Malaysia
Acrylamide	Amersham Biosciences, UK
Ammonium persulfate	Sigma Aldrich, UK
Ammonium sulfate	R & M Marketing, UK
Benchmark protein ladder	Thermo Fisher Scientific, USA
Bis-acrylamide	Amresco, U.S.
Ethylenediaminetetraacetic acid (EDTA)	QRec, Malaysia
Glycine	Merck, Germany
Hydrochloric acid	VWR Prolabo, Singapore
Luria Bertani agar	Laboratorios Conda, Spain
Luria Bertani broth	Laboratorios Conda, Spain
Lysozyme	Bio Basic Canada Inc., Canada
Methanol	Merck, Germany

Polyethylene glycol 8000 (PEG 8000)	Fisher Scientific, U.S.
Sodium chloride	Bio Basic Canada Inc., Canada
Sodium dodecyl sulphate (SDS)	Bio Basic Canada Inc., Canada
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific, USA
Tris	Bio Basic Canada Inc., Canada

APPENDIX B

LIST OF EQUIPMENT AND LABORATORY WARES

Equipment/ Laboratory Wares	Manufacturers, Country
Autoclave machine	Hirayama, Japan
Cellulose acetate 0.45 μm syringe filter	Thermo Scientific, USA
Centrifuge tubes (15 mL and 50 mL)	TRP, Europe
High speed centrifuge	Sigma 2-16PK Sartorius, Germany
Incubator	Memmert, Germany
Laminar flow cabinet	Esco Micro Pte. Ltd., Singapore
Media bottles	Kimax, Germanyy
Microsep™ advance centrifuge devices	Pall Corp., U.S.
Microcentrifuge machine	Thermo Scientific, USA
Microcentrifuge tubes (0.2 mL and 1.5 mL)	Greiner Bio-One, Austri

Microwave oven	Sharp, Japan
Petri dishes	Nest Biotechnology Co, Ltd, China
pH meter	Sartorius, Germany
Shaking incubator	Yihder Co, Ltd, Taiwan
SnakeSkin Pleated Dialysis Tubing	Thermo Scientific, USA
Spectrophotometer and cuvettes	Biochrom, USA
Syringe (l mL/cc)	Muzamal Sdn. Bhd., Malaysia
Table-top microcentrifuge machine	Sigma 2-16PK Sartorius, Germany
Ultracentrifuge machine	Thermo Scientific, USA
UV transilluminator	UVP, Canada
Vortex mixer	Gemmy Industrial Corp., Taiwan
Water bath	Corpens Scientific, Malaysia
Weighing balance	Sartorius, Germany

APPENDIX C

PREPARATION OF BUFFERS AND MEDIA

Preparation of Luria Bertani Broth

By adding 10 g of LB broth powder into 500 mL of deionized water, 500 mL of Luria Bertani (LB) broth was prepared. The medium was mixed well and autoclaved for 15 min at 121^{o}C, 975kPa. Then, the LB broth was stored at room temperature until use.

Preparation of LB agar

By adding 17.5 g of LB agar powder into 500 mL of deionized water, 500 mL of Luria Bertani (LB) agar was prepared. The medium was mixed well and autoclaved. The agar was then poured into sterile Petri dishes and allowed to solidify at room temperature. The solidified agar plates were stored at 4^{o}C until use.

Preparation of LB Top Agar

Top agar was prepared by adding 1.5 g of LB broth powder and 2.6 g of LB agar powder into 150 mL of deionized water. The mixture was mixed well and

autoclaved. Then, the mixture was kept at room temperature. Prior to use, the agar was melted using a microwave oven.

Preparation of Tris-buffered saline (TBS) (pH 7.6)

Tris-buffered saline solution (50mM Tris-base, 150 mM NaCl) was prepared by adding 2.42 g of Tris and 3.5 g NaCl into 300 mL of deionized water. The pH of the solution was adjusted to 7.6 by using 1 M HCl. Once the desired pH was obtained, the solution was topped up to 400 mL with deionized water and autoclaved before use.

Preparation of 0.02 M Tris-HCl

To prepare 300 mL of 0.02 M Tris-base, 0.946 g of Tris-base was dissolved in 200 mL of deionized water. The mixture was thoroughly mixed and the pH was adjusted to pH 8.0 using 1 M HCl. The volume of the mixture was then topped up to 300 mL. The mixture was mixed well and autoclaved.

Preparation of 0.02 M Tris-HCl Containing 0.1 M EDTA

Three hundred milliliters of 0.02 M Tris-base containing 0.1 M EDTA was prepared by dissolving 0.946 g Tris-base and 11.405 g of EDTA into 200 mL of deionized water. The mixture was thoroughly mixed and the pH was adjusted to pH 8.0 using 1 M HCl. The volume of the mixture was then topped up to 300 mL. Lastly, the mixture was mixed well and autoclaved.

APPENDIX D

PREPARATION OF RESOLVING AND STACKING GEL SOLUTION

List of Components Required to Prepare the Resolving Gel

Components	Volume (µL)
30% Bis-acrylamide (30% T, 2.67% C)	1875
Sterile deionized water	940
4X resolving gel buffer	940
10% Ammonium Persulfate (APS)	23.5
Tetramethylethylenediamine (TEMED)	3.8
Total Volume	3782.5

List of Components Required to Prepare the Stacking Gel

Components	Volume (µL)
30% Bis-acrylamide (30% T, 2.67% C)	415
Sterile deionized water	1460
4X resolving gel buffer	625
10% Ammonium Persulfate (APS)	16.7
Tetramethylethylenediamine (TEMED)	3.5
Total Volume	2520.2