SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Pseudomonas aeruginosa* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY

By

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ABSTRACT

SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Pseudomonas aeruginosa* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY

Lee Ying Xian

Pseudomonas aeruginosa is a nosocomial pathogen which causes a number of nosocomial infections. Moreover, it is resistant to most of the commonly used antibiotics as it exhibits both intrinsic and acquired resistance towards multiple classes of antibiotics. Therefore, alternative antibacterial agents are urgently required. Antibacterial peptides may serve as the potential alternative agent to treat bacterial infections. This study aims to isolate antibacterial peptide(s) against *P. aeruginosa* using 12-mer phage-displayed peptide library. Briefly, Ph D. 12 phage-displayed peptide library (New England Biolabs) was panned against *P. aeruginosa* strain ATCC 27853 using solution panning. The randomly selected individual phage clones from the third and fourth rounds of biopanning were tested for the bacteria binding selectivity using ELISA, as well as antibacterial activity through microtiter broth assay. Phage clones with positive antibacterial activity or high binding selectivity towards the bacteria were subjected to DNA sequencing. In total, 70 of the selected individual clones showed positive binding selectivity to *P. aeruginosa*, indicated the enrichment of high affinity-binding phage clones throughout the four rounds of

biopanning. However, all the selected clones showed no inhibition against the bacteria. Two peptides with the highest binding selectivity were GPVNKSSTILRM and GLHTSATNLYLH. The peptide analysis revealed a consensus sequence of -Ser-X-Thr-X-Leu- (X = any amino acid residues) which may serve as critical residues binding to the bacteria. In conclusion, all the selected phage-displayed peptides showed high binding selectivity but negative antibacterial activity towards *P. aeruginosa*. Moreover, cationicity and hydrophobicity of the peptides are essential for the binding of peptides towards the bacteria.

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DECLARATION

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

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LEE YING XIAN

APPROVAL SHEET

This project report entitled "**SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Pseudomonas aeruginosa* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY**" was prepared by LEE YING XIAN and submitted as partial fulfillment of the requirements for degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **LEE YING XIAN** (ID No: **09ADB06462**) has completed this final year project entitled "**SCREENING AND ISOLATION OF ANTIBACTERIAL PEPTIDE AGAINST *Pseudomonas aeruginosa* USING 12-MER PHAGE-DISPLAYED PEPTIDE LIBRARY**" under the supervision of Mr. Yuen Hawk Leong from the Department of Biomedical Science, Faculty of Science.

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

Yours truly,

_____

(Lee Ying Xian)

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AIDS	Acquired Immunodeficiency Syndrome
Asn	Asparagine
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
Gly	Glycine
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
ICU	Intensive Care Unit
IPTG	Isopropyl-β-D-thiogalactoside
LB	Luria-Bertani
Leu	Leucine
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
NaCl	Sodium Chloride
NEB	New England Biolabs
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optimal Density

PEG	Polyethylene glycol
PFU	Plaque Forming Units
Ph.D	Phage Display
Phe	Phenylalanine
Ser	Serine
TAE	Tris- Acetic Acid- EDTA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline - Tween 20
TE	Tris-EDTA
Tet	Tetracycline
Thr	Threonine
Xgal	5-Bromo-4-chloro-3-indolyl-β-galactoside

CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa has been known as one of the most common clinical isolates that causes several infections in human. Undeniable, *P. aeruginosa* is an opportunistic pathogen that provokes persistent infection especially in immunocompromised individual (Hakki et al., 2007). In addition, it is also notorious as one of the nosocomial pathogens among the patients, causing infection through medical devices used during therapy of the patient.

P. aeruginosa infects human tissue through several entry sites, including respiratory tract, gastrointestinal tract, urinary tract, skin, eyes, ears, bones and joints. Among all, respiratory tract infection is the most severe especially in patient with cystic fibrosis as it might result in pneumonia (Fujitani et al., 2011). Furthermore, it may also enter systemic circulation thereby causing bacteraemia and septicaemia after primary infection. Upon infection, host immune system will initiate inflammatory responses. This might detriment vital function of body organ which can be fatal.

Similar to other bacterial infections, antibiotics are the primary treatment for *P. aeruginosa* infections. Unfortunately, *P. aeruginosa* exhibits intrinsic antibiotics resistant mechanism as well as other acquired resistant mechanism which make it a powerful infectious bacterium (Strateva and Yordanov 2009). As it is resistant to multiple classes of drug simultaneously, thus it has been

known as multidrug resistant pathogen. Consequently, antibiotic is no longer an effective treatment for *P. aeruginosa* infections. However, as medical science develops greatly, there are potential alternative treatments to solve the problem of multidrug resistant *P. aeruginosa*.

Antibacterial peptide is one of the potential alternative treatments to *P. aeruginosa* infections. Antibacterial peptide not only initiates a direct killing to pathogen but also boost up human innate and acquired immunities to eradicate *P. aeruginosa* (Mookherjee and Hancock 2007). In addition, it also acts as an immunomodulatory system by selectively modulating pathogen-induced inflammatory responses.

The objectives of this study include:

1. To screen and isolate antibacterial peptide(s) using 12-mer phage-displayed peptide library.
2. To examine the binding selectivity of individual phage-displayed peptides toward *P. aeruginosa*.
3. To characterize the physiochemical properties of the selected phage-displayed peptides.

CHAPTER 2

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

2.1.1 Characteristics of *Pseudomonas aeruginosa*

In particular, *Pseudomonas aeruginosa* is a Gram-negative bacillus (rod-shaped). It consists of a single polar flagellum for its motility and multiple pili on its cell surface that allow it to attach to other particles or surfaces. Besides that, its mucoid exopolysaccharide slime layer facilitates the formation of biofilms to increase its pathogenicity through quorum sensing mechanism (de Kievet 2009). *P. aeruginosa* colony is easier to be identified because of its pigment secretion properties. Three types of soluble pigments produced by *P.aeruginosa* are pyoverdin (yellow-green pigment), pyocyanin (blue-green pigment) and pyorubin (red brown pigment). *P. aeruginosa* is a facultative anaerobe and it is capable of surviving and reproducing at minimal nutrient conditions (Frangipani et al., 2008).

2.1.2 Clinical Significant of *Pseudomonas aeruginosa*

Basically, *P. aeruginosa* is a nosocomial pathogen which causes a number of nosocomial infections. A research which was conducted in the period between 1981 and 1982 in Malaysia showed that 70% of total 2456 non-fermentative Gram-negative bacillus clinical isolates are *P. aeruginosa* (Lim 1985). Another study in Malaysia had revealed that *P. aeruginosa* was one of the bacteria that

highly associated with nosocomial infections (Katherson et al., 2008). In addition, *P. aeruginosa* infections have been accounting for about 10% of all hospital-acquired infections in worldwide (National Nosocomial Infections Surveillance System 2004). According to the Center for Disease Control and Prevention, the total incidence of *P. aeruginosa* infection in hospitals of the United States was approximately 0.4% (Lessnau 2012). On the other hand, in London, *P. aeruginosa* strains composed of 90% of all the cases of *Pseudomonas* infection (BBC 2012). In short, *P. aeruginosa* infections are not restricted in Malaysia but also worldwide.

P. aeruginosa has been considered as an emerging opportunistic pathogen as well as hospital-acquired pathogen. It initiates its attack to the host when their immune system is weak. *P. aeruginosa* infection has a high mortality rate in the immunocompromised host with neutropenia, cystic fibrosis (CF), burns, organ transplant and acquired immunodeficiency syndrome (AIDS) patients (Hakki et al., 2007). Particularly, *P. aeruginosa* infection in CF patients has become the concern among the researchers. Likewise, patients who have been admitted to the Intensive Care Unit (ICU) have high probability to be infected by *P. aeruginosa* through the utilization of contaminated medical devices such as urinary catheters, dialysis catheters, endotracheal tube and intravenous line. In addition, implantation of prosthetic heart valves and organ transplantation also increase the risk of hospital-acquired infections.

P. aeruginosa infects almost all types of tissues in the human body. It causes primary infections in eyes, ears, bones and joints, skin epithelial cells,

respiratory tract, heart, gastrointestinal tract and urinary tract. Respiratory tract infection caused by *P. aeruginosa* may result in pneumonia especially in patients with cystic fibrosis (Fujitani et al., 2011). In addition, skin infection (Douglas et al., 2011) and urinary tract infection (Mittal et al., 2009) that are caused by *P. aeruginosa* are commonly seen in nosocomial infections.

Subsequently, the bacteria may lead to secondary infections such as bacteraemia and septicaemia by spreading through central nervous system and systemic circulation (Lin and Chen 2006). Bacteraemia might cause no symptoms but septicaemia can rapidly progress to respiratory distress syndrome, septic shock and death. *P. aeruginosa* infection may lead to serious problems particularly in hospitalized patient with CF, AIDS, burns and cancer. In addition, various systemic infections of *P. aeruginosa* in these patients might be lethal with fatality rate of nearly 50 percent (Loureiro et al., 2002).

2.1.3 Multidrug Resistant in *Pseudomonas aeruginosa*

Antibiotics are the common treatment against *P. aeruginosa* infections over the decades. However, the bacteria exhibits intrinsic resistance and possess remarkable ability to acquire further resistant mechanisms toward multiple classes of antibiotics (Strateva and Yordanov 2009). Practically, it manifests all the known enzymatic and mutational mechanisms of bacterial resistance (Pechere and Kohler 1999). Hence, *P. aeruginosa* infections lead to increased morbidity and mortality in the infected patients (Obritsch et al., 2004). According to Esther et al. (2012), in a hospital located in Spain, a total of 183

patients were colonized or infected with multidrug-resistant *P. aeruginosa* during 2007-2010 and the prevalence had increased from 2.8% to 15.3% over this period. In Malaysia, the incidence of multidrug resistance is relatively high with 19.6% of total cases of *P. aeruginosa* infections (Pathmanathan et al., 2009).

A research by Javiya and his colleagues (2008) in India showed a remarkable resistance of *P. aeruginosa* to cephalosporin class of antibiotics where there were 67.86% resistance towards ceftazidime and 94.64% resistance towards cephalexin. In addition, the bacteria showed 71.43% resistance towards aztreonem compare to other classes of antibiotics. Jayiya et al. (2008) also showed that 19.64% of the bacteria were resistant to carbapenem. In addition, carbapenem-resistant strains producing imipenem-type metallo-*β*-lactamases had been reported (Senda et al., 1996).

P. aeruginosa might exhibit three resistant mechanisms to *β*-lactam, which are low permeability of the outer membrane (Livermore 1984), constitutive expression of various efflux pumps (Livermore 2001) and naturally occurring *β*-lactamases (Nordmann and Guibert 1998). In nature, *P. aeruginosa* is resistant to penicillin G, aminopenicillins as well as the first and second generation of cephalosporins (Strateva and Yordanov 2009). *P. aeruginosa* can be divided to four phenotypes in terms of their resistance to specific class of antibiotics, (i) "intrinsic resistant to carbenicillin", (ii) resistant to all *β*-lactams except cephem and carbapenems, (iii) resistant to penicillin and (iv) increased MICs to carbapenems (Strateva and Yordanov 2009).

A study showed that antibiotic susceptibility of *P. aeruginosa* is influenced by three conditions, which are aerobic, anaerobic and the presence or absence of biofilm. The antibiotics susceptibility decreases significantly under anaerobic and the presence of biofilm (Hill et al., 2005). These two conditions resemble the case in patient with cystic fibrosis where *P. aeruginosa* are growing in mucus clog of respiratory tract with low oxygen availability and in the presence of biofilm. Therefore, multidrug resistant *P. aeruginosa* is harmful to host cell especially for CF patients.

2.1.4 Alternative Treatments for *Pseudomonas aeruginosa* Infections

Several alternative treatments for multidrug resistant *P. aeruginosa* infections are available nowadays. Single antibiotic administration is not effective against the resistance mechanisms of the bacteria. However, a combination of double or triple antibiotics with different modes of action can reduce the likelihood of resistance and increase the efficacy of drug (Rahal 2006).

Study of Javiya et al. (2008) indicated that the combination of ticarcillin and piperacillin demonstrated significantly greater antibacterial activity against *P. aeruginosa*. Double antibiotics combinations, meropenem-tobramycin and meropenem-ciprofloxacin, are the most effective drug to *P. aeruginosa* under aerobic, anaerobic and biofilm condition (Hill et al., 2005). In addition, Zuravleftt et al. (1984) demonstrated that *P. aeruginosa* were killed by the addition of rifampin to ticarcillin and tobramycin which were resistant alone by the bacteria and in combination by the bacteria previously.

Antibiotics combination therapy does bring additional advantages. It is used to improve the clinical outcome by achieving the synergistic effect using two or more antibiotics drugs. Besides that, this approach lowers the dosage of antibiotic thus reducing the toxicity effect. It is believed that the use of two antibiotics might prevent the emergence of resistance to the antibiotics (Rahal 2006). Nonetheless, the susceptibility of antibiotics combination may not effective over a long period of time. Moreover, antagonism effect is common in antibiotics combination. This is indicated by the growth of bacteria when an additional antibiotic is added to a previously bactericidal single antibiotic or combination of antibiotics (Hill et al., 2005). Therefore, combination of antibiotics is not the best solution to the multidrug-resistant bacterial infection.

Despite combination of antibiotics, antibacterial peptide may be the potential alternative antibacterial agent to complement antibiotics treatment. Unlike antibiotics, antibacterial peptide possesses completely different modes of killing action against the bacteria (Ulvatne et al., 2001). Therefore, it can evade the antibiotic resistance mechanisms and exhibits antibacterial effect against the bacteria.

2.2 Antibacterial Peptides

2.2.1 Origin of Antibacterial Peptides

Antibacterial peptides are widely distributed in insects, plants as well as highly evolved animal with more sophisticated immune systems (Mookherjee and Hancock 2007). These peptides are produced by the immune cells such as

neutrophils, dendritic cell and monocytes/macrophages to rapidly neutralize a broad range of bacteria (Wetering et al., 2004). Nevertheless, antibacterial peptides can be chemically synthesized nowadays.

2.2.2 Physiochemical Properties of Antibacterial Peptides

Antibacterial peptides are generally positively charged at neutral pH (Lee 2002). Hence, they also known as cationic host defence peptides (Mookherjee and Hancock 2007). Generally, they are amphipathic with hydrophilic patches on the surface of hydrophobic, charged peptides. They are small peptides ranges from 12-50 amino acid residues and have at least two positive charged residues either arginine or lysine residue (Bulet et al., 2004). Cationic host defence peptides are gene-encoded and they are constitutively expressed or strongly induced by microbial antigen, inflammation or tissue injury.

Based on the diverse structures, antibacterial peptides can be divided into four classes, (i) amphipathic α helical, (ii) β-sheet structures, (iii) extended structures, and (iv) loop structures. Only amphipathic peptides with hydrophobicity and net positive charge, conformational flexibility and secondary structure are capable to interact with and insert into the phospholipids bilayer membrane (Jenssen et al., 2006).

2.2.3 Modes of Action of Antibacterial Peptides

Antibacterial peptide targets the bacterial cell membrane, thus it has to pass through the negatively-charged outer surface membrane of Gram-negative bacteria. According to Sawyer et al. (1988) and his coworker, peptides firstly displace the divalent polycations, which cross-bridge the adjacent lipopolysaccharides, and then neutralize the lipopolysaccharides. As a consequence, the outer membrane is disrupted. Therefore, the peptides can bind to negatively-charged phospholipid bilayer of cytoplasmic membrane through electrostatic and hydrophobic interaction. On the other hand, peptides might pass across the membrane and target on intracellular component, leading to cytoplasmic vacuolization and degeneration of cellular organization (Bera et al., 2003).

There are two models of action for antibacterial peptides on membrane activity based on their permeation method, (i) transmembrane pore model and (ii) nonpore model. Transmembrane pore model is characterized by formation of a pore or channel as a pathway for peptides permeation. It can be divided into barrel stave and toroidal model. In the barrel stave pore model, membrane-bound peptides interact and recognize the peptides that located laterally to each other, forming a transmembrane pore with at least three peptide molecules (Rapaport and Shai 1991). In order to insert into the phospholipids membrane, the peptides should possess a structure with amphipathic α-helix, hydrophobic α-helix, β-sheet or both α-helix and β-sheet (Shai and Oren 2001). In toroidal model, specific peptide interaction is not necessary (Ludtke et al., 1996) in

which peptides change the local curvature of the membrane in order to form a high curvature toroid.

In contrast, nonpore models enhance the membrane permeability by perturbation of lipid bilayer without forming pores. Under this model, there are carpet model, detergent model, lipid clustering model, molecular shape model and interfacial activity model. In carpet model, peptides cover the membrane surface with parallel orientation to the membrane (Wimley and Hristova 2011). According to Wimley and Hristova (2011), peptides permeate membrane via global bilayer destabilization when their threshold concentration is reached. On the other hand, the membrane integrity is destroyed at high peptide concentration through catastrophic collapse as described in detergent model (Hristova et al, 1997). In lipid clustering model, peptides stimulate clustering or phase separation of lipids (Epand and Epand 2009). Consequently, the boundary defects result in membrane leakage. In addition, Bechinger and Lohner (2006) described the molecular shape model in which the peptide-lipid interaction may disrupt the lipid packing within the membrane. Lastly, interfacial activity model explains the propensity of an imperfectly amphipathic peptide to separate into bilayer interface and drive the vertical rearrangement of the lipid polar and non-polar groups which causes membrane permeabilization (Wimmley, 2010).

2.2.4 Functions of Antibacterial Peptides

Antibacterial peptides have direct killing actions against the bacteria (Hancock and Diamond 2000). However, direct killing only occur at a relatively high concentration of peptides because they have limited bactericidal activity under modest concentration due to strong antagonism resulted from the physiological salt concentration in the host tissues (Bowdish et al., 2005). The anionic host factors such as glycosaminoglycans like heparin and high concentration of cations in the body surfaces and fluid may interfere with antibacterial activity of the peptides (Bals 1998, Bowdish et al., 2005). These substances may compete with antibacterial peptides by either binding to bacterial membrane or antibacterial peptide. Hence, they inhibit the antibacterial activity of the peptides. Anyway, these strong antagonism conditions usually only occur when peptides in modest to low concentrations.

Cationic antibacterial peptides are broadly involved in innate immunity with the ability to confer protection against a variety of pathogens as well as limiting sepsis. Moreover, they are also served as potential novel cytotoxic agents against certain types of cancers (Mookherjee and Hancock 2007). Thus, expression of these peptides is upregulated during infection or inflammation. For instance, human β-defensin-2 is expressed in monocytes, epithelial cells and keratinocytes upon stimulation by different bacterial components (Proud et al., 2004). These components interact with the Toll-like receptor from immune cell thereby activate nuclear factor (NF)-κB pathway that stimulate the innate and adaptive immune responses to infection (Vora et al., 2004).

Even though antibacterial peptides might not optimally act on bacteria at low concentration, however, they can balance and selectively modulate the host's innate immune functions and subsequently transition to adaptive immunity (Mookherjee and Hancock 2007). Firstly, antibacterial peptides exhibit anti-endotoxin properties by balancing, regulating and preventing exacerbated inflammatory response. These peptides can selectively repress proinflammatory response by suppressing endotoxin-induced pro-inflammatory gene expression, protein secretion of inflammatory mediators and endotoxin-induced nuclear translocation of NF-κB subunits while insure other pro-inflammatory responses such as production and release of several chemokines (Bowdish and Hancock 2005).

Second, antibacterial peptides exhibit chemotactic activity to accelerate the immune responses upon infections. Host defence peptides are released upon the pathogenic infections. The secreted peptides are then directly or indirectly promoting further recruitment of effector cells, for example, neutrophils, immature dendritic cells, macrophages, and T cells (Zanetti 2003). This creates a positive feedback loop that recruits sufficient effector cells until the pathogens are eradicated. At slightly higher concentration, antibacterial peptides exhibit a direct chemokine activity by interacting with chemokine receptor of the host immune cells (Tjabringa et al., 2006). This is followed by homing of immune effector cells to infection site for innate immune response as well as subsequent adaptive immune response. At low to modest concentration, antibacterial peptides may induce chemotaxis indirectly by stimulating the production of chemokines (Bowdish et al., 2005).

Besides than chemotactic activity, antibacterial peptides also promote other immunological aspects by inducing cellular differentiation and proliferation, extending neutrophils lifespan via the suppression of apoptosis, activating and degranulating mast cell, wound repair, stimulating angiogenesis, and enhancing the ability of dendritic cells to uptake and present antigen (Davidson et al., 2004; Koczulla et. al., 2003; Chen et al., 2006; Nagaoka et al., 2006). These peptides do not solely exhibit immune responses instead they work cooperatively with the host's immune system to eradicate pathogen efficiently and effectively.

2.2.5 Strategies for Antibacterial Peptides Isolation and Development

Lee (2002) had proposed a few steps for developing a potent, short antibacterial peptide. Firstly, the host defence peptide is isolated from the animal sources to analyze the complete sequence and identify the essential region with antibacterial activity. The region is then used to produce active fragments and its analog with improved biological activity. Secondly, based on the absolute interaction between peptides and bacterial membrane, novel antibacterial peptides can be artificially designed and synthesized.

In addition, antibacterial peptides can be isolated from animals or plants through crude extraction method (Treffers et al., 2005). It is a conventional method for isolation of antibacterial peptide. However, this method is tedious and time-consuming. In addition, only limited number of peptides can be isolated through this method. Nevertheless, the introduction of phage display

technology by Smith in 1985 has eased the screening and isolation of antibacterial peptide. The novel antibacterial peptides can be isolated from combinatorial peptide libraries (Lee 2002).

2.3 Phage-Displayed Peptide Library

2.3.1 Background and Principles of Phage-Displayed Peptide Library

Phage display is pioneered by Smith (1985), who had great interest in filamentous phage study. Smith and Petrenko (1997) defined phage-displayed peptide library as "a heterogeneous mixture of such phage clones, each carrying a different foreign DNA and therefore displaying a different peptide on its surface". The foreign peptides to be displayed by phage are fused into phage coat proteins through random foreign DNA insertion into the phage genome, particularly in the N-terminus of pIII coat protein between the vector-insert and insert-vector junctions. Consequently, coat protein and foreign peptide becomes a hybrid fusion protein that is exposed to the exterior environment. pIII, a minor coat protein, is located at one tip of the phage with five copies. Hence, theoretically, foreign peptides that fused with pIII protein are displayed in five copies as well. In addition to pIII, pVIII major coat protein and pVI minor coat protein have been used to fuse with the foreign peptides. Nonetheless, pIII display system is much more common because it allows different foreign DNA insertion sites (N-terminus of pIII, middle of pIII or replaces N-terminal domain or C terminal via leucine zipper) while pVI only allows fusion of foreign peptide at C-terminal and pVIII at N terminal. According to Smith and Petrenko (1997), numerous of pVIII displayed

peptides, which comprise a substantial fraction of the phage's mass, might dramatically alter the physical and biological properties of the phage.

Selection of the antibacterial peptide is started through affinity biopanning using phage-displayed peptide library. The target can react with the phage-displayed peptides in solution, followed by the affinity capture of phage-target complexes. After first round of selection, only peptides with strong binding affinity to bacteria are captured while unbound phages are washed away. The captured phages are then eluted and amplified to generate more copies of each selected individual phage in order to secure an ever-fitter subsets of peptides in further rounds of selection. At the end of the selection, the selected phages should be low in yield but high in stringency, with the specific and tight binding phage-displayed peptides (Zhang et al., 2012). Individual phage is subsequently isolated, followed by the DNA sequencing for the inserted DNA region that encodes the displayed peptide. Based on the DNA sequences, peptide sequences can be deduced.

2.3.2 Ph.D 12 Peptide Library

Ph.D. 12 peptide library consists of linear peptide with 12 amino acid residues. Phage display is not applicable to larger polypeptide in which the fusions might lead to detrimental effects on the function of coat protein (Christensen et al., 2001). According to New England BioLabs, each phage has short linker sequence (Gly-Gly-Gly-Ser) that connects the peptide to pIII coat protein. The phage displays 12 amino acids on the pIII minor coat protein with five copies

per phage. It fulfils the minimum requirement of antibacterial peptide of 12-50 amino acids. Therefore, it is believed to have good binding efficacy to the target bacterial membrane. Lastly, Ph.D 12 from New England Biolabs is supplied with large population of phage clone, approximately 10^9 phage clones each with unique displayed peptide and 10^{11} phage particles in total.

2.3.3 Applications of Phage Display Technology in Antibacterial Peptide Discovery

Phage display technology is significantly useful in antibacterial drug discovery. There were several previous studies using this technology with successful isolation of peptides with antibacterial effect. Antibacterial peptides against *Escherichia coli* using 10-mer phage-displayed library was isolated by Pini and his colleague in 2005. At the same time, Bishop-Hurley and his colleagues (2005) had successfully isolated 15-mer peptide with bactericidal properties towards virulent strain of *Haemophilus influenzae*. A few years later, he again isolated the antibacterial peptides against *Campylobacter jejuni* (Bishop-Hurley et al., 2010). These few studies showed that phage display technology is highly potential tool for isolation of antibacterial peptides.

2.4.5 Advantages of using Phage-Displayed Peptide Library for Screening of Antibacterial Peptide

Undoubtedly, phage-displayed peptide library has significant advantages over other screening methods. The main advantage of phage display is the enormous diversity of sequences that can be displayed on phage (Beghetto and Gargano

2011). Phage display allows screening of large numbers of clones at once. Displayed peptides with more than 10^9 complexities can be screened easily by using phage display. Additionally, phage clones can be amplified by infecting *E. coli* for multiple rounds of selection to obtain the peptides with the greatest affinity binding to the target bacteria. In comparison to those conventional bacterial expression and lambda phage-based methods, which are more laborious and time-consuming, phage display is faster and permits more efficient selection in small volumes (Huang et al., 2012). Moreover, phage display method does not require direct ligand labeling and ligand purification which are necessary in other conventional screening methods.

CHAPTER 3

MATERIALS AND METHODS

3.1 General Experimental Design

The 12-mer phage-displayed peptide library was panned against the target bacteria, *Pseudomonas aeruginosa*. A total of four rounds solution biopanning was carried out. Total of 70 random selected phage clones from the third and the fourth round of biopanning were amplified individually. Each individual phage clones were tested for their binding selectivity and antibacterial activity against the bacteria. The DNA of those clones which show high binding selectivity ratio or positive antibacterial activity was extracted and sequenced. Lastly, DNA sequence was deduced into peptide sequence and the physiochemical properties of the peptide was examined. The experiment flow was summarized in Figure 3.1.

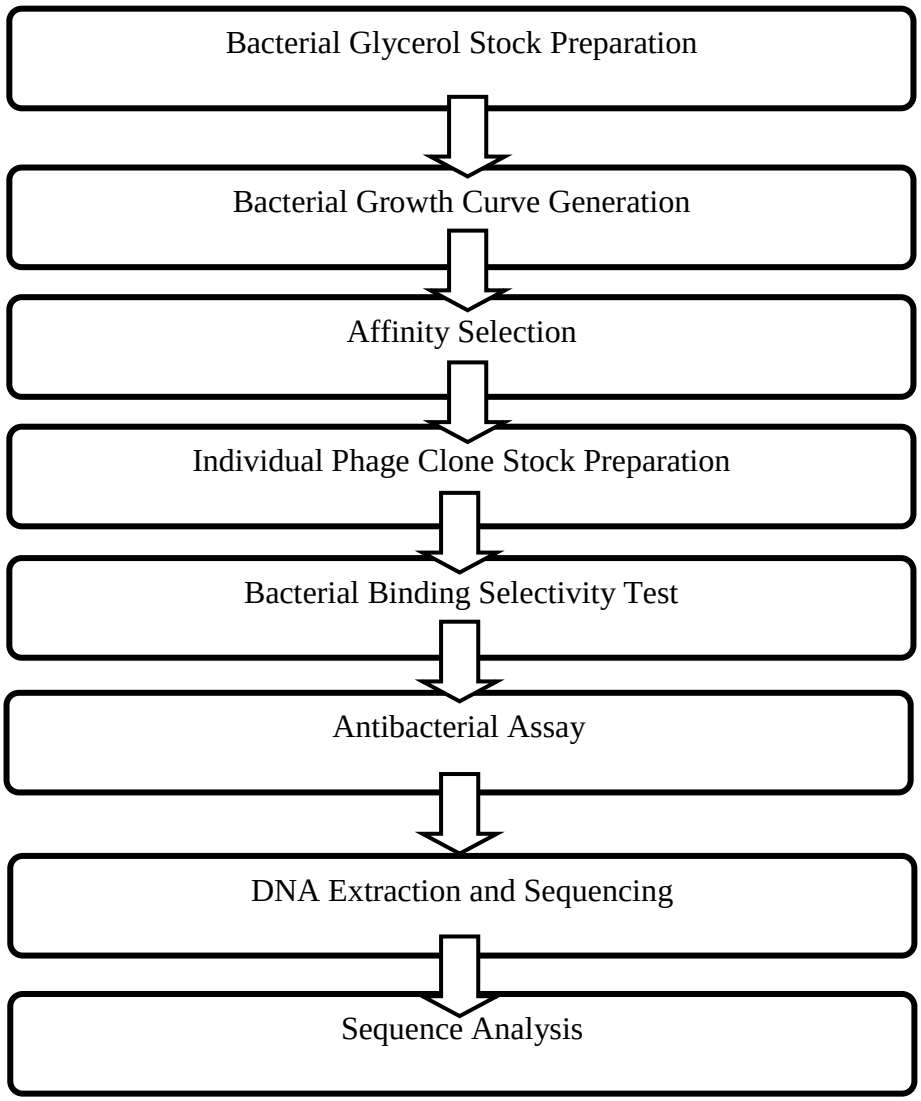

Figure 3.1: General experimental design.

3.2 Materials

3.2.1 Bacterial Strains

Escherichia coli ER2738 (F' proA+B+ lacIq Δ (lacZ) M15 *zzf::Tn10*(TetR)/thuA2 glnV Δ(lac-proAB) Δ(*hsdMS-mcrB*)5 [rk-mk-McrBC-]) from New England Biolabs (NEB) was used for M13 phage propagation. It was grown on Luria agar supplemented with tetracycline (20 mg/ml in 1:1 ethanol:water) and incubated overnight at 37 ℃.

Pseudomonas aeruginosa strain ATCC 27853 was used as target bacteria for affinity selection. It was plated on either MacConkey agar or Muller-Hilton agar. It was incubated overnight at 37 ℃ and kept as master culture at 4 ℃ after grown. The master culture can keep for one week and new master culture has to be maintained to ensure the freshness of the colonies.

3.2.2 Phage-Displayed Peptide Library

The 12-mer phage-displayed peptide library from New England Biolabs was used for biopanning against *P. aeruginosa*. In this library, five copies of peptides with 12 amino acids are displayed by the phages. The peptides are encoded by the random foreign DNA insertion in phage genome. They are linked with phage coat protein pIII via a short linker sequence, which is Gly-Gly-Gly-Ser. The library contains 1×10^{13} plaque forming units per millilitre (PFU/ml) with 1.28×10^{9} complexities. It was stored at -20 ℃ and thawed on ice before used to avoid heat shock reaction.

3.2.3 Buffers and Reagents

See Appendices.

3.3 Protocols

3.3.1 Bacterial Glycerol Stock Preparation

The procedure of bacterial glycerol stock preparation for both *E. coli* ER2738 and *P. aeruginosa* 27853 were similar. Bacteria were cultured on respective agar as described above. Upon overnight incubation, a loop of single colony of bacteria was added into 20 ml of Luria Bertani (LB) broth and incubated at 37℃ with rotation at 200 rpm. After 4½ hours incubation, the bacteria was then transferred into 50 ml centrifuge tube and added with 12 ml of 80% glycerol. The bacterial suspension was mixed well using vortex. After that, 500 µl of the suspension was dispensed into microcentrifuge tubes. The bacterial glycerol stock was stored at -80℃. When new master culture was needed, bacterial stock was thawed on ice before plating on the agar.

3.3.2 Bacterial Growth Curve Generation

A loop of single colony of *P. aeruginosa* was added into 20 ml of LB medium and placed on shaking incubator at 37℃, 200 rpm. The OD reading of the bacteria was measured at 600 nm for every one hour interval until completion of the incubation at 4½ hours. At each time interval, 100 µl of the bacteria suspension was transferred to 900 µl Tris-buffered saline (TBS) solution to perform 10-fold serial dilution. The serially diluted bacteria were plated out on

MacConkey agar to determine the bacteria titer in colony forming unit (CFU). Growth curve of the bacteria were plotted as measured in OD_{600} and bacteria titer (CFU/ml).

3.3.3 Affinity Selection

3.3.3.1 Bacteria Suspension Preparation

A loop of single colony of *P. aeruginosa* was added into 20 ml of LB medium and incubated for 4½ hours at 37 ℃ with 200 rpm agitation. The bacteria was centrifuged at 5000 g, 4 ℃ for five minutes. The supernatant was discarded and the bacteria pellet was washed with 2 ml of TBS. Upon washing, the bacteria was resuspended with 1 ml TBS and kept at 4 ℃.

Serial dilution of the bacteria was performed. One hundred microlitres of the serially diluted bacteria was plated on MacConkey agar to determine the bacteria titer. According to the bacteria titer determined on the next day, the dilution which contains about 10^9 CFU/ml will be selected for biopanning. The bacterial suspension with the desired concentration of bacteria was then diluted with 2 ml of TBS.

3.3.3.2 Phage Display Peptide Library Dilution

Ten microlitres of the 12-mer phage-displayed peptide library was diluted with 990 µl of TBS to a total volume of 1 ml.

3.3.3.3 Biopannings

The diluted bacteria described in section 3.3.3.1 was poured into a 60 mm petri dish. The diluted phages mentioned in section3.3.3.2 was then added to the bacteria. The bacteria-phage mixture was incubated at room temperature with gentle agitation on a rotary shaker. After an hour, the bacteria and phages were precipitated by spinning at 5,000 g, 4℃ for five minutes. The pellet was washed with 2 ml of 0.1 % of TBS/Tween buffer [50 mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% (v/v) Tween 20] for 10 times to remove unbound phages. The concentration of Tween 20 (v/v) was increased gradually from 0.1% in the first round, 0.3% in the second round to 0.5% in both the third round and the fourth round of biopanning. Two hundred microliters of elution buffer [0.2 M glycine-HCl (pH 2.2)] was added to resuspend the pellet right after the 10^{th} washing completed. The tube was incubated at room temperature on a rotary shaker for 10 minutes. Then, 30 µl of neutralization buffer [1 M Tris-HCl (pH 9.1)] was added immediately to the tube. Ten microlitres of the phage eluate was tittered and the remaining phages were amplified to enrich the phage clones for subsequent rounds of biopanning.

3.3.3.4 Phage Amplification

A loop of single colony of *E. coli* ER2738 was added into 10 ml of LB medium supplemented with tetracycline (20 mg/ml in 1:1 ethanol:water) and incubated overnight at 37℃. The next day, 400 µl of the overnight culture was added into 20 ml of fresh LB medium in 250 ml conical flask and incubated for about half an hour in shaking incubator until the OD_{600} of the culture reached

approximately 0.05. The remaining phage eluate mentioned in section 3.3.3.3 was added into the *E. coli* for propagation at 37℃, 200 rpm. After 4½ hours incubation, the culture was transferred into a 50 ml polypropylene tube and centrifuged at 12,000 g, 4℃ for 10 minutes. The supernatant was transferred into a fresh tube and respun. Eighty percent of the upper supernatant (16 ml) was transferred into a fresh tube that was filled with 1/6 volume of 20% (v/v) PEG/2.5 M NaCl (3 ml). The mixture was mixed well and incubated overnight at 4℃ to precipitate the phages. The next day, the mixture was spun down at 12,000 g, 4℃ for 15 minutes. The supernatant was discarded and the tube was respun to completely remove the residual supernatant. The precipitated whitish pellet was resuspended with 1 ml of TBS and transferred to a fresh microcentrifuge tube. The suspended phage was centrifuged for five minutes and the 80% of the upper supernatant (800 µl) was transferred into fresh microcentrifuge tube that was filled with 150 µl of PEG/NaCl. The tube was stood on ice for one hour, followed by centrifugation at 12,000 g, 4℃ for 10 minutes. The supernatant was discarded and the tube was briefly respun. After removing the residual supernatant, the whitish pellet was resuspended with 200 µl of TBS. This is the amplified eluate. Like the pre-amplified eluate in section 3.3.3.3, 10 µl of the amplified eluate was tittered to determine the input titer for the subsequent rounds of biopanning.

3.3.3.4 Phage Titering

A loop of single colony of *E. coli* ER2738 was added into 10 ml of LB medium supplemented with tetracycline (20mg/ml in 1:1 ethanol:water) in a 50 ml

centrifuge tube and incubated for three hours in shaking incubator. Once the OD_{600} of the bacteria reached it's mid-log phase (approximately 0.5), the bacteria can be used for phage infection. Pre-amplified phage eluate was serially diluted to 10^{-4} while amplified phage eluate was diluted to 10^{-10}. Ten microlitres of the diluted phage from last three dilution tubes, which are 10^{-2}, 10^{-3} and 10^{-4} from the pre-amplified phage eluate and 10^{-8}, 10^{-9} and 10^{-10} from the amplified phage eluate, were added to 200 µl of *E. coli* ER2738. After five minutes of infection in room temperature, the phage-infected *E. coli* ER2738 was transferred to 3 ml of melted top agar and briefly mixed well before pouring onto LB agar supplemented with IPTG/Xgal [1.25 g IPTG and 1 g Xgal in 25 ml DMF]. The plate was incubated overnight at 37 ℃. The number blue plaque was counted and recorded as plaque forming unit (PFU)/ml on the next day.

3.3.4 Individual Phage Clones Stock Preparation

Forty clones from the third round of biopanning and 30 clones from the fourth round of biopanning were selected and amplified individually. Small scale amplification was carried out to make individual phage clone stock. Overnight *E. coli* ER 2738 was diluted according to the dilution factor 1:100 in LB medium. One millilitre of the diluted culture was aliquot into the microcentrifuge tube. Single, well-separated blue plaque from pre-amplified phage titer plate was picked up using sterilized cut blue tips by stabbing on the agar then slowly aspirated the agar over the plaque. The culture together with plaque was incubated at 37 ℃, 200 rpm for 4½ hours. The culture was

centrifuged at 12,000 g for 30 seconds. The supernatant was transferred into new microcentrifuge tube and respun. The upper 600 µl of the supernatant was then mixed with 600 µl of 50% glycerol in another new tube. Individual phage clone stock was kept at -20 ℃.

Large scale amplification was carried out to increase the individual phage clone to a sufficient titer for subsequent assays. The phage clone stock was thawed on ice before adding into bacterial culture. The remaining procedure is similar to the phage amplification as mentioned in section 3.3.3.4. However, instead of the adding phage eluate, 100 µl of individual phage clone stock was added into 20 ml of diluted bacterial culture.

3.3.5 Bacterial Binding Selectivity Test

Each individual phage clone was tested for binding selectivity to the target bacteria using ELISA. For each test, the 96-microtitre plate well was coated with 150 µl of diluted *P. aeruginosa* (1 x 10^8 CFU/ml). Negative control well was inoculated with 150 µl of BSA blocking buffer. The plate was incubated overnight at 4 ℃ in air-tight humidified box. The next day, 350 µl of blocking buffer was added into the coated well after removing the content in the well. The plate was incubated at 4 ℃. After an hour, the well was washed with 300 µl of 0.5% (v/v) TBST on shaking incubator. The washing step was repeated for another five times. Once washing was completed, 150 µl of four folds diluted phage (2.5 x $10^{11\text{-}12}$ PFU/ml) was added into both negative control and sample wells and the plate was incubated at room temperature with gentle

agitation on a rotary shaker. Likewise, six times washing was carried out after one hour of incubation with phage. This was followed by addition of 200 µl of horseradish peroxidase (HRP)-linked anti-M13 monoclonal antibody that had been diluted 1:1000 in blocking buffer. The wells were washed for another six times after one hour of incubation. Lastly, 200 µl of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) with 30% of H_2O_2 was added into the wells and incubated on rotary shaker in dark for one hour. The absorbance was read at OD_{405} after 20 minutes of incubation. The ratio of OD_{405} reading of negative control to sample was calculated to determine the binding selectivity of phage to the target bacteria.

3.3.6 Antibacterial Assay

Antibacterial test was conducted in 96 wells microtitre plate. Negative control was run concurrently with the sample for each individual phage clone to be tested. In both negative control and sample wells, 50 µl of diluted *P. aeruginosa* (10^3 CFU/ml) in minimal liquid test medium (LTM; 1% of LB medium in TBS) was added. In negative control well, 150 µl of TBS was added. In sample well, TBS was replaced by individual amplified phage clone ($1 \times 10^{11\text{-}13}$ PFU/ml). The phage was incubated at 65 ℃ in water bath before added into the sample well in order to kill any residues *E. coli* ER2738 that might not completely being removed from the phage clone during the precipitation process described in section 3.3.3.4. After four hours of incubation at 37 ℃, 50 µl of LB medium was added into wells and the plate was further incubated for another 12-14 hours at 37 ℃. The comparison

between the content in the negative control and sample well is listed in Table 3.1.

Upon incubation, the plate was placed on a multiplate absorbance reader to measure the OD_{600} of each well. The sample well that has lower OD_{600} value than its correspondent control well indicated antibacterial effect of the individual phage clone. The test was duplicated if phage clone showed antibacterial activity.

Table 3.1: Comparisons of the control and sample well in antibacterial assay.

Content	Control Well	Sample Well
Diluted Bacteria	50 µl	50 µl
TBS	150 µl	-
Individual Phage Clone	-	150 µl
LB medium	50 µl	50 µl

3.3.7 DNA Extraction and Sequencing

One millilitre of diluted overnight *E. coli* ER2738 was coincubated with 100 µl of individual phage clone stock at 37 ℃, 200 rpm for 4½ hours. After spinning down the culture at 12, 000 g for one minute at 4 ℃, 800 µl of the supernatant was then mixed with 200 µl of 20% (v/v) PEG/ 2.5 M NaCl. After one hour standing on ice, the mixture was centrifuged at 12,000 g for 10 minutes at 4 ℃. Pellet was resuspended with 100 µl of iodide buffer by gently tapping the tube.

Then, 250 µl of absolute ethanol was added and mixed. The suspension was incubated at room temperature for 20 minutes. Later, the suspension was spun down and washed with 0.5 ml of cold 70% ethanol. The supernatant was removed completely while the pellet was dried in vacuum at 37℃ for 30 minutes and resuspended with 30 µl of TE buffer.

Upon extraction, the DNA concentration was measured using NanoDrop 2000 spectrophotometer. The DNA was then analysed in 1% agarose gel electrophoresis at 60 V for 1 hour in 1 X TAE buffer. After that, the gel was stained with 0.5 µg/ml of ethidium bromide for five minutes and destained in distilled water for 10 minutes. The gel image was viewed under UV light. Dense and uniform band was considered as purified DNA. DNA was sequenced using 96 gIII sequencing primer 5´- HOCCC TCA TAG TTA GCG TAA CG –3´by 1st Base Sequencing Company.

3.3.8 Sequence Analysis

The DNA sequence was viewed using Sequence Scanner v1.0. The conserved region "ATGGGATTTTGCTAAACAACTTTCAACAGTTTCGGCCGA" of the phage genome was identified using Molecular Evolutionary Genetic Analysis 5.1 (MEGA5.1). The oligonucleotide that encodes the displayed peptide was located after the conserved region. The oligonucleotide was then translated into peptide sequence using ExPASy DNA translate tool (available at http://web.expasy.org/translate/). The physiochemical properties of the peptide were examined using GeneDoc. The peptide sequence is further examined

through the Collections of Antimicrobial Peptide (CAMP) (available at http://www.bicnirrh.res.in/antimicrobial/) and NCBI protein database (available at http://blast.ncbi.nlm.nih.gov/).

CHAPTER 4

RESULTS

4.1 Bacterial Growth Curve

Bacterial growth curve for *P. aeruginosa* was constructed based on the OD_{600} reading and bacterial titer in CFU/ml. The OD_{600} and bacterial titer (CFU/ml) of *P. aeruginosa* at various time intervals were tabulated in Table 4.1 and the growth curves were plotted as shown in Figure 4.1. The curve in blue line shows the increment of OD_{600} reading over the time. The bacteria grew exponentially from the first hour to the third hour, and growth of the bacteria was reduced from the fourth hour. On the other hand, the growth curve in bacterial titer (CFU/ml) is represented by the red line in Figure 4.1. The growth trend in bacterial titer was not coincided with the trend in OD_{600} where lag phase was presented in the first two hours and exponential phase happened from the second to the fourth hour. Nevertheless, both results showed that the bacteria reached to titer of 10^9 CFU/ml after 4½ hours, which is the sufficient titer for biopanning.

Table 4.1: The OD_{600} reading and bacterial titer (CFU/ml) of *P. aeruginosa* at various time intervals.

Time (hours)	OD_{600}	Bacteria Titer (CFU/ml)
1	0.218	1.19×10^8
2	0.689	1.33×10^8
3	1.266	9.80×10^8
4	1.438	3.70×10^9
4.5	1.502	4.10×10^9

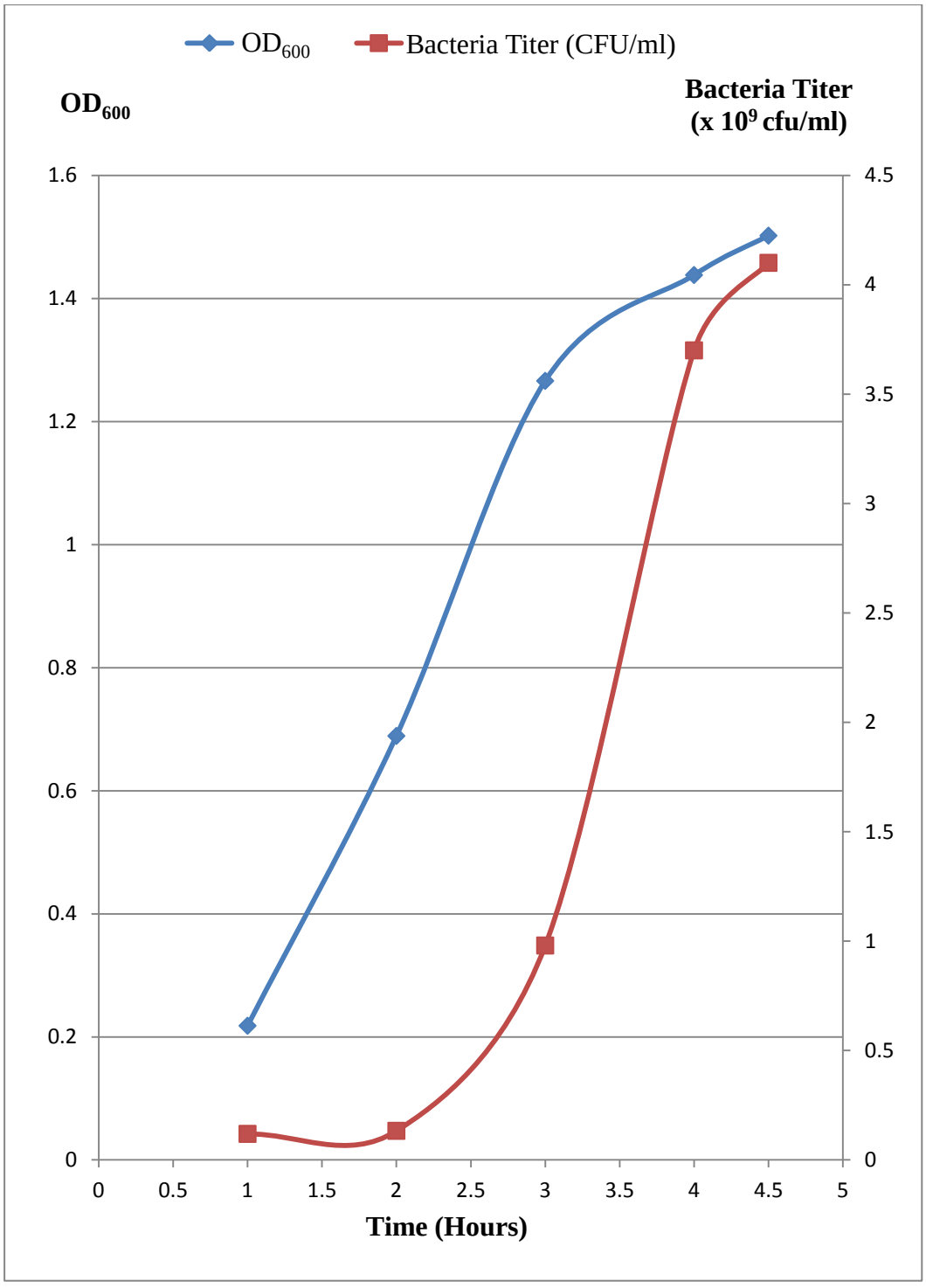

Figure 4.1: Growth curve of *P. aeruginosa* throughout 4½ hours as measured in OD_{600} and bacterial titer (CFU/ml).

4.2 Selection of Phage-Displayed Peptides Binding to *Pseudomonas aeruginosa*

Figure 4.2 shows the titer of eluted phage from the biopanning. The blue plaque forming units indicate that the affinity-selected phages from the biopanning were derived from the library with special *lac Z α* gene insertion. At the same time, the titer plate suggested that the phage eluate had no contaminant as no colourless plaque was seen.

According to Table 4.2, the ouput titer, which was also the phage eluate titer, was ranged in between 10^5-10^6 PFU/ml over the four rounds of biopanning. The eluted phage from the first round of biopanning had the lowest input titer with 1.23 x 10^5 PFU/ml while the eluted phage from the third round of biopanning had the highest output titer which is 1.6 x 10^6 PFU/ml. This indicated that the output titer was increased from the first round to the third round of biopanning and decreased in the fourth round of biopanning. On the other hand, input titer was maintained at 10^{11}-10^{12} PFU/ml throughout the four rounds of biopanning.

The yield of selection (Table 4.2) of phages for each round of biopanning was calculated as the percentage of the output titer divided by the input titer. It was maintained in between the percentage of 10^{-6} and 10^{-7}. This indicated that only certain numbers of the input phage was being selected in the biopanning. The yield of selection was increased from the first round (1.23 x 10^{-7}%) to the third round (1.86 x 10^{-6}%) of biopanning and then decreased at the fourth round (4.2 x 10^{-8}%) of biopanning. Lastly, a total of 70 individual phage clones were

randomly selected from the third and the fourth round of biopanning for subsequent assays.

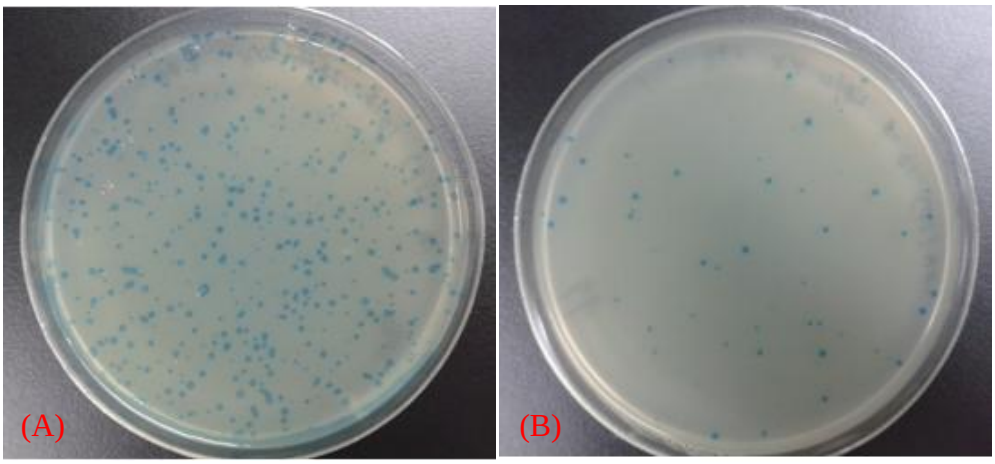

Figure 4.2: Phage titer of eluted phage on IPTG/Xgal plate. Figure (A) shows the phage titer with 10^{-1} dilution, and Figure (B) shows the phage titer with 10^{-2} dilution where the phage titer was 10^5 PFU/ml.

Table 4.2: Yield of selection for four rounds of biopanning.

Rounds of biopanning	Input titer (PFU/ml)	Output titer (PFU/ml)	Yield of Selection[a] (%)
1	1×10^{12}	1.23×10^{5}	1.23×10^{-7}
2	4.9×10^{11}	4.6×10^{5}	9.39×10^{-7}
3	8.6×10^{11}	1.6×10^{6}	1.86×10^{-6}
4	8.3×10^{12}	3.5×10^{5}	4.2×10^{-8}

[a]Yield of Selection (%) was calculated by the formula: [(Output titer / Input titer) x 100%].

4.3 Determination of Binding Selectivity of Phage-Displayed Peptide to *Pseudomonas aeruginosa*

Figure 4.3 shows the colour development in negative control and sample well after 20 minutes of incubation with ABTS substrate. The negative control wells were in very light green colour while the sample wells were in green colour with varying intensities. This indicated that greater numbers of phage displaying peptides bound to the sample well that were coated with the target bacteria compared to the negative control well that only filled with bovine serum albumin (BSA) blocking buffer. The binding of phage-displayed peptides toward the target bacteria was indicated by OD_{405} reading which is shown in Figure 4.4 and 4.5. All of the samples showed higher OD_{405} compare to their corresponding negative control. The OD_{405} reading of all the negative controls was less than 0.2. Phage clone 3PA34 demonstrated the highest OD_{405} reading at 2.5338 while phage clone 4PA13 had the lowest OD_{405} reading at 0.8021. The binding selectivity of phage displaying peptides to the bacteria was calculated based on the ratio of the sample OD_{405} to the negative control OD_{405} reading. Phage clone 3PA26 showed the highest binding selectivity ratio of 29.13 among the 40 clones selected from the third round of biopanning. Additionally, phage clone 4PA4 showed the highest binding selectivity ratio of 22.84 compare to other selected clones from the fourth round of biopanning. In contrast, phage clone 4PA24 demonstrated the lowest binding selectivity ratio of 5.47. Nevertheless, most of the clones showed a relatively high binding selectivity towards *P. aeruginosa* with the ratio ranging from 10 to 20.

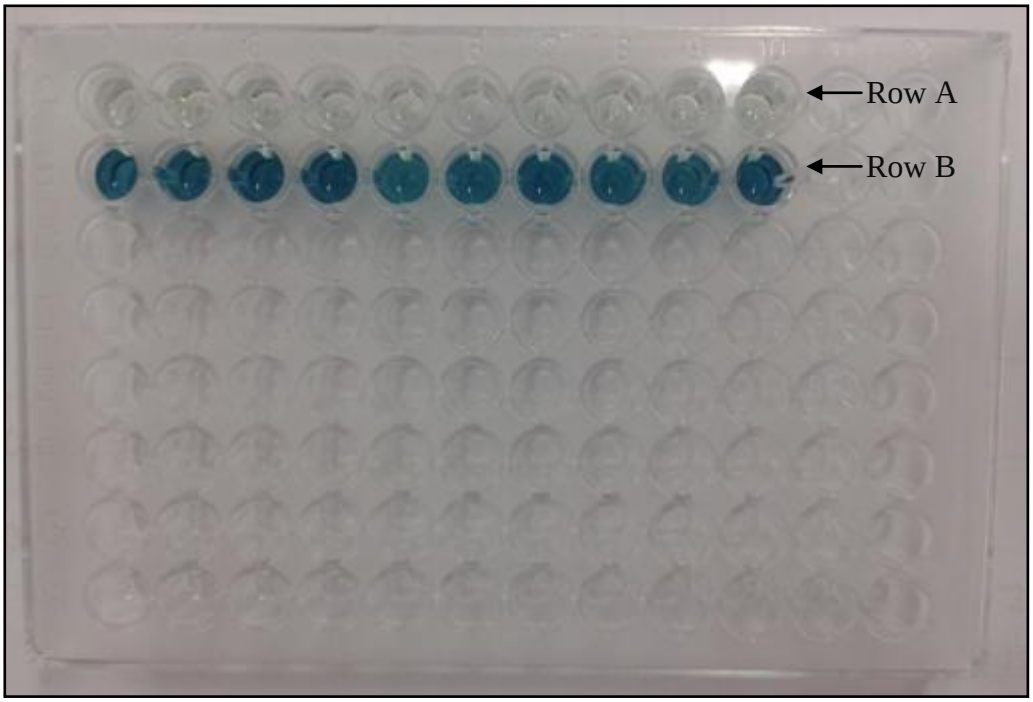

Figure 4.3: Development of colour in negative control and sample wells after 20 minutes ABTS substrate incubation. Row A shows negative control wells while Row B shows sample wells.

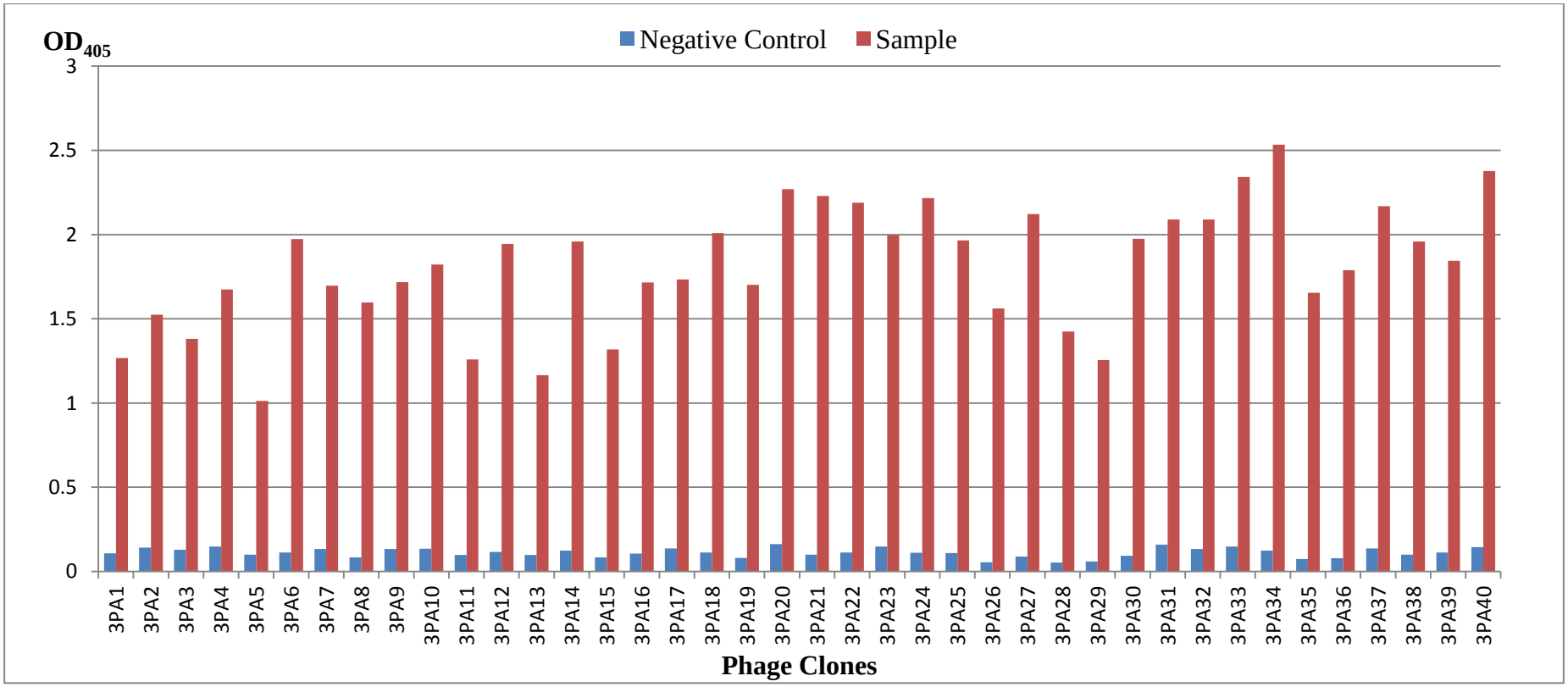

Figure 4.4: Bar chart of ELISA reactivity for the selected phage-displayed peptides of phage clones 3PA1 to 3PA40. Negative control represents the wells coated with BSA while sample represents the wells coated with bacteria.

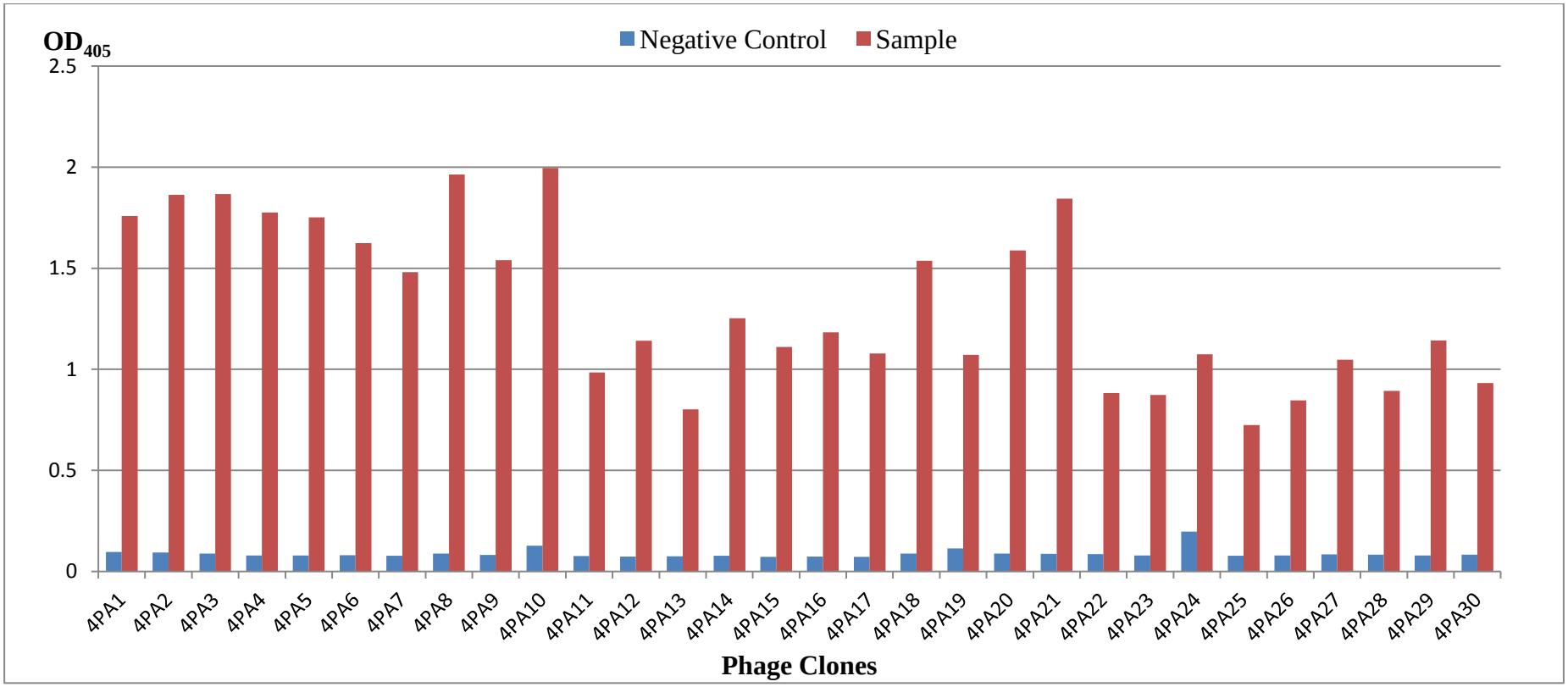

Figure 4.5: Bar chart of ELISA reactivity for the selected phage-displayed peptides of phage clones 4PA1 to 4PA30. Negative control represents the wells coated with BSA while sample represents the wells coated with bacteria.

Table 4.3: OD_{405} reading of ELISA reactivity for the selected phage-displayed peptides and their corresponding ratio of binding selectivity.

Phage Clone	Negative Control[a] (OD_{405})	Sample[b] (OD_{405})	Binding Selectivity Ratio[c]
3PA1	0.1081	1.2663	11.71
3PA2	0.1416	1.5239	10.76
3PA3	0.1287	1.3803	10.72
3PA4	0.1474	1.6736	11.35
3PA5	0.0987	1.0127	10.26
3PA6	0.1118	1.9734	17.65
3PA7	0.1325	1.6958	12.80
3PA8	0.0838	1.596	19.05
3PA9	0.1337	1.7173	12.84
3PA10	0.1354	1.8229	13.46
3PA11	0.0974	1.2591	12.9
3PA12	0.1161	1.9451	16.75
3PA13	0.0983	1.1653	11.85
3PA14	0.1231	1.959	15.91
3PA15	0.0826	1.3174	15.95
3PA16	0.1052	1.7165	16.32
3PA17	0.1361	1.7343	12.74
3PA18	0.1119	2.0089	17.95
3PA19	0.0809	1.7012	21.03
3PA20	0.1628	2.2694	13.94

[a]Negative control represents the well coated with BSA.
[b]Sample represents the well coated with bacteria.
[c]Binding selectivity ratio was calculated by the formula of : (Sample/Negative control).

Table 4.3 Continued:

Phage Clone	Negative Control[a] (OD_{405})	Sample[b] (OD_{405})	Binding Selectivity Ratio[c]
3PA21	0.0998	2.2287	22.33
3PA22	0.1117	2.1888	19.60
3PA23	0.1476	1.9952	13.52
3PA24	0.1104	2.2158	20.07
3PA25	0.1091	1.9661	18.02
3PA26	0.0536	1.5612	29.13
3PA27	0.0882	2.1211	24.05
3PA28	0.0524	1.4247	27.19
3PA29	0.0598	1.2562	21.01
3PA30	0.0934	1.9746	21.14
3PA31	0.1592	2.0892	13.12
3PA32	0.1335	2.0899	15.65
3PA33	0.1475	2.3422	15.88
3PA34	0.1237	2.5338	20.48
3PA35	0.0739	1.655	22.40
3PA36	0.0783	1.7885	22.84
3PA37	0.1359	2.1679	15.95
3PA38	0.0996	1.9586	19.66
3PA39	0.1129	1.8445	16.34
3PA40	0.1439	2.3783	16.53

[a]Negative control represents the well coated with BSA.
[b]Sample represents the well coated with bacteria.
[c]Binding selectivity ratio was calculated by the formula of : (Sample/Negative control).

Table 4.3 continued:

Phage Clone	Negative control[a] (OD_{405})	Sample[b] (OD_{405})	Binding Selectivity Ratio[c]
4PA1	0.0959	1.7589	18.34
4PA2	0.0931	1.8637	20.02
4PA3	0.0881	1.8672	21.19
4PA4	0.0786	1.7765	22.60
4PA5	0.0786	1.7516	22.28
4PA6	0.0798	1.6239	20.34
4PA7	0.0779	1.4804	19.00
4PA8	0.0885	1.9642	22.19
4PA9	0.082	1.5394	18.77
4PA10	0.1269	1.9959	15.73
4PA11	0.0766	0.9845	12.85
4PA12	0.0735	1.1419	15.54
4PA13	0.0743	0.8021	10.79
4PA14	0.0776	1.2531	16.15
4PA15	0.0722	1.1111	15.39
4PA16	0.0733	1.1824	16.13
4PA17	0.0727	1.0782	14.83
4PA18	0.0877	1.5374	17.53
4PA19	0.114	1.0721	9.04
4PA20	0.0885	1.5878	17.94

[a]Negative control represents the well coated with BSA.
[b]Sample represents the well coated with bacteria.
[c]Binding selectivity ratio was calculated by the formula of : (Sample/Negative control).

Table 4.3 continued:

Phage Clone	Negative control[a] (OD_{405})	Sample[b] (OD_{405})	Binding Selectivity Ratio[c]
4PA21	0.0863	1.8447	21.38
4PA22	0.0854	0.8821	10.33
4PA23	0.0788	0.8725	11.07
4PA24	0.1965	1.0739	5.47
4PA25	0.077	0.724	9.40
4PA26	0.0794	0.8456	10.65
4PA27	0.0847	1.0479	12.37
4PA28	0.0833	0.8936	10.72
4PA29	0.079	1.1429	14.47
4PA30	0.0825	0.9315	11.29

[a]Negative control represents the well coated with BSA.
[b]Sample represents the well coated with bacteria.
[c]Binding selectivity ratio was calculated by the formula of : (Sample/Negative control).

4.4 Antibacterial Activity of Phage-Displayed Peptides

The antibacterial activity of individual phage-displayed peptides was indicated by the lower OD_{600} in the sample that harbored the mixture of phage-displayed peptides and *P. aeruginosa* than the negative control that only harbored the target bacteria only where the OD_{600} is a measurement of the amount of the bacteria. All of the 70 selected clones of phage-displayed peptides did not exhibit any antibacterial activity towards *P. aeruginosa* as demonstrated by the higher optical density of the sample than the negative control (Figure 4.6 and 4.7). Although there were a few clones of sample that had an OD_{600} reading lower than the negative control, but the degree of inhibition were very low. These clones were 3PA35, 4PA10 and 4PA12 which exhibited the degree of inhibition of 1.67%, 0.88% and 2.30%, respectively (Table 4.4). The low degree of inhibition shown by these clones was insufficient to be concluded as positive antibacterial activity. Therefore, there was no isolation of phage-displayed peptides with antibacterial effect in this study.

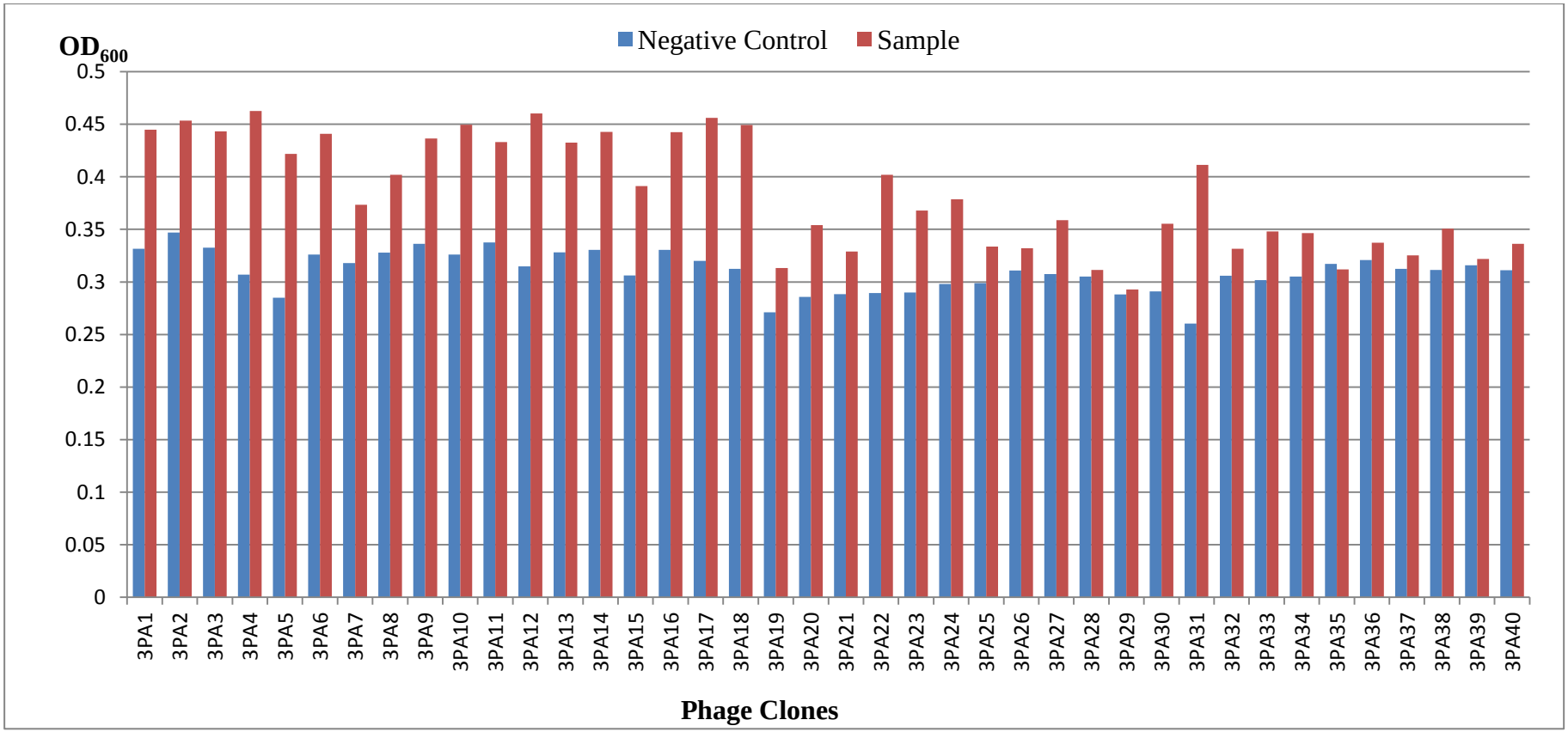

Figure 4.6: Bar chart of antibacterial activity of phage clones 3PA1 to 3PA40. Negative control represents the well with only the target bacteria while sample represents the well with phage and bacteria.

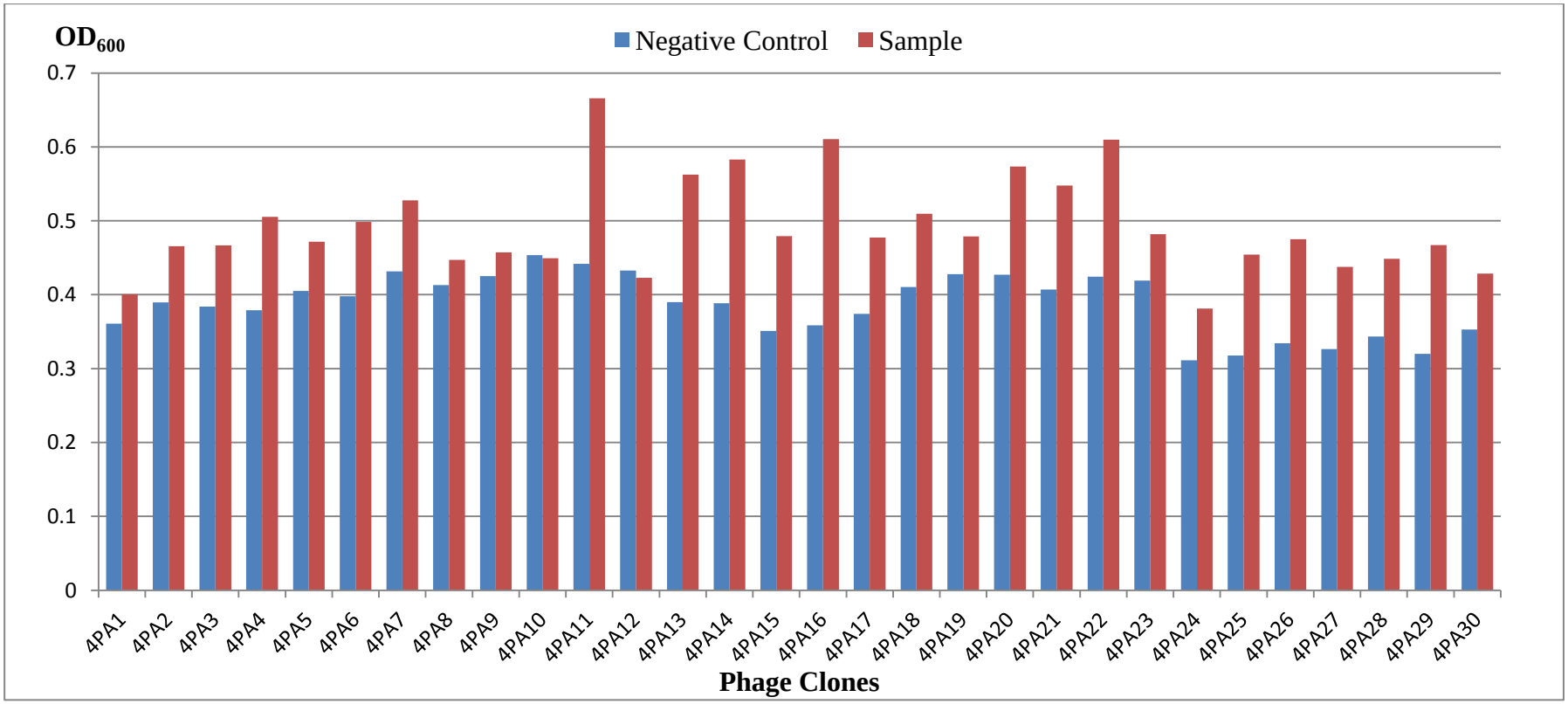

Figure 4.7: Bar chart of antibacterial activity of phage clones 4PA1 to 4PA30. Negative control represents the well with only the target bacteria while sample represents the well with phage and bacteria.

Table 4.4: OD_{600} reading of bacterial culture for selected clones with low degree of inhibition in antibacterial assay.

Phage clone	Negative control[a] OD_{600}	Sample[b] OD_{600}	Degree of Inhibition[c] (%)
3PA35	0.3172	0.3119	1.67
4PA10	0.4534	0.4494	0.88
4PA12	0.4328	0.4229	2.29

[a]Negative control represents the well with only bacteria.
[b]Sample represents the well with bacteria and phage
[c]Degree of inhibition in percentage was calculated by the formula:[100-{(Sample/Negative control) x 100}].

4.5 Purity of the Extracted Phage Genomic DNA

Phage genomic DNA was extracted from five phage clones 3PA26, 3PA28, 4PA44, 4PA45 and 4PA48 which showed the highest binding selectivity ratio towards *P. aeruginosa* as determined in section 4.3. The concentrations of the five DNA samples were varied from each other as shown in Table 4.5, with the lowest concentration at 18.9 ng/µl and the highest concentration at 218.2 ng/µl. The five extracted genomic ssDNAs (L1 to L5 which represents 3PA26, 3PA28, 4PA44, 4PA45 and 4PA 48, respectively), together with 1kb DNA ladder (lane M), were examined by 1% of agarose gel electrophoresis as shown in Figure 4.8. Single clear band was observed at the lanes labeled as L1, L2, L3, L4 and L5, indicating that the DNAs were extracted. All of the five DNAs were positioned at 6,000 bp. However, DNA of M13 phage from the library comprises of approximately 7,250 nucleotides which were not lined up with the double-stranded DNA ladder of the same length.

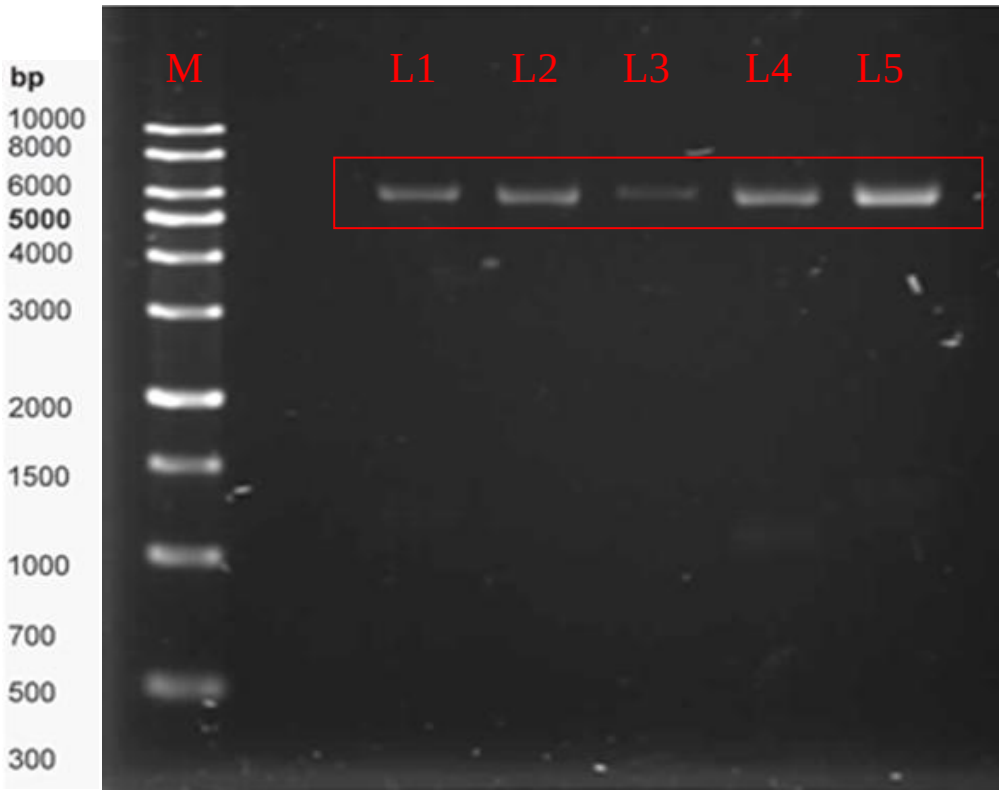

Figure 4.8: Agarose gel electrophoresis analysis of phage genomic DNAs. Lane M shows the 1kb DNA ladder. L1 to L5 show the genomic DNAs from the phage clone 3PA26, 3PA28, 4PA44, 4PA45 and 4PA 48, respectively.

Table 4.5: Concentration of extracted ssDNAs from selected phage clones.

Phage Clone	DNA Concentration (ng/µl)
3PA26	18.9
3PA28	23.2
4PA44	33.0
4PA45	56.1
4PA48	218.2

4.6 Peptide Sequence Analysis

Five DNAs from the clones mentioned in section 4.5 were sequenced for the inserted oligonucleotide. However, only three oligonucleotides from clones 3PA26, 4PA4 and 4PA5 were successfully sequenced. Figure 4.9(a), (c) and (d) show a good quality of DNA sequencing for clones 3PA26, 4PA4 and 4PA5, respectively. DNA of clone 4PA8 was poorly sequenced as shown in Figure 4.9(e), resulted in unsuccessful identification of the oligonucleotide insert region. Clone 3PA28 showed a good DNA sequencing signal [(Figure 4.9(b)]. However, the DNA sequence possessed a deletion of the library insert nucleotides (ACCTCCACC) and the oligonucleotides located at the peptide insert region which is indicated by dashes in the sequence alignment as shown in Figure 4.10. Therefore, peptide sequence of clone 3PA28 and 4PA8 were failed to be revealed. The conserved region of "ATGGGATTTTGCTAAACAACTTTCAACAGTTTCGGCCGA" which is part of the phage genome located before the insert of interest is highlighted in yellow in the sequences alignment (Figure 4.10). The 36-nucleotides region located right after the yellow DNA region and conserved region ACCTCCACC is the insert region that encodes for the displayed peptide. The reverse complement sequence (Table 4.6) of the oligonucleotide is determined through reverse complement tool. The sequences were translated into peptide sequence as tabulated in Table 4.7.

Peptides of clones 4PA4 and 4PA5 are identical, which is GLHTSATNLYLH. On the other hand, peptide of clone 3PA26 is GPVNKSSTILRM. The net ionic charge and hydrophobicity of the peptides are listed in Table 4.7, where

GPVNKSSTILRM is a positively charged peptide while GLHTSATNLYLH is a zwitterionic peptide. Peptide GPVNKSSTILRM showed the common features to the typical antimicrobial peptide where it consists of a net positive charge (+2). In addition, the peptide consists of a 33.33% of total residue hydrophobicity. Besides, the peptide GLHTSATNLYLH showed a total hydrophobicity of 41.67%. A consensus motif of –Ser-X-Thr-X-Leu- , where 'X' can be any amino acid residues, was revealed in peptides GPVNKSSTILRM and GLHTSATNLYLH as shown in Figure 4.11. The physiochemical properties of the peptides were summarized in Figure 4.12. Hydrophobic amino acids are conserved at position 2, 3, 10 and 12 while small amino acids are conserved at position 4, 7, and 8. In addition, polar amino acid is conserved at position 5 of both the peptides. However, no matches were found when GLHTSATNLYLH and GPVNKSSTILRM were blasted against Collections of Antimicrobial Peptides (CAMP) and NCBI protein database.

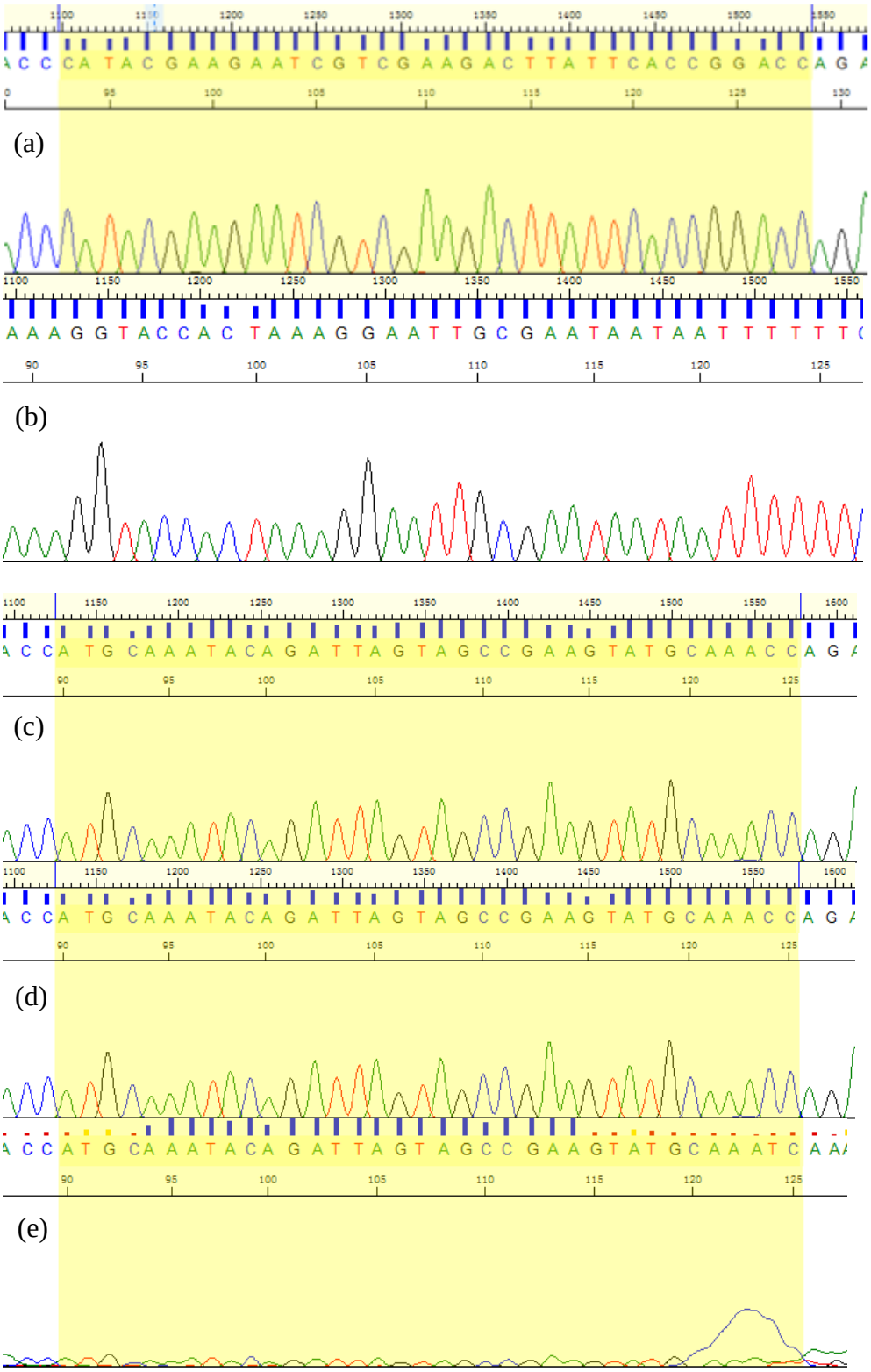

Figure 4.9: DNA sequence electropherogram of the phage inserted oligonucleotides (yellow region) encoding the displayed peptides. The alphabet represents the selected clones respectively**,** (a) 3PA26, (b) 3PA28, (c) 4PA4, (d) 4PA5 and (d) 4PA8**.**

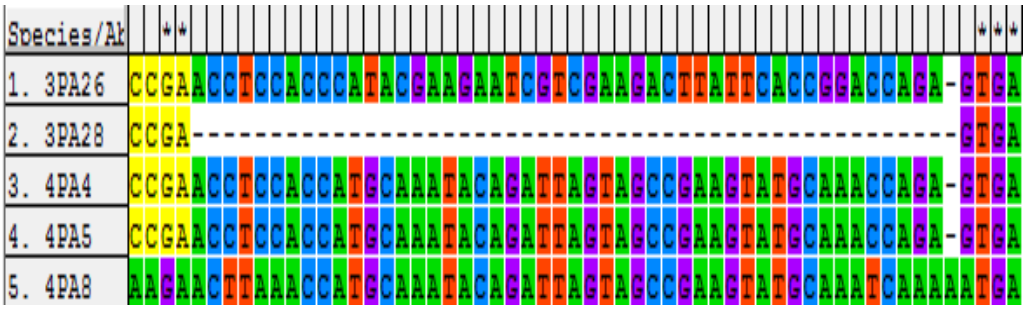

Figure 4.10: Alignment of the DNA sequences using ClustalW.

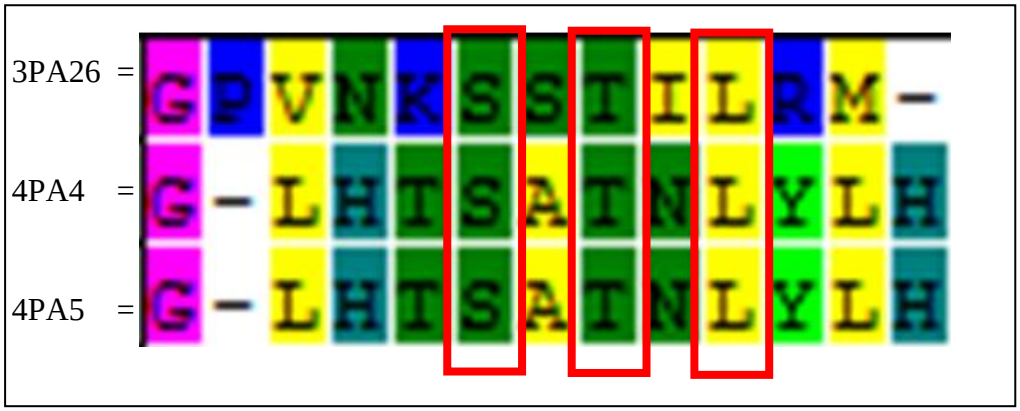

Figure 4.11: Alignment of the peptide sequences using ClustalW. Peptide sequences alignment revealed a consensus sequence of Ser-X-Thr-X-Leu (X= any amino acid residues).

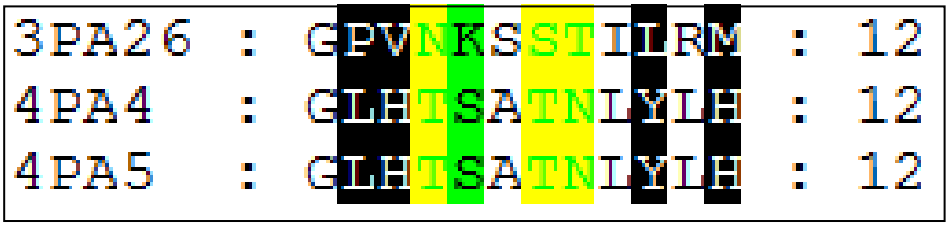

Figure 4.12: Alignment of peptide sequences in GeneDoc. The peptide sequences of clones 3PA26, 4PA4 and 4PA5 were aligned. The physical-chemical mode of GeneDoc was used to assign columns of alignment to one of the 12 pre-defined groups of physiochemical properties. White writing on a black background indicates hydrophobic amino acids; green writing on a yellow background indicates small amino acids; black writing on a green background indicates polar amino acids.

Table 4.6: Reverse complement sequence of the 36 nucleotides insert region.

Phage Clone	Reverse complement sequence
3PA26	GGTCCGGTGAATAAGTCTTCGACGATTCTTCGTATG
4PA4	GGTTTGCATACTTCGGCTACTAATCTGTATTTGCAT
4PA5	GGTTTGCATACTTCGGCTACTAATCTGTATTTGCAT

Table 4.7: Sequence and physiochemical properties of the isolated phage-displayed peptide.

Phage Clone	Peptide Sequence	Net Charges	Hydrophobicity
3PA26	G P V N K S S T I L R M	+2	33.33%
4PA4	G L H T S A T N L Y L H	0	41.67%
4PA5	G L H T S A T N L Y L H	0	41.67%

CHAPTER 5

DISCUSSION

Emergence of multi-drug resistant strain of *P. aeruginosa* threatens the effectiveness of antibiotics in infection treatment. Therefore, alternative treatments have to be discovered to complement with the antibiotic treatment. Antibacterial peptides may be a potential alternative antibacterial agent. In this study, a phage-displayed peptide library was used to screen for peptides with antibacterial properties. Phage display technology has been successfully used in antibacterial peptides isolation over the years (Bishop-Hurley et al., 2005; Pini et al., 2005; Bishop-Hurley et al., 2010). It is fast and cost-effective by allowing the screening of large numbers of peptides at once. Thus, it is reasonable to use phage display as the tool for screening and isolation of antibacterial peptide against *P. aeruginosa.*

5.1 Selection of Phage-Displayed Peptides Binding to *Pseudomonas aeruginosa*

Dodecapeptide-bearing phage display library was panned against the whole bacterial cells of *Pseudomonas aeruginosa*. Bacterial cell membrane surface possesses diverse epitopes that might be bound by various types of phage-displayed peptides. Hence, panning with whole bacterial cells probably may isolate as many types of phage-displayed peptides as possible especially in the first round of biopanning. Solution biopanning approach was chosen so that the entire bacterial cell membrane surface was exposed to phage-displayed peptides in all dimensions during the biopanning. Thus, this might increase the probability of selecting different types of antibacterial peptides which target at various surface molecules of the bacteria.

Selection of target-specific phage-displayed peptides was optimized by washing away weak binding phage-displayed peptides and retained only those peptides with high affinity binding which are represented by the output phage eluate. This was achieved by stepwise increasing the concentration of Tween 20 in the washing buffer (TBST) in the subsequent round of biopanning. Tween 20 works as a detergent to reduce non-specific hydrophobic interactions between the phage-displayed peptide and the target epitope of the bacteria (Caberoy et al., 2009). Hence, with each round of biopanning and amplification, the pool of phage was enriched in favour to the peptides that bind to the target bacteria. This was indicated by the increased percentage of phage clones yield from the first round to the third round of biopanning. The number of phage clone that bind selectively toward the bacteria increases after every round of the biopanning. However, in the fourth round of biopanning, the percentage of

phage clones yield was decreased. This might be due to the greater input phage titer compare to the second and the third round of biopanning (refer to Table 4.2). Furthermore, output titer of the biopanning was limited to the range of between 10^5 and 10^6 PFU/ml which is similar to the result of the study of Abi-Ghanem et al. (2008). This might be due to the saturation of phage clones enrichment after four rounds of biopanning. As the result, increasing input titer did not increase the output titer in the last round of biopanning.

5.2 Binding Selectivity of Phage-Displayed Peptide to *Pseudomonas aeruginosa*

The binding selectivity should always be emphasized since strong interactions between phage-displayed peptide and bacteria are the basic requirement for the antibacterial activity (Gofman et al., 2010). Positive ELISA signal which is demonstrated by the greater OD_{405} of the sample than the negative control indicates that the individual selected phage-displayed peptides were selectively bound to the bacteria. This was also reflected by the enrichment of high affinity-binding phage clones after four rounds of biopanning. Theoretically, the titer of eluted phages with high binding selectivity to *P. aeruginosa* should be gradually increased in the subsequent rounds of biopanning. Thus, certain phage clones selected from the fourth round of biopanning were supposed to show higher binding selectivity ratio compare to the phage clones from the third round of biopanning. However, as shown in Table 4.3, binding selectivity ratio of phage clone selected from the third round of biopanning showed the highest ratio of 29.13. In contrast, the highest ratio of phage clone in the fourth round of biopanning was only 22.84. The repeated biopanning does not

increase the binding strength of the phage clone towards the bacteria but only enrich the pool of the phage clones with high binding affinity and selectivity. This condition was supported by the studies of Vodnik et al. (2011). Therefore, in this study, it is possible that the phage clone isolated from the third round of biopanning had higher binding affinity than phage clone isolated from the fourth round of biopanning. This is because of the random selection of phage clones from the titer plates, which may have selected phage clones with different binding affinity. Individual phage clones were randomly selected from an IPTG/Xgal plate with approximately 100 plaque forming unit of the phage clones. Out of the 10^5 PFU/ml of the phage clones, only a small fraction of phage clones in which 40 clones from the third round and 30 clones from the fourth round eluate were selected. Therefore, phage clones with the higher binding selectivity does not always present in last round of biopanning.

Determination of binding affinity of phage-displayed peptide to *P. aeruginosa* was conducted with ELISA method, in which washing is a critical step to produce valid results. The duration and frequency of washing, volume of washing buffer and strength applied to both negative control and sample wells should be standardized. Moreover, the OD_{405} reading of negative control should be as low as possible. The binding selectivity ratio decreased when OD_{405} of the negative control was high ($OD_{405} > 1$). This occurred when light green colour was observed in the negative control well. The background signal in the negative control may be due to a certain number of the phage clones that bound to the bovine serum albumin (BSA) coated in the negative control well. This has been described through the studies of Revelen et al. (2000). Therefore,

the ratio may reflect the binding selectivity of the peptide towards *P. aeruginosa* by excluding the OD_{405} contributed by the BSA-bound phage-displayed peptides through the formula described in Table 4.3.

5.3 Assay on Antibacterial Activity by Selected Phage-Displayed Peptides

There is no observable antibacterial activity demonstrated by the 70 phage-displayed peptides selected from the third and the fourth round of biopanning. This might be due to several reasons. These might include low cationicity of the selected peptides, inadequacy of pIII display system, low affinity-binding of phage clones and resistance mechanism of *P. aeruginosa* towards antibacterial peptides.

Typically, most antimicrobial peptides are highly cationic with the net positive charge in the range between +2 and +9 (Hancock et al., 2006). Therefore, peptides with low cationicity or zwitterionic might not be able to exert antibacterial activity even though the peptides were bound to the bacteria with high affinity. Besides, anionic peptides do not bind strongly to the bacteria as the peptides and the bacteria may repel each other by the same charge condition. In addition, the phage- displayed peptides from New England Biolabs are linked to pIII coat protein. Unlike pVIII coat protein display system with thousand copies of displayed peptides, pIII system displayed only three to five copies of peptides by each phage particle (Loset et al., 2011). Therefore, the low concentration of the displayed peptides might not be sufficient to inhibit or kill the bacteria (Liao et al., 2005). Moreover, Chauhan

and Varma (2009) suggested that polyvalent displayed peptide on pIII may result in steric hindrance which reduce the binding of the peptide to the bacteria. Besides that, phage particle itself may interfere with the carpeting mechanisms of the peptides on the surface of outer membrane or the penetration of peptides into cytoplasmic membrane of the bacteria (Pini et al., 2005). Therefore, the antibacterial activity of the peptides was interrupted.

The first round of biopanning is the critical step in phage clones selection as there are 10^9 of diverse phage-displayed peptides being panned against the bacteria. As the concentration of Tween 20 increased in the subsequent rounds of biopanning, more phage-displayed peptides with low binding affinity to the bacteria would be washed away. However, certain antibacterial peptide may have low affinity binding to the bacteria especially at the membrane with highly zwitterion (Shai, 1999). In addition, the result of this study had shown that peptides with high binding affinity may not exert antibacterial activity. This indicates that binding affinity of phage-displayed peptide to the bacteria does not directly correlate with the antibacterial activity (Soares et al., 2004). Therefore, certain weak binding antibacterial peptide might be washed away during the repeated biopannings in this study, resulting in failure of selecting the promising antibacterial peptides.

Bacterial resistance to antibiotics was reported since past decades. Likewise, bacterial resistance to antibacterial peptides has been discussed over the years (Nizet 2006). The fundamental of antibacterial effect of these peptides is the electrostatic interaction between the cationic peptides with the anionic cell

membrane surface of the bacteria. However, *P. aeruginosa* is capable of producing anionic exopolysaccharide which has been known as bacterial decoy for antibacterial peptides (Llobet et al., 2008). It is a protein substance that forms biofilm to facilitate *P. aeruginosa* colonization on host cell surface such as mucosal lining of respiratory tract in patient with cystic fibrosis (Hoiby et al., 2010). Besides that, the alginate produced by *P. aeruginosa* was identified as an opaque layer above the bacteria pellet in the bacteria suspension after several times of washing as shown in Figure 5.1. This alginate slime layer might trap and reduce the number of phage-displayed peptides that bind to the bacteria during biopanning. Phage clones with high binding affinity to the bacteria membrane might be washed away as complexes with the bacteria alginate during the biopanning. Consequently, the numbers of phage-displayed peptide with possible antibacterial activity might be greatly reduced during the biopanning process. Moreover, the alginate acts as sticky layer covering the outer membrane surface of the bacteria. The anionic alginate may prevent the activity of antibacterial peptide on the bacterial membrane by interacting with those cationic peptides through electrostatic interaction. Thus, it traps and prevents the peptides from reaching bacterial cell membrane surface in order to prevent killing of *P. aeruginosa* by these antibacterial peptides. Llobet et al. (2008) showed that 10^4 CFU/ml of bacteria can produce sufficient exopolysaccharide to achieve protection against antibacterial peptides. In comparison to the antibacterial assay carried out in this study, 10^3 CFU/ml of bacteria was incubated with phage-displayed peptide. It can be assumed that plenty of exopolysaccharide was produced to protect the bacteria from those antibacterial peptides.

In addition to trapping mechanisms, *P. aeruginosa* may resist antibacterial peptides by altering the bacterial cell membrane. Bacterial cell membrane is the major target of antibacterial peptides, thereby their interaction was crucial to determine the antibacterial activity of the peptides. According to Nizet (2006), charge patterns of the bacterial outer membrane determine the degree of binding of antibacterial peptide to membrane surface. The negative charge of bacterial membrane can be reduced through modification of lipid A component of lipopolyssacharide on the outer cell membrane (Moskowitz et al., 2004). Aminoarabinose, a cationic amino sugar residue, is added to lipid A component of lipopolysaccharide by certain bacteria to reduce the anionicity of the outer membrane. As a result, antibacterial peptides are repelled by the same charge of lipid A before reaching bacterial cytoplasmic membrane for their antibacterial activity. Moreover, this modification is regulated by two-component regulators PhoP-PhoQ and PmrA-PmrB which upregulate the genes involved in the addition of 4-aminoarabinose to lipid A of lipopolysaccharide (Fernandez et al., 2010). This adaption occurs in response to low concentrations of divalent ions, for instance, Mg^{2+} and Ca^{2+} cations. In contrast to *in vivo* condition, antibacterial assay was conducted in 96-well microtiter plate with LB broth, phage clones in TBS and the bacteria where the divalent cations mentioned above were absent. Therefore, it is possible that the two-component regulators might be activated to protect the bacteria from the antibacterial activity.

Lastly, *P. aeruginosa* may resist antibacterial peptides by producing elastase which cleaves Asn-Leu and Asp-Phe of antibacterial peptides (Schmidtchen et.

al., 2002). The proteolytic degradation interrupts the conserved region of the antibacterial peptides and disables them to bind to the bacterial membrane thereby disabling their antibacterial activity. In this study, two phage-displayed peptides, 4PA4 and 4PA8 with the peptide GLHTSATNLYLH also possess this Asn-Leu region. Therefore, undetectable antibacterial activity of 4PA4 and 4PA8 might be due to the elastase degradation at Asn-Leu region of the peptide.

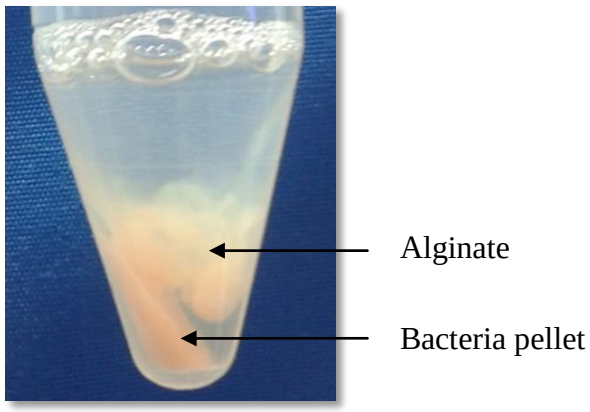

Figure 5.1: Alginate of *Pseudomonas aeruginosa*. It is produced as separate opaque layer after several rounds of washing.

5.4 Strong Binding Affinity of Phage-Displayed Peptides

Even though the peptides failed to show antibacterial activity, its strong binding affinity to *P. aeruginosa* may be beneficial to host system. Antibacterial peptides are not limited to direct killing of bacteria. It is also known to modulate host innate immunity (Hancock and Diamond 2000). Activation of certain innate immunity requires the binding of antibacterial peptide to the bacteria. For example, antibacterial peptides mark the bacteria to enhance phagocytosis by phagocytes (Yiva 2009). In addition, antibacterial peptides may become the barriers to bacterial infection. The binding of

antibacterial peptides to bacteria can cover up the membrane adhesion molecules on the bacterial surface membrane, thereby blocking the adherence and colonization of bacteria to host cell surface (Tsai et al., 2011). Moreover, antibacterial peptides have anti-endotoxin effect by inhibiting exaggerated inflammatory reaction that causes severe tissue damage. Binding of antibacterial peptides to lipopolysaccharides (LPS) can block the interaction between LPS and LPS binding protein (LBP), preventing macrophage activation induced by LPS (Hancock and Scott, 2000; Scott et. al., 2000). Consequently antibacterial peptides antagonise massive cytokine release by macrophage, thereby balancing and regulating proper inflammatory response.

5.5 DNA Extraction and Sequencing

Five DNAs samples were extracted from clones 3PA26, 3PA28, 4PA4, 4PA5 and 4PA8. The difference of DNA concentration across the phage clones may be due to the different phage clone stock titer. Individual phage clones were selected from blue plaques on IPTG/Xgal plate of the third and the fourth rounds of eluted phage. Blue plaques may differ in size, where plaques with smaller diameter have lesser yield while plaques with larger diameter produce higher yield in phage clone stock. However, the DNA concentration yield did not affect the sequencing since all the DNA samples were successfully sequenced.

Nevertheless, only three peptide sequences can be obtained as shown in Table 4.7 while peptide sequences of clones 3PA28 and 4PA5 were not obtained. In

clone 3PA28, there was no oligonucleotide found in the region that encodes the phage-displayed peptide. It is assumed that the clone did not carry any inserts. Furthermore, individual phage clones were amplified in small scale for 4½ hours for stock production and amplified for another 4½ hours for DNA extraction which resulted in total of 9 hours incubation time. According to New England Biolabs, long incubation time may result in deletion of the inserted oligonucleotide of certain phage clones. In contrast, clone 4PA8 showed weak and noisy sequence in the DNA sequencing electropherogram, which may due to insufficient DNA concentration or presence of contaminants from the surrounding environment.

5.6 Evaluation of Peptide Sequence

In this study, two peptides with high binding selectivity to *P. aeruginosa*, were GPVNKSSTILRM from clone 3P26 and GLHTSATNLYLH from clone 4PA4 as well as 4PA5. GPVNKSSTILRM is positively charged contributed by arginine and lysine. The cationicity of this peptide might contribute to the binding of this peptide to *P. aeruginosa* through the electrostatic interaction between the anionic bacteria membrane and the cationic peptide (Majerle et al., 2003). On the other hand, despite the zwitterionic nature of peptide GLHTSATNLYLH, it was still able to bind to *P. aeruginosa*. The binding of this peptide to the bacteria may be contributed by the polar residues. Although there are 12 amino acid residues in the peptide, several amino acid residues may be the critical region for binding of the peptide to the bacteria. Both peptides revealed a consensus motif of –Ser-X-Thr-X-Leu-, where X can be

any amino acid residues, indicates the probability that these amino acid residues contribute to the strong binding to the bacteria. It is likely that both peptides were targeted on the same epitope of the bacteria. In addition, diversity of the two peptide sequences suggested that not all the amino acid residues were involved in bacteria binding. However, other amino acid residues may serve for other functions. Hydrophobic amino acids are essential for non-polar interaction with the lipid bilayer hydrophobic core of bacterial plasma membrane to drive the biological activity of the peptide (Oren et al., 2002). Shai and Oren (2001) suggested that approximately 50% of hydrophobic amino acids are characteristics of antibacterial peptide. Therefore, the two peptides isolated in this study, with 33.33% and 41.67% of hydrophobicity, respectively, may be the potential antibacterial peptides. In addition, a relatively low peptide hydrophobicity may prevent peptide self association which hinders the peptide passing through the membrane (Chen et al., 2007).

Peptides GPVNKSSTILRM and GLHTSATNLYLH were compared with the peptides from other studies that showed inhibition effect against *P. aeruginosa* such as P-113_D (Sajjan et al., 2001), LL-37 (Deslouches et al., 2005), Pexiganan (Ge et al., 1999) and HBCPα-2 (Zhang et al., 2005). These peptides possess at least a glycine residue as shown in Table 5.1. Likewise, both peptides GPVNKSSTILRM and GLHTSATNLYLH also consist of a glycine residue at the peptide N-terminus. Glycine residue may be important for inducing α-helicity of antibacterial peptide (Lee et al., 2002). In addition, Lee et al. (2002) also suggested that incorporation of glycine at the N-terminus may protect the peptide from losing their helicity. According to Azad et al. (2011)

and Chen et al. (2005), helicity plays an important role in antibacterial activity. Hence, both peptides GPVNKSSTILRM and GLHTSATNLYLH with N-terminal glycine residues may be the potential antibacterial peptides against *P. aeruginosa.*

Table 5.1: Sequence of other antibacterial peptides from previous studies.

Antibacterial Peptide	Peptide Sequence
P-113_D[a]	AKRHHGYKRKFH
LL-37[b]	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
Pexiganan[c]	GIGKFLKKAKKFGKAFVKILKK
HBCPα-2[d]	KWKKFIKKIGIGAVLKVLTTGLPALKLTKK

[a] Sajjan et al., 2001, [b]Deslouches et al., 2005, [c]Ge et al., 1999, [d] Zhang et al., 2005.

5.7 Future Studies

In this study, alginate might appear as the major factor that resulted in the undetectable antibacterial activity of the selected phage-displayed peptides. Besides that, it may also greatly reduce the selection of phage-displayed peptides in the beginning step of biopanning. Therefore, in order to increase the number of possible clones that display peptides with high binding affinity and positive antibacterial activity, alginate-free bacteria suspension shall be used in the biopanning and microtiter broth assay. It can be prepared by separating and removing the alginate completely from the bacteria through several rounds of washing with TBS buffer solution. Alternatively, a freshly prepared bacteria suspension with no alginate production can be used. As the result, this may

increase the probability of selecting phage-displayed peptides with antibacterial activity towards *P. aeruginosa*.

In another aspect, two peptides with high binding selectivity from this study can be chemically synthesized as linear peptides. Synthetic peptides without phage particle may enhance diffusion of peptides across the alginate of the bacteria (Yin 2012). Consequently, peptides may be able to act on the bacterial surface membrane to exert antibacterial activity. On the other hand, synthetic peptides can be tested for blocking adherence of the bacteria to the host cell in cell culture. Similar to one of the mechanisms of human antibodies, binding of peptides to the bacteria covers up the bacterial surface adhesion molecules preventing it from adhering to host cell to cause infection. Therefore, this approach may complement the host immunity to prevent *P. aeruginosa* infection.

CHAPTER 6

CONCLUSION

In conclusion, 12-mer phage-displayed library is a convenient tool that can be used for screening potential peptides that have high binding selectivity as well as antibacterial activity. In this study, the selected phage-displayed peptides showed high binding selectivity towards *Pseudomonas aeruginosa*. However, none of them demonstrated antibacterial effect. Nevertheless, the two isolated peptides GPVNKSSTILRM and GLHTSATNLYLH which possessed highest binding selectivity towards *P. aeruginosa* may be beneficial to host defence system. The cationicity of the peptides is believed the essential feature for electrostatic interaction with bacterial outer surface membrane while hydrophobicity of the peptides allows non-polar interaction with the bacterial lipid bilayer membrane. In addition, the peptide analysis revealed a consensus sequence of –Ser-X-Thr-X-Leu- (X = any amino acid residues) which may be the crucial residues for the binding of the peptides towards *P. aeruginosa*.

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APPENDICES

Preparation of media

LB broth (Lennox) was prepared by dissolving 14 g of LB broth powder in 700 ml of distilled water. The medium was then autoclaved at 121 ℃ for 15 minutes.

LB agar (Lennox) was prepared by dissolving 6 g of LB agar powder in 300 ml of distilled water. The medium was then autoclaved at 121 ℃ for 15 minutes. The autoclaved medium was poured into petri dish and kept at 4 ℃.

LB agar with tetracycline was prepared by adding 300 µl of Tetracycline stock solution into 300 ml of autoclaved LB agar. The plates were kept at 4 ℃ in the dark.

LB agar with isopropyl-β-D-thiogalactoside (IPTG)/5-Bromo-4-chloro-3-indolyl-β-galactoside (Xgal) was prepared by 300 µl of IPTG/Xgal stock solution into 300 ml of autoclaved LB agar. The plates were kept at 4 ℃ in the dark.

Top agar was prepared by dissolving 2 g of LB broth and 0.35 g of agarose (1^{st} Base) in 50 ml of distilled water. The medium was then autoclaved at 121 ℃ for 15 minutes.

MacConkey agar (Oxoid) was prepared by dissolving 15.6 g of MacConkey agar powder in 300 ml of distilled water. The medium was then autoclaved at 121 ℃ for 15 minutes. The autoclaved medium was poured into petri dish and kept at 4 ℃.

Preparation of stock solution

Tetracycline stock solution was prepared by dissolving 0.2 g of Tetracycline Hydrochloride powder (Bio Basic) in 10 ml of 1:1 ethanol:water. The solution was filtered sterilized using 0.22 µm pore filter and was kept at -20 ℃ in dark.

IPTG/Xgal stock solution was prepared by dissolving 0.5 g of IPTG (Promega) and 0.4 g of Xgal (Bio Basic) in 10 ml of dimethyl formamide (DMF) (Lab Scan) solution. The solution was filtered sterilized using 0.22 µm pore filter and was kept at -20 ℃ in dark.

0.5 M of Tris-HCl pH 7.5 stock solution was prepared by dissolving 30.29 g of Tris (Bio Basic) in 500 ml of distilled water. The pH of the solution was adjusted with 37% of HCl to reach pH 7.5. The solution was then autoclaved at 121 ℃ for 15 minutes.

1.5 M of NaCl stock solution was prepared by dissolving 43.83 g of sodium chloride (Merck) in 500 ml of distilled water. The solution was then autoclaved at 121 ℃ for 15 minutes.

Preparation of buffer solution

TBS buffer solution was prepared by mixing 50 ml of 0.5 M of Tris-HCl pH 7.5 stock solution and 50 ml of 1.5 M of NaCl stock solution, and then topped up to 500 ml. The solution was then sent for autoclave.

TBST buffer solutions were prepared by mixing adding 40 µl [0.1% (v/v)], 120 µl [0.3% (v/v)], and 200 µl [0.5% (v/v)] of Tween 20 (Chemfur) in 40 ml of TBS buffer respectively.

Elution buffer solution was prepared by dissolving 0.15014 g of glycine (Fisher Scientific) in 10 ml of distilled water. The pH of the solution was adjusted with HCl to reach pH 2.2. The solution was then added with 10 mg 0.01 g of bovine serum albumin (Merck). The solution was filtered sterilized using 0.22 µm pore filter.

Neutralization buffer solution was prepared by dissolving 1.2114 g of Tris (Bio Basic) in 10 ml of distilled water. The pH of the solution was adjusted with NaOH to reach pH 9.1. The solution was then autoclaved at 121 ℃ for 15 minutes.

20% PEG/ 2.5 M NaCl was prepared by dissolving 60 g of PEG 8000 (BioBasic) and 43.83 g of NaCl (Merck) in 300 ml of distilled water. The solution was then autoclaved at 121 ℃ for 15 minutes.

Coating buffer solution was prepared by dissolving 0.8401 g of $NaHCO_3$ (Bendosen) in 100 ml of distilled water. The pH of the solution was adjusted with NaOH to reach pH 8.6. The solution was then autoclaved at 121 ℃ for 15 minutes.

Blocking buffer solution was prepared by dissolving 0.8401 g of $NaHCO_3$ (Bendosen) in 100 ml of distilled water. The pH of the solution was adjusted with NaOH to reach pH 8.6. The solution was then mixed with 0.5 g of BSA (Merck) and 0.02 g of NaN_3 (Sigma). The solution was filtered sterilized using 0.22 µm pore filter.

ABTS substrate solution was prepared by dissolving 1.4705g of in sodium citrate in 100 ml of distilled water. The pH of the solution was adjusted with HCl to reach pH 4.0. The solution was then mixed with 0.022g of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma). The solution was filtered sterilized using 0.22 µm pore filter.