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Design and Evaluation of Bacteriophage Production for Potential Commercial Use

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: *Date: September 2011*

For my parents, Sunan - Pasana Boonmee,

and my husband, Sutipon Promukson

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Abbreviations

Standard Units

% - percent

ºC - degree Celsius

µg/ml - microgram per millilitre

µl - microlitre

µm - micrometre

cells/ml - cells per millilitre

cfu - colony forming unit

cm - centimetre

cm/min - centimetre per minute

dm^3min^{-1} - cubic decimeter per minute

g - gram

g/L - gram per litre

g/ml - gram per millilitre

hr - hour

hr^{-1} - per hour

kb - kilobase

L - litre

litres/min - litre per minute

M - Molar

min - minute

ml - millilitre

ml/L - millilitre per litre

mm - millimetre

mM - millimolar

mM - millimole

mol L^{-1} - mole per litre

nm - nanometre

pfu - plaque forming unit

pfu/ml - plaque forming unit per millilitre

rpm - round per minute

DNA Bases

A - Adenine

C - Cytosine

G - Guanine

T - Thymine

U - Uracil

Textual abbreviations

ANOVA - Analysis of Variance

APS - Alternative Protein Source

APTS - aminopropyltriethoxy-silane

Asp-tRNA - aspartic acid transfer Ribonucleic acid

ATP - adenine triphosphate

bp - base pair

BSA - bovine serum albumin

BSE - Bovine Spongiform Encephalopathy

CHAP - cysteine, histidine-dependent amidohydrolases/peptidases

DMRT - Duncan's multiple range test

DNA - Deoxyribonucleic acid

dO_2 - dissolved oxygen

dsDNA - double stranded Deoxyribonucleic acid

dsRNA - double stranded Ribonucleic acid

ESBL - Extended-spectrum β-lactamase

FBP - Fructose biphosphate pathway

FDA - Food and Drug Administration

G + C content - guanine/cytocine content

GM - Glucose media

GMP - Good Manufacturing Practices

GPPW - glucose phosphate peptone water

GPW - glucose peptone water

ICTV - International Committee for Taxonomy of Viruses

IgG - Immunoglobulin G

LA - Luria Agar

LA - Luria Agar

LB - Luria broth

LB - Luria broth

M9 - minimal medium

Met-tRNA - methionine-tRNA

MMP - mechanically mixed phage

moi - multiplicity of infection

MOPS - (3-(N-Morpholino) propanesulfonic acid

mRNA - messenger ribonucleic acid

MRSA - methicillin-resistant *Staphylococcus aureus*

MSG - monosodium glutamate

NAG - β-1,4-*N*-acetylglucosamine

NAM - *N*-acetyl-muramic acid

NB - Nutrient broth

NBM - Nutrient broth media

NTA - the Nanoparticle Tracking Analysis

OD - optical density

OMP - original mixed phage

ORF - Open reading frames

PBP - penicillin-binding protein

PBS - Phosphate Buffer Saline

PCR - polymerase chain reaction

PCU - primary control unit

Phe-tRNA - phenylalanine transfer Ribonucleic acid

PHP - protein hydrolysate powder

pol - polymerase

PP - the pentose phosphate pathway

PSS - protein-synthesising system

RABIT - the Rapid Automated Bacterial Impedance Technique

RLU - relative light output (RLU)

RNA - ribonucleic acid

RNase - ribonuclease

rRNA - ribosomal Ribonucleic acid

SB - Super Broth

SCCmec - the Staphylococcal Cassette Chromosome *mec*

SD - standard deviation

SM - Storage Medium

ssDNA - single stranded Deoxyribonucleic acid

ssRNA - single stranded Ribonucleic acid

TA - teichoic acid

TCA - the tricarboxylic acid cycle

TH - Todd-Hewitt broth

tRNA - transfer Ribonucleic acid

VRE - vancomycin-resistant *Enterococcus faecium*

VRSA - vancomycin-resistant *Staphylococcus aureus*

vvm - volume of air per volume of culture per minute

WTA - wall teichoic acid

Equation Symbols

x - the concentration of bacteria cell

t - time

μ - the specific growth rate

x_o - the original concentration of bacteria cell

x_t - the concentration of bacteria cell after the time interval

e - the base of the natural logarithm

S - the concentration of limiting substrate

μ_{max} - the maximum specific growth rate

K_s - the substrate utilization constant

t_d - doubling time

ln - natural logarithm

D - dilution rate

F - flow rate

V - volume

τ - residence time

Abstract

The purpose of this research was to evaluate the production of bacteriophages in a commercial plant. Bacteriophage K, a broad host range bacteriophage which is active against a wide range of staphylococci including MRSA, was used as a typical bacteriophage. There has been increased interest in the potential use of bacteriophage in pharmaceutical, agricultural and other areas. There are several techniques used for bacteriophage growth on a small-scale, however, large scale production has not been examined.

In these studies, the production of bacteriophage K in *S. aureus* 8588 has been investigated. Medium optimization, the bacterial host growth conditions and infection and lysis conditions were investigated. The optimal condition for the growth of bacterial host were in a medium containing amino acids as carbon sources, in aerobic condition, at 37°C; these conditions were also the optimal conditions for infection and lysis. By feeding continuously bacterial host cells in their stationary phase into a bacteriophage lysate, bacteriophages were produced at all dilution rates (0.5-2.61 hr^{-1}) and remained in a steady state. Moreover, there was no wash out observed, even at the time exceeding the average bacteriophage growth period.

In addition, immobilization of the bacteriophage on nylon membrane by corona discharge was examined for use as a potential wound dressing. The result showed that the bacteriophages were immobilized on the nylon strip and remained active.

CHAPTER 1
INTRODUCTION

1.1 General Introduction

The growing problem of antibiotic resistance is increasingly leading to untreatable infections (Gould, 2008) whereas the number of new antimicrobials being developed has decreased (Alanis, 2005). The situation has provided strong motivation to discover new antibiotics and alternative therapies. Such an alternative is bacteriophage therapy (Marks & Sharp, 2000; Sulakvelidze, 2005; Taylor, Stapleton, & Luzio, 2002; Weld, 2000; Wilson & Salyers, 2002).

Bacteriophages have been advocated for the treatment and prevention of bacterial infection, which holds greater potential in clinical and environmental applications than in the past due to increased understanding of the difficulties (Levin & Bull, 2004). Re-appraisal and review of the potential of bacteriophages for the prophylaxis and therapy of bacterial infections has led to an increase in the literature (Abhilash, Vidya, & Jagadevi, 2009; Hanlon, 2007; Housby & Mann, 2009; Kutateladze & Adamia, 2010; Mattey & Spencer, 2008; Monk, Rees, Barrow, Hagens, & Harper, 2010; Skurnik, Pajunen, & Kiljunen, 2007; Sulakvelidze, Alavidze, & Morris, 2001). Moreover, many new companies involved in bacteriophage research or bacteriophage therapy have been established around the world (Housby & Mann, 2009; Kropinski, 2006; Monk et al., 2010; Thiel, 2004).

Borysowski & Gorski (2008) have reported that in the last decade there have been dramatic developments in bacteriophage therapy, which are motivating greater interesting in the West in using bacteriophages as an alternative to antibiotics for treatment bacterial infection, including multidrug resistance. This is because of the following reasons first, major advances in phage biology, genomics, and biotechnology have lead to the development of purified preparations of well-characterized and effective bacteriophages. Second, there have been many high-quality experimental studies to demonstrate the high efficacy of phage therapy of

antibiotic-resistant infections caused by strains of different clinically relevant bacterial species, including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*, and imipenem-resistant *P. aeruginosa*. Third, in 2005 the Centre for Phage Therapy was opened in Wroclaw, Poland. This is the first time since the 1950's that patients infected with a wide range of antibiotic-resistant bacteria have been able to receive treatment with bacteriophages in the European Union. Furthermore, the first placebo-controlled safety test was performed at the Nestle Research Centre, Switzerland, The preliminarily result confirmed no toxicity of bacteriophage preparation following oral administration. Finally, the approval of the use a bacteriophage preparation containing six different bacteriophages specific against *Listeria monocytogenes* by the US Food and Drug Administration (FDA) in 2006 for use as food additive.

Moreover, there have been many reports regarding the successful use of bacteriophage therapy e.g. in human for treatment of cancer and septicaemia (Weber-Dabrowska, Mulczyk, & Gorski, 2001, 2003); particularly in children for treatment of various bacterial infections such as diarrhoea, dysentery, respiratory infection, suppurative wound staphylococcal skin diseases and septicemia (Fortuna, Miedzybrodzki, Weber-Dabrowska, & Gorski, 2008); in humans for treatment of Staphylococcal infection including MRSA (Miedzybrodzki, Fortuna, Weber-Dabrowska, & Gorski, 2007); in humans to reduce nasal colonisation of *S. aureus* including MRSA infection and experiment study inducing *S. aureus* infection in animals (Mann, 2008); treatment of *Escherichia coli* infection in humans, animals and crops of agricultural importance (Brussow, 2005); treatment of *Escherichia coli* and *Salmonella* infection in chicken, calves and pigs, treatment of *Salmonella* and *Campylobacter jejuni* in chickens and *E. coli* O157:H7 in cattle (Johnson et al., 2008) and the review by Sulakvelidze, Alavidze, & Morris (2001) state the several effective studies on phage therapy in human performed in Poland and the former Soviet Union, where bacteriophage therapy has continued to be used for treatment in humans.

In all the studies reported bacteriophages were prepared on a relatively small scale whether on semi-solid medium or in liquid medium. However, large-scale production of bacteriophages is still limited.

Previous research has shown that lytic bacteriophages can be immobilised onto surfaces while retaining their biological activity (Mattey *et al.* 2010, personal communication). The immobilised bacteriophages were more stable than non-immobilised bacteriophages with respect to temperature, humidity and other environmental parameters and their activity is greatly prolonged. This will enable bacteriophage to be incorporated into products and materials with useful commercial shelf life. Bacteriophage active against MRSA and other pathogens have been isolated. However, large scale production has not been examined.

This research was concerned with three areas of the production of bacteriophages, the production of host bacteria including medium optimisation, the infection of host bacteria and the large-scale production of bacteriophages in continuous culture. In addition, the bacteriophage immobilization was examined, which was carried out by physical method.

1.1.1 Thesis objects

This research objects are as follows:

1) To investigate the optimal condition for production of the bacterial host, *Staphylococcus aureus* 8588 including medium optimisation.

2) To examine the procedures of bacterial host infection including factors effect on infection and lysis of bacteriophages.

3) To investigate the production of bacteriophage K in continuous culture.

4) To examine the way to immobilise bacteriophages on nylon membrane.

1.2 Bacteriophage

Bacteriophages or “phages” are prokaryote viruses which include viruses of eubacteria and archaea (Ackermann, 2007, 2009). They are the most numerous biological entities in the biosphere and are ubiquitous, particularly in the ocean (Brussow & Kutter, 2005; Wommack & Colwell, 2000). They were independently discovered by Frederick William Twort, a British doctor in 1915 and then in 1917 by Felix D. Herelle, a French-Canadian microbiologist who coined the term “bacteriophage” (Duckworth, 1976).

1.2.1 Classification and taxonomy

Since the discovery of bacteriophages, no universal method for classification has been devised (Ackermann, 2009). With the advent of the electron microscopes, differences in morphology have been used as a basis for classification. One of the most important studies was by Bradley in 1967, which is still used as the basis of the present bacteriophage classification system (Ackermann, 2003; Ackermann, 2009) (Figure 1), and is defined by morphology and the nature of the nucleic acid of bacteriophage. The six basic phage morphological types are tailed bacteriophages, filamentous bacteriophages, and cubic bacteriophages with dsDNA or ssDNA or dsRNA or ssRNA (Bradley, 1967). In the first report, the ICTV or International Committee for Taxonomy of Viruses classified bacteriophages into six genera corresponding to Bradley’s basic types (Ackermann, 2003). Recently, the ICTV taxonomical system has been based on the culture of bacteriophages and the measurement of physical parameters of the free virion (Rohwer & Edwards, 2002).

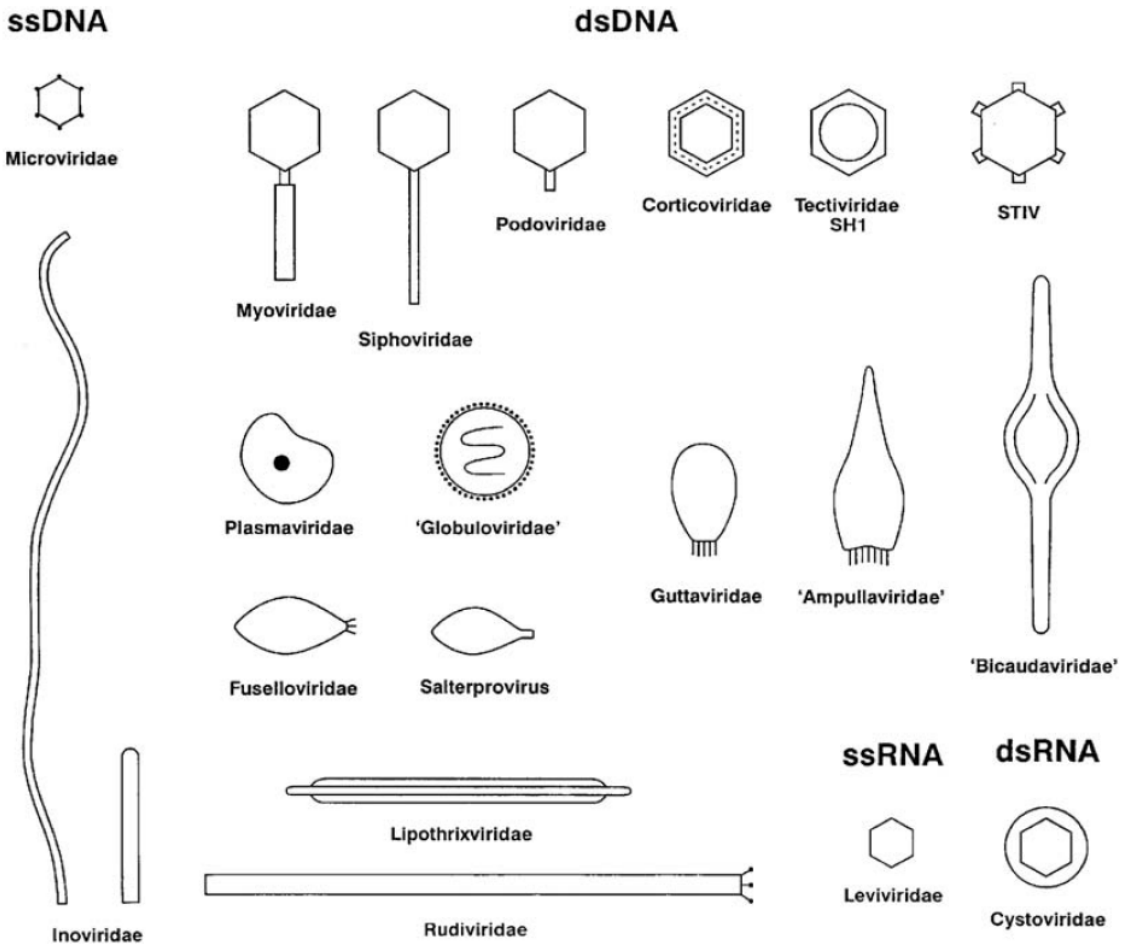


Figure 1 Schematic representation of major bacteriophage groups (Ackermann, 2009).

Taxonomic names of orders, families, and genera are basically constructed from Latin or Greek roots and end in *-virales*, *-viridae*, and *-virus*, respectively. Most genera of "cubic", filamentous, and pleomorphic phages have latinized names. To dates, tailed bacteriophage genera have vernacular names only (e.g., "T4-like viruses") (Ackermann, 2003).

Bacteriophages are classified by morphotype and host genus (Ackermann, 1996, 2001). To date, at least 5568 phages have been examined in the electron microscope since negative staining introduced in 1959. Bacteriophages infect at least 154 host

genera, mostly of the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. Most bacteriophages (96%) are tailed. Only 208 phages (4%) are polyhedral, filamentous, or pleomorphic (PFP). Bacteriophages belong to 17 families which include 14 officially accepted families and three "floating" groups. Tailed bacteriophages fall into three families, the *Myoviridae* (24.5%), *Siphoviridae* (61%), and *Podoviridae* (14%) which constitute the order *Caudovirales* (Ackermann, 2007, 2009).

The vast majority of bacteriophages contain double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) are a minority (Ackermann, 2007, 2009).

Tailed bacteriophages infect Eubacteria and Archaea and are the oldest known group of virus. They contain dsDNA and have icosahedral or elongated heads. Tails are helical, provided with fixation structures (baseplates, spikes, fibers) without an envelope. They adsorb to their hosts and infect them from the outside. The progeny are assembled via complex pathways, with phage DNA entering preformed capsids. They are extremely varied in size and structure, DNA content and composition, genome structure, proteins, antigenic and biological properties. They are virulent or temperate (see section 2.3.2). Tailed bacteriophages are divided into three families (Ackermann, 2009):

Myoviridae: Tails consist of a neck, a contractile sheath, and a central tube. *Myoviruses* are larger than other groups and include some of the largest and most highly evolved tailed phages (approximately 1300 known, 25% of tailed phages). Secondly, *Siphoviridae*: Tails are simple, non contractile, flexible or rigid tubes. *Siphoviruses* are the most abundant of tailed phages (over 3,200 known, 61%). Thirdly, *Podoviridae*: Tails are short and non contractile. *Podoviruses* may be more related to *Siphoviruses* than to *Myoviruses* (~750 known, 14.5%) (Ackermann, 2009).

Rohwer & Edwards (2002) presented the "Phage Proteomic Tree," which is based on the overall similarity of 105 completely sequenced genomes of bacteriophage. It was found that there is no single gene found in all phages that can be used as the basis for

a classification system. An alternative, a new taxonomic system based on the predicted phage proteome was present the “Phage Proteomic Tree”.

1.2.2 Life cycle bacteriophage

Bacteriophages are categorized into virulent (lytic) and temperate (lysogenic) bacteriophage based on the type of life cycle in its host. The two types of bacteriophage life cycle are the lytic life cycle and lysogenic life cycle, respectively.

Virulent bacteriophages lead to the lysis and death of the bacterial host cell when new phage progeny are released, whereas in the lysogenic cycle the bacteriophage genome is integrated into the genome of the host cell and will be replicated when the host genome replicates. Therefore, the daughter cells will inherit the bacteriophage genome (Guttman, Raya, & Kutter, 2005; Hanlon, 2007; Madigan & Martinko, 2006; Skurnik & Strauch, 2006).

Lytic bacteriophages are the only useful type for therapy. This is because they kill their target host cells rapidly, increase their numbers rapidly and transduction is rare. Transduction would result in high levels of horizontal gene transfer within the bacterial population. DNA sequencing of bacteriophage genomes is now used to confirm both identity and the absence of undesirable elements such as functional lysogenic components or bacterial toxins. Such toxins are known to be associated with some bacteriophages, for instance the Shiga toxins of *Escherichia coli*. (Monk et al., 2010).

The life cycle of a lytic bacteriophage is illustrated in Figure 2 (Guttman et al., 2005; Hanlon, 2007; Madigan & Martinko, 2006; Skurnik & Strauch, 2006); (1) Adsorption (attachment); the bacteriophage attaches to the bacterial host cell via a specific receptor on the bacterial cell wall which may be one of a variety of cell surface componentse.g. protein, oligosaccharide, teichoic acid, peptidoglycan or lipopolysaccharide (2) Penetration (injection); the bacteriophage genome is injected into the bacterium, while the capsid, which is a protein coat the virion, remains outside the host cell. In general, the tail tip has an enzymatic mechanism for

penetration of the peptidoglycan layer and then penetrates the inner membrane to release the DNA directly into the cell. (3) Expression of the bacteriophage early genes; transcription of bacteriophage genome, then producing early mRNA, particularly encoding proteins which shut off bacterial host cell metabolic machinery. In some case, the early proteins also degrade the bacterial host chromosome. (4) Expression of the bacteriophage late protein; the bacteriophage takes over the metabolic machinery of the bacterium. Late proteins are involved in the synthesis of new bacteriophage components and the lysis of the host cell. (5) Assembly; the bacteriophage components are assembled into the complete bacteriophage particles (6) Lysis; the bacterial cell lyses and releases the new bacteriophage progeny.

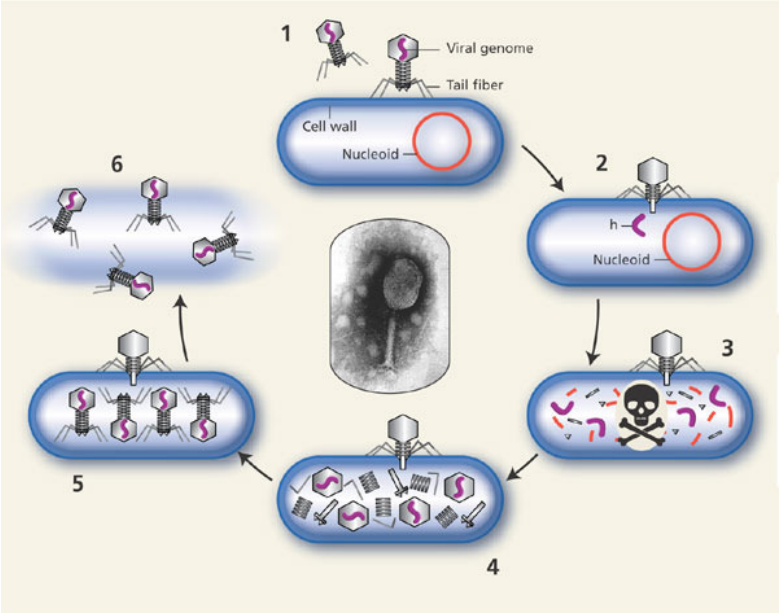


Figure 2 Bacteriophage life cycle; lytic cycle (Thiel, 2004)

Bacteriophage growth can be monitored on the laboratory with a growth curve. The growth curve of bacteriophage refers to *the one-step growth curve* illustrated in Figure 3. In the first few minutes after infection is the eclipse phase during this period the bacteriophage nucleic acid is separated from its protein coat. Maturation begins as the new bacteriophage appears within the host cell, as well as the virions increase dramatically. The eclipse and maturation periods are called the latent period. Latent period can be defined as the time between adsorption of the phage to the host cell and the lysis of the host cell with release of progeny bacteriophage, whereas the eclipse period is the delay time from adsorption until the first phage is completed inside the bacterium. At the end of maturation, mature bacteriophages are released, as a result of bacterial host cell lysis. The number of bacteriophage release per cell, called the burst size, varies with the particular bacteriophage and the particular bacterial host (Ellis & Delbruck, 1939; Madigan & Martinko, 2006; Moat, Foster, & Spector, 2002).

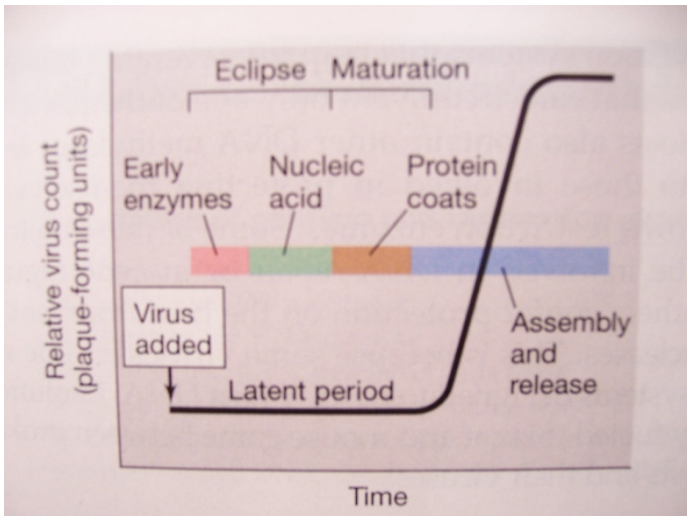


Figure 3 The one-step growth curve (Madigan & Martinko, 2006)

Double-stranded DNA bacteriophages of both Gram-positive and Gram-negative bacteria use a holin–endolysin system to achieve lysis of bacterial host cell. Two components, which are an endolysin (lysin) and holin, are essential for lysis of bacteriophage. First, an endolysin, which is a muralytic enzyme, can cleave one of the key bonds in the peptidoglycan layer. To degrade cell wall peptidoglycan, a lysin requires holins. Holins are small proteins which are accumulated in the cell membrane and control the lysis timing (Grundling, Manson, & Young, 2001) at the appropriate time to allow the lysin to reach the peptidoglycan layer and lysis of the bacterium (Wang, Smith, & Young, 2000; Young, Wang, & Roof, 2000).

1.2.3 Bacteriophage K

Phage K is a polyvalent Myoviridae bacteriophage which is active against a wide range of staphylococci (O'Flaherty et al., 2004). This phage is in serological group D, lyses both coagulase-positive and coagulase-negative (Rountree, 1949) and uses *N*-acetylglucosamine in cell wall teichoic acid for phage adsorption (Chatterjee, 1996). Phage K has a G + C content of 30.6% (Kwan, Liu, DuBow, Gros, & Pelletier, 2005).

1.2.3.1 Bacteriophage K morphology

The morphology and dimensions of bacteriophage K particles were examined by electron microscopy. This bacteriophage has an icosahedral head with a diameter approximately 70 nm. The tail of the bacteriophage was 210 nm; long and 15 nm; thin. The contractile tail terminated in a complex basal appendage. (Rees & Fry, 1981).

The bouyant densities of the bacteriophage K particle with DNA was 1.479 g/ml and 1.689 g/ml respectively. The DNA contour length was 16.1 μm and the molecular weight of it DNA was 33×10^6. There was no unusual bases such as hydroxyl-methylcytosine or hydroxymethyluracil. (Rees & Fry, 1981).

1.2.3.2 DNA metabolism in infected *S. aureus* of bacteriophage K

With *Staphylococcus aureus* NCTC 9318 as host, the latent period of this bacteriophage was 25 minutes, the eclipse period of 14 minutes and the average burst size of 60 pfu per bacterium. Infection with bacteriophage K resulted in immediately inhibition of host DNA synthesis and degradation of the bacterial DNA within the first 5 minutes of infection. The host DNA degradation products were used for the synthesis of bacteriophage DNA. There was no initial cessation of DNA synthesis in the infected bacteria (Rees & Fry, 1981). It was found that DNA replication of this bacteriophage occurred attached to the cytoplasmic membrane of the bacterial host (Rees & Fry, 1983)

1.2.3.3 Genome of Staphylococcal bacteriophage K

1.2.3.3.1 General features of the genome of bacteriophage K

The genome of bacteriophage K is a linear double-stranded DNA of 127,395 base pairs, which encodes 118 putative open reading frames (ORFs). The majority of ORFs (112) initiate translation with the AUG start codon. Bioinformatics analysis of ORFs found that the majority exhibited low identities with proteins from the database. The genome is able to be divided into two distinct regions, which are divergently transcribed. In this respect, of the 118 ORFs, 85 are transcribed in one orientation (right direction of transcription) and 33 are transcribed in the opposite orientation (left direction of transcription), with all of the latter grouped together in the first 30 kb (Figure 4) (O'Flaherty et al., 2004).

1.2.3.3.2 Bacteriophage K has its genes arranged in modules

The bacteriophage K genome is organized in a modular forms. Each module contains a set of genes which carry out a biological function encoding modules for lysis, structural region, DNA replication and transcription. The lysis module of bacteriophage K lines up separately from the structural region. Bacteriophage K lacks of intergenic regions between the structural and DNA replication and transcription modules (Figure 4) (O'Flaherty et al., 2004).

1.2.3.3.3 Lack of restriction sites for host-encoded endonucleases

Analysis of the bacteriophage genome exhibited no significant homology to any DNA methylases. However, the bacteriophage K genome completely lacks GATC sites. As a result, the bacteriophages are unable be restricted by Sau3A1, BamHI, PvuI, and DpnI (O'Flaherty et al., 2004). This is because *S. aureus* encodes a Sau3A1 restriction-modification system which recognizes the 5-GATC-3 DNA sequence (Sussenbach, Monfoort, Schiphof, & Stobberingh, 1976). Another site-specific endonuclease from *S. aureus*, which recognizes the sequence 5-GGNCC-3, was identified (Sussenbach, Steenbergh, Rost, vanLeeuwen, & vanEmbden, 1978). It also found that there was only one sequence 5-GGTCC-3 site in the bacteriophage K genome whereas none of the other possible recognition site combinations, which are 5-GGGCC-3, 5-GGACC-3, or 5-GGCCC-3, are present. Therefore, these reasons suggest that bacteriophage K has a very efficient mechanism of counter defence against these specific endonucleases (Figure 4) (O'Flaherty et al., 2004).

1.2.3.3.4 Promoters and tRNAs are located in an intergenic region

The 4.5-kb region between divergently translated ORFs 33 and 34, does not carry ORFs. However, this region is located in two putative promoters (Figure 4) (O'Flaherty et al., 2004).

In the intergenic region from bp 30,600 to 30,370 is located in three regions tRNAs which encodes Asp-tRNA, Phe-tRNA, and a pseudo-tRNA gene (Figure 4). A fourth

tRNA gene between ORFs 7 and ORF 8 (bp 7,222 to 7151), encodes Met-tRNA gene, is located in a non coding region (Figure 4). These tRNA genes are typically in large bacteriophages such as coliphage T4, vibriophage KVP40, and *Pseudomonas aeruginosa* phage phi KZ. These three lytic bacteriophage also has tRNA genes located in the intergenic regions (O'Flaherty et al., 2004).

1.2.3.3.5 Lysis module is located in the first divergently transcribed 30 kb

The lysis module (ORFs 30 to 33) is located at the end of the first 30 kb of the genome. The ORF 33 encodes a putative holin (167 amino acids). The bacteriophage K putative holin, which functions by generating pores in the cell membrane of bacteria, demonstrated 61% identity with a holin from bacteriophage Twort. The lysine, which is spliced products of ORFs 30 and 32, contains the CHAP domain, which is characterized by three conserved motifs. The putative *N*-acetylmuramoyl-L-alanine amidase domain is located in the centre of the protein as well as a second amidase domain is located in the N terminus (Figure 4; O'Flaherty et al., 2004).

1.2.3.3.6 Bacteriophage K may encode its own replisome and sigma factors

Bacteriophage K has most of the proteins required for its own replisome; for example, DNA ligase (ORF 21), primase (ORF 76), helicase (ORF 69 and 71), polymerase (spliced products of ORFs 86, 88, and 90), RNase H (ORF 24), and DNA binding proteins (ORFs 17 and 85) (Fig. 1 and Table 1). Further ORFs include those encoding two exonucleases (ORFs 72 and 74), an integration host factor (ORF 85), enzymes required for nucleotide metabolism (ORFs 79, 80, and 81), and a thioredoxin protein (ORF 83), which could function in post-translational modification or act as a chaperone (Figure 4). Indeed, of the 52 ORFs assigned a putative function, approximately one-third are involved in DNA replication, metabolism, and repair. The majority of these proteins exhibit homology to bacterial but not bacteriophage proteins. Therefore, bacteriophage K has an advantage in that it can potentially replicate its DNA without too much reliance on host functions. This

may suggest that bacteriophage K has evolved to a broader host range. Bacteriophage K is one of large bacteriophage genomes that encode so many DNA replication proteins (O'Flaherty et al., 2004).

When bacteriophages infect the host bacterial cells, they take control of many of the host proteins which are used to their advantage, one of these being RNA polymerase. Bacteriophage K carries a putative sigma-like factor ORF (ORF 94), which encodes a protein of 220 amino acids. None of the unknown proteins shared homology with the ORF 94 of bacteriophage K genome. Reverse transcription-PCR analysis indicated that this protein is expressed at the same levels 10, 20, and 30 min after bacteriophage infection. This sigma factor (ORF 94) could function to modify the host core RNA polymerase to recognize bacteriophage promoter regions, thereby regulating gene expression to express bacteriophage genes rather than host genes (O'Flaherty et al., 2004).

1.2.3.3.7 Introns with ORFs interrupting genes with crucial enzymatic functions

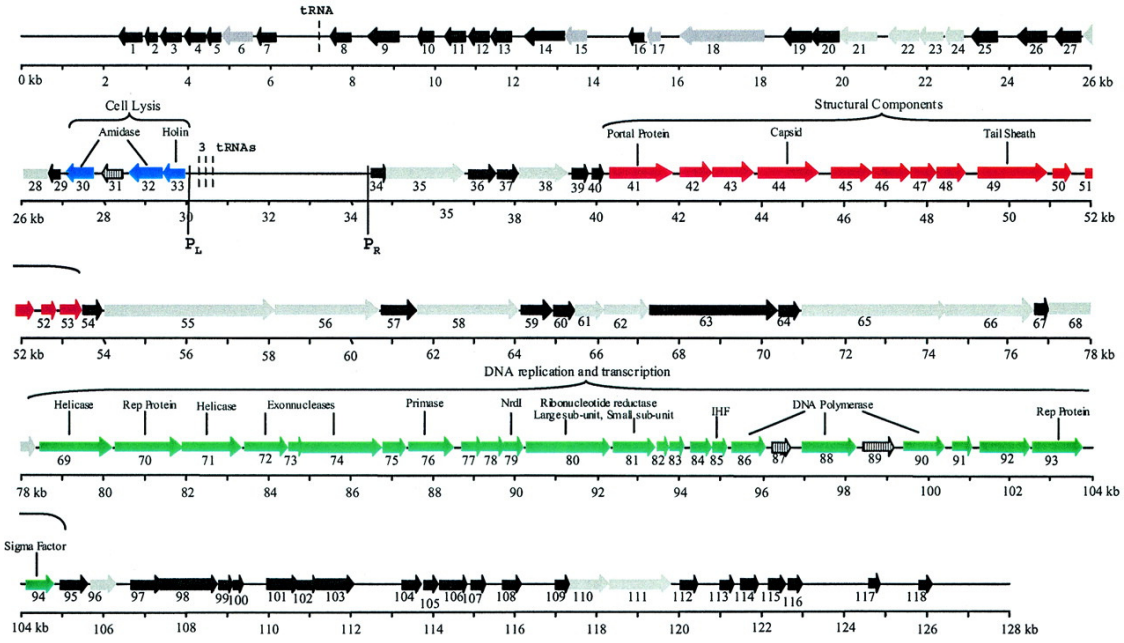
Analysis of the genome found that both the putative polymerase and lysin genetic determinants contained intron-like sequences. The polymerase gene contained two putative structures (*pol*-I2 and *pol*-I3) each encoding endonucleases; ORF 87 [I-*Ksa*II] and ORF 89 [I-*Ksa*III], respectively. In contrast, the lysin gene contained one intron-like sequence (*lys*-I1), which also encodes a endonuclease; ORF 31 [I-*Ksa*I] (Figure 4). Both I-*Ksa*I and I*Ksa*III show homology to HNH endonuclease and contain a HNH motif. (O'Flaherty et al., 2004).

1.2.3.3.8 Taxonomy and comparative genomics

Based on the current bacteriophage genome and proteome taxonomy, bacteriophage K does not classify within any defined group. This confirms that bacteriophage K is the member of a new taxonomic group. Bacteriophage K falls closest to the PZA-like *Podoviridae* (O'Flaherty et al., 2004) (Figure 5).

1.2.3.3.9 Overview of relationship to other bacteriophage: bacteriophage K and *Listeria* phage A511 have similar structural modules.

It was found that the structural modules of bacteriophage K similar to that of *Listeria* bacteriophage A511. This suggests that bacteriophage K and *Listeria* bacteriophage A511 are related and could constitute a new lineage of *Myoviridae* infecting low-G+C-content gram-positive bacteria. (O'Flaherty et al., 2004).



P_L 29,996 ACCGACCTACTGTTATATTATTGTTAGAAATAAATATAATAGAAA**GGTCGG**TTTTTTA**ATG** 29,935

P_R 34,448 AATTAACAAGAAAAAGTTAGAAGAAGAGGATACAAGAAAATATATAGCT**GATGGG**TTT**ATG** 34,509

Figure 4 ORF organization of phage K. ORFs 1 to 118 are indicated by arrows; Blue arrows, putative lysis module; red arrows, structural module; green arrows, DNA replication and transcription module; grey arrows, proteins with a putative function; black arrows, hypothetical proteins. Arrows with black vertical lines indicate three intron-carried ORFs. Arrows are roughly drawn to scale. Vertical lines mark two putative promoters. L and R, direction of transcription (left or right). Start codons and ribosome binding sites are indicated in boldface, and putative _10 and _35 sites are underlined. Dashed vertical lines represent the positions of four putative tRNAs (O'Flaherty et al., 2004).

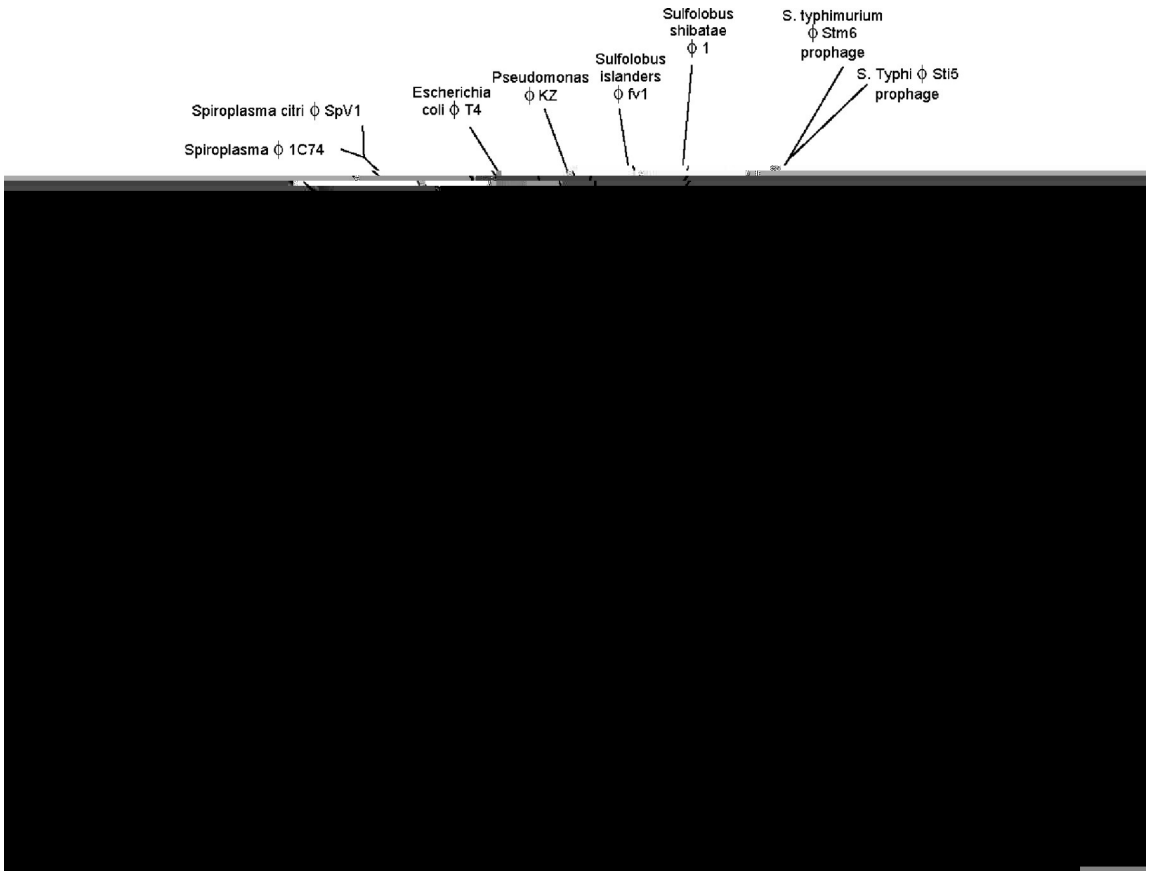


Figure 5 Section of the phage proteomic tree illustrating the relationship between phage K and other sequenced *Myoviridae*. The tree is based on 375 sequenced phage genomes and prophages. Only the section of the tree corresponding to phage K is shown for clarity. The tree joins the remainder of the phages at the dashed line. Phage K is weakly related to both the PZA-like *Podoviridae* and the *Borrelia burgdorferi* plasmid prophage, which form unique taxonomic groups (O'Flaherty et al., 2004).

1.2.4 Bacteriophage production

In general, there are two different methods for bacteriophage propagation: (1) in a liquid medium and (2) on the surface of a solid medium. Broth propagation is simpler method whereas when higher titres are required propagation on solid medium is preferable and it can be used for all bacteriophages (Blair & Williams, 1961; Wentworth, 1963).

There are several methods for propagation on solid medium; the soft-agar layer method, the freeze-and-thaw method, the cellophane method and a surface method. These different methods were compared at the Staphylococcus Reference Laboratory. As a result shown, the soft-agar layer method was the easiest to perform and the highest titre yield (Blair & Williams, 1961).

In all studies reported, preparation of bacteriophage whether in laboratory or clinical applications is carried out on semi-solid medium (double agar layer method) or in liquid medium in a small scale of production. Large-scale production would be required for commercial scale use of bacteriophages and liquid culture in fermenters would be the only practical method for such production of bacteriophages.

In general, the term “large-scale production” tends to have working volumes of 1,000 litres and more (Macauley-Patrick & Finn, 2008; Metzenberg, 2010). The term “pilot-scale fermenters” tend to have working volumes of 20, 100 and 1,000 litres (Burke, 2008) or 20-100L (Macauley-Patrick & Finn, 2008). The term “laboratory fermenters” tend to have working volumes of 500ml-5litres (Burke, 2008) or 1-2L (Macauley-Patrick & Finn, 2008). The term “shake flask scale” refers to production in volume 50-500 ml (Burke, 2008).

Furthermore, many publications have reported the use of bacteriophage cocktails, which contain several difference bacteriophages, for clinical applications (Leverentz et al., 2003; McVay, Velasquez, & Fralick, 2007; Merabishvili et al., 2009; Monk et al., 2010; O'Flynn, Coffey, Fitzgerald, & Ross, 2006; O'Flynn, Ross, Fitzgerald, & Coffey, 2004; Tanji et al., 2005; Y. Tanji et al., 2004; Yoichi et al., 2004). This is because using a cocktail of bacteriophages expands the host range and helps to

prevent the emergence of bacterial resistance to bacteriophage (Cairns & Payne, 2008; Kutateladze & Adamia, 2010; Skurnik et al., 2007). The production of such a cocktail of bacteriophages would need more than one fermenter.

The costs of fermentation processes fall into two categories, the capital costs of the plant and the operational costs such as medium, energy and staff salaries. The exact distribution of these costs depends on the scale of the process and the labour costs of the geographical locality. Clearly the larger fermenters used the more capital cost investment.

1.2.4.1 Method for large scale bacteriophage production

There are two methods for large-scale bacteriophage preparation; infection at low multiplicity and infection at high multiplicity (Sambrook & Russell, 2001).

For infection at low multiplicity, a large volume of medium is inoculated with bacteriophages at less than a 1:1 ratio. At the beginning, small numbers of bacterial cell are infected and the uninfected bacterial cells in the culture continue to divide for several hours. However, successive rounds of growth and infection bring about the production of increasing quantities of bacteriophages. Finally, all of the bacteria are infected and complete lysis of the culture occurs. This method requires care because small changes in the ratio of the bacterial cells to bacteriophage particles in the initial infection greatly affect the final yield of bacteriophage particles. Moreover, the optimum ratio varies for different strains of bacteriophage and bacteria. However, with little effort, the method can be adapted for use with most combinations of virus and host cells.

For the second method, high multiplicity, large quantities of bacteriophages are required for infection of the bacterial culture. During infection at high multiplicity, the majority of the bacterial culture is infected from the initial inoculation. Therefore, growth of bacteriophages is completed in a short period of time (usually 3-5 hours), rapidly leading to complete lysis of the bacterial culture. However, for the maximum

yield of any strain of bacteriophage it may be necessary to adjust the multiplicity of infection or the incubation length.

1.2.4.2 Study on bacteriophage production

Some reports exist about the production of bacteriophage either in shake flask culture [Blair & Williams (1961), Schade & Caroline (1943)], in batch culture [Sergeant & Yeo (1966), Siquet-Descans & Calberg-Bacq (1973), Grieco, Lee, Dunbar, MacGillivray & Curtis (2009)], in continuous culture; in chemostat [De Haan, Winkler & Felix (1955), Mizoguchi et al. (2003)] and in turbidostat [Schwienhorst, Lindemann & Eigen (1996)]. In addition, only literature by Bujanover (2004) reported on bacteriophage production for large scale commercial production on semi-solid medium used.

Blair & Williams (1961) reported technique for bacteriophage typing of staphylococci and also mentioned regarding to propagation of bacteriophage for typing. Propagation on solid medium was preferable; broth propagation can be used as an alternative method for all bacteriophages. Each bacteriophage type was propagated on a suitable single type strain of staphylococcus. The culture media used for bacteriophage propagation were trypticase soy broth/ agar and nutrient broth/agar. Incubating temperature of almost all cultures was at 37ºC a few cultures were incubated at 30ºC. The bacteriophage yield was a concentration between 5×10^{8} and 10^{10} particles per millilitre. However, technique was used for the propagation of Staphylococci bacteriophages, the optimal bacteriophage inoculums and time and temperature of incubation varied with each bacteriophage.

Schade & Caroline (1943) reported the production of polyvalent dysentery bacterio-phages. The propagation medium used was yeast-casein hydrolysate medium which supported bacteriophage production in high titres, equivalent to the result obtained in meat extract broth. The production of a mixture of six polyvalent dysentery bacteriophages was investigated in two different ways. The first way involved in the mechanical mixing of 5-litre batches of each of six bacteriophages. The preparation of the 6 bacteriophages were done individually in 9 litres of Pyrex serum storage

bottle containing 5 litres of the medium by inoculating the bacterial host suspension from an overnight slant culture and immediately seeding with bacteriophages. Culture was for 48 hr at 37ºC. At the end of this time, cultures were filtered separately. The filtrates were mechanically mixed by measuring equal quantities of the 6 bacteriophages into a large bottle, followed by re-filtering and storing at 4°C. This type of mixture was termed the mechanically mixed phage or MMP. Alternatively, a mixture of 7 bacteriophages was achieved by inoculating the growth medium with appropriate amount of the 7 bacterial strains and seeding it with their 7 respective bacteriophages. Five litres of the medium in 9 litres of Pyrex serum storage bottle was inoculated with a bacterial host suspension containing appropriate amount of each bacterial strain, immediately adding the mixed bacteriophage stock. This culture was then incubated for 48 hr at 37ºC before filtering and storing at 4°C. This type of mixture was termed the original mixed phage or OMP. The result of tests for polyvalency with all of the available stock bacterial cultures, 30 of 31 strains were lysed with MMP and the same result was observed with the OMP. These results suggested that the preparation of the OMP was a practicable one which warrants serious consideration for possible large scale polyvalent bacteriophage production.

Sergeant & Yeo (1966) studied the production of bacteriophage μ2 in three series of experiments, 3-L, 20-L and 150-L bacterial culture which were grown in stirred tank. The mainly culture medium used in this study contained 3.0% casein hydrolysate, 2.0% glycerol, 0.1% yeast extract, 0.5% KH_2PO_4 and 0.1% $MgSO_4.7H_2O$. The average bacterial cell densities were 71×10^8, 63×10^8 and 43×10^8 cfu/ml in 3-L, 20-L and 150-L bacterial culture, respectively. When infection with the number of bacteriophage particles per bacterium more than 1 the average yields of bacteriophages were 21×10^{12}, 16.8×10^{12} and 12.6×10^{12} pfu/ml of culture lysates in 3-L, 20-L and 150-L, respectively.

In 3-L experiments to determine the influence of bacterial cell density at infection on the bacteriophage yield, the carbon dioxide concentration was used as a guide to the viable bacteria count, and a series of experiments was carried out to investigate the optimum stage at which bacteriophages should be introduced into the growing bacterial cell culture for the best yield of bacteriophages. The result shows that the

lysates having higher titres were obtained from denser bacterial cultures, but a dramatic drop in yield was observed if infection was delayed until the bacterial cell density exceeded 100×10^8 cfu/ml. This indicated that for the best results, the infection of bacterial culture should be timed that carbon dioxide evolution rate never reached its potential maximum level. Therefore, in production work in 3-L, 20-L and 150-L culture, bacteriophages were introduced into growing bacterial cultures as late as possible.

In 3-L experiments to determine the influence of multiplicity of infection (moi) on bacteriophage yield, it was found that variation of moi on bacteriophage infection between 1.8 and 410, there was no systematic variation in bacteriophage yield.

The 20-L culture were grown in a vessel which had a lower capacity to dissolve oxygen than the 3-L vessel and the 150-L culture had an even lower capacity to dissolve oxygen than the 20-L vessel. The 20-L and 150-L culture gave culture lysate containing, on average, fewer bacteriophage particles per ml than the 3-L culture. The decline was in proportion to the decline in the number of bacterial cells infected with bacteriophages. This showed that higher yields of bacteriophages per millilitre obtained by infecting bacterial cultures of higher cell density although yields per bacterial cell were similar. Moreover, the aeration capacity of the culture vessel used was the major factor influencing the bacterial, and hence bacteriophages, yields.

In conclusion, it was found that aeration of the bacterial host culture and timing of the addition of bacteriophages was critical to obtaining maximum yields.

Siquet-Descans & Calberg-Bacq (1973) studied on large-scale production of bacterio-phage Φx174 in stirred aerated fermenters of 20 and 200 litre. Fermenters were inoculated with an 18 hr culture of *E. Coli* C (5 ml/L) and were operated at 37ºC with vigorous aeration (3 or 50 litres/min) and stirring (120 rpm). Three hour after inoculation (5×10^8 bacteria/ml), infection of bacteriopghage Φx174 was performed with $2\text{-}3\times10^7$ pfu/ml. Four hour after infection, the culture was lysed and the bacteriophage concentration was 2.36×10^{11} pfu/ml and 7.4×10^{10} pfu/ml in the 20 litre fermenter (10 litre working volume) and the 200 litre fermenter (100 litre working volume), respectively. In some experiments for bacteriophage production in

a 20 litre fermenter, the aeration was increased from 3-10 litres/min. The production was not be improved by the increased aeration. However, these experiments showed that it was possible to obtain good production of in volumes as high as 100 litres in fermenters but required only 1/10 to 1/100 the multiplicity of infection.

Grieco, Lee, Dunbar, MacGillivray, & Curtis (2009) studied on a production of filamentous bacteriophage M13. The *E. coli* strain K91 was used as the bacterial host strain. The fermentations were performed in 1 litre of medium using a 3 litre computer-controlled fermenter. By measuring bacteriophage yields and bacterial growth while changing the growth medium, pH and dissolved oxygen concentration; four different fermentation conditions were assembled (1) NZY medium as control, which NZY broth was prepared as follows: 10 g NZ-Amine, 5 g yeast extract, 5 g NaCl in l L distilled H2O, and the pH was adjusted to 7.4, (2) NZY medium with pH maintained at 7.4, (3) NZY medium with pH maintained at 7.4 and decreased dO_2 level to 20%, and (4) Super broth (SB) medium with pH controlled at 7.4; SB was prepared as follows: 10 g MOPS (3(N-Morpholino) propanesulfonic acid, 30 g tryptone, 20 g yeast extract in 1 L distilled water, and the pH adjusted to 7.4. It was found that the optimal conditions for bacteriophage yield were NZY medium with pH maintained at 7.4, the dO_2 held at 100% and agitation at 800 rpm. These computer-controlled fermentations resulted in a minimum of a tenfold higher bacteriophage production compared to standard shake flask conditions.

De Haan, Winkler, & Felix (1955) studied the relationship between growth rate of bacterial host and growth rate (latent period) of the bacteriophage in continuous culture. *E. coli* strain B and bacteriophages T_1 to T_7 were used in this study. A simple medium consisting of glucose, ammonium chloride and salts buffered with phosphate was used. Bacteriophage propagation was studied under 2 different flow rates; at generation time of 50 and 150 minutes, and under 2 different glucose concentrations; in with glucose in excess (20 g/L) and in with glucose as a limiting factor (0.1 g/L). With glucose in excess the number of bacteria in the vessel was constant at 4×10^8/ml for a generation time of 50 minutes and at 8×10^8 /ml for a generation time of 150 minutes whereas in with limiting glucose medium for the generation time of 50 and 150 minutes the number of bacteria was much lower (1.2×10^7 /ml). Bacteriophage

was added after the bacterial host culture reached a steady state. The result showed that in the glucose in excess medium, increase of bacterial generation time resulted in an increase of the latent period for bacteriophages T_4 T_6 T_7 and to a minor extent for T_2. The latent periods of T_1, T_3 and T_5 were much less affected. In a glucose-limited culture the increase of the bacterial generation time from 50 to 150 minutes had the same effects. Comparison of the curves for the same generation times in the media showed that the propagation of bacteriophages T_2, T_4, T_6 and T_7 is slower in the glucose limited medium than in the medium with glucose in excess. This means that the longest latent periods were found in the medium with small amounts of glucose. The bacteriophages T_1, T_3 and T_5, which were synthesized mainly from substances already present in the bacterial host cells and only to a small extent from constituents of the medium, were much less affected by the slowing down of bacterial metabolism and the reduced energy- and carbon-supply. In conclusion, the propagation rate (as measured by the latent period) of bacteriophages T_2, T_4, T_6 and T_7 decreased with longer bacterial generation times and with reduced energy- and carbon-supply. In contrast the production of bacteriophages T_1, T_3 and T_5 (which were mainly synthesized from the contents of the host cell) was unaffected by the slowing down of bacterial metabolism.

Mizoguchi et al. (2003) studied on the interaction between *Escherichia coli* O157:H7 and its specific bacteriophage PP01 in batch culture and in chemostat continuous culture using LB broth. In batch culture, an overnight culture of bacterial host (1%) was inoculated into 30 ml of fresh LB broth. When the culture reaching an OD_{600nm} of 0.1, bacteriophage infection was performed with an moi of 2. The culture turbidity of bacterial host decreased after the addition of bacteriophage and the bacteriophage concentration increased relatively. In chemostat continuous culture, two different dilution rates (D); 0.327 and 0.876 hr^{-1}, were investigated. The culture volume was 30 ml. The bacteriophage PPO1 was added into the culture with an moi of 0.01 when establishing a steady-state condition. The results showed that the concentration of bacterial host cell and bacteriophage dropped and oscillated. The oscillation amplitude at D of 0.876 hr^{-1} was larger than that of the culture at 0.327 hr^{-1}. Eventually, the oscillation in the bacteriophage concentration synchronised with the

oscillation in the bacterial host cell concentration. At equilibrium condition, the bacteriophage concentration at D of 0.327 hr^{-1} was higher than that of 0.876 hr^{-1}.

Schwienhorst, Lindemann, & Eigen (1996) studied on production of lytic coliphage Qβ in continuous culture using the "cellstat" apparatus. The cellstat consists of a turbidostat serving an exponentially growing host cells and a number of stirred flow reactors connected to the turbidostat outlet. The bacterial host cells, passing in a continuous stream through the reactor, are used to propagate bacteriophages. The flow rate of bacterial host cells was adjusted to a value that keeps the mean residence time of bacteria in the reactor. Turbidostat fermentation medium used contained Turbidostat fermentation medium contained 100 mM Tris-HCl, pH 7.4, 27 mM KCl, 38 mM NH4Cl, 1 mM K_2HP0_4, 2 mM trisodium citrate, 2.5 mM $MgS0_4$, 1 mg/L $FeCl_2$.4 H_20, 3 g/L glycerol, 1 g/L casamino acids, and 2 ml/L vitamin solution. The culture volume of bacterial host cells within the infection reactor was kept constant at 20 ml. The dilution rate of 7.48 hr^{-1} was chosen to compensate for the bacteriophage growth rate. As a resulted shown, the oscillation amplitude of the bacteriophage concentration was observed. The numbers of bacteriophage produced fluctuated between 10^6- 10^7 pfu/ml. Moreover, total washout of bacteriophage particles was not observed, not even at dilution rates exceeding the growth rate of bacteriophage (D=10.14 hr^{-1}).

Bujanover (2009) reported a method for intermediate to large scale commercial bacteriophage production for the use of bacteriophage to treat infectious diseases. The method reduced the production volume and elevated production yields. The bacteriophage production was carried out in a semi-solid medium by adapting technique used on a laboratory scale. It was found that the titer obtainable and the ease of recovery of bacteriophage from media was enhanced if the semi-solid medium comprised less than 0.5% hydrocolloid, preferably less than 0.3% hydrocolloid. The bacteriophage yields obtained from the method were 10^{15}-10^{16} bacteriophages per one litre of semi-solid culture medium, typically extracted in a volume of 10-50 litres.

The procedure for bacteriophage preparation was: Tray preparation; spread 250 ml of rich medium at 1.5% agar on 60×60×5 cm plastic trays. Preparation of semi-solid

composition: 60 ml of rich medium at 0.27% agar was added with 10 isolated bacteriophage plaques and 10^9 to 10^{10} cfu of the matching bacterial strain. Phage growth: pour 60 ml of the semi-solid culture medium over the solid phase on each tray, then all trays (25 trays) were incubated in an industrial incubator at 37ºC for 16-18 hr. Collecting phage lysate: the semi-solid composition from each tray was scraped and mixed with 200 ml of rich medium before transferring into new tube. The slurry (about 300 ml) was mixed vigorously for 30 second and then was centrifuged at 11G for 30 min at 4ºC. The supernatant comprising the crude bacteriophage extract was collected and the pellet was subjected to multiple serial extractions. The total volume (from 25 trays) of the crude phage extract was about 55 litres at a titer of 10^{12} pfu.

Merabishvili et al. (2009) described quality control of a well-defined bacteriophage cocktail production for use in human clinical trials. The production of bacteriophage cocktail consisting of exclusively lytic bacteriophages, designed for the treatment of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections in burn wound patients, was carried out in small-scale/laboratory-based production by using the double-agar overlay method. Quality control included stability (shelf life), determination of pyrogenicity, sterility and cytotoxicity, confirmation of the absence of temperate bacteriophages and transmission electron microscopy-based confirmation of the presence of the expected virion morphologic particles as well as of their specific interaction with the target bacteria. Bacteriophage genome and proteome analysis confirmed the lytic nature of the bacteriophages, the absence of toxin-coding genes. This bacteriophage cocktail, consisting of *P. aeruginosa* phages 14/1 (Myoviridae) and PNM (Podoviridae) and *S. aureus* phage ISP (Myoviridae) was produced and purified to remove endotoxin. The bacteriophage cocktail is currently being evaluated in a pilot clinical study by a leading Medical Ethical Committee. According to the current regulatory framework, bacteriophages are not approved because of the lack of clinical trials. This development of a well-characterized bacteriophage preparation using GMP (Good Manufacturing Practices)-like procedures was warranted for scientific and medico-legal reasons. Moreover, as a bacterial growth medium certified to be free of animal proteins then

soy hydrolysate and yeast extract used as components of the Alternative Protein Source (APS) LB broth base used for bacterial growth and bacteriophage production.

1.2.4.3 Medium for bacteriophage production

A series of papers during the 1940s by Schade & Caroline (1943, 1944a, 1944b) reported the production of polyvalent dysentery bacteriophages. They suggested the choice of medium using for large-scale production. These following factors should be taken into consideration; (1) stability of the bacterial host growth, (2) enhancement the production of bacteriophage, (3) stability of the production of bacteriophage, (4) proposed method of administration of phage therapy, (5) ease of preparation, (6) cost. The result showed the stability of the yeast-casein hydrolysate medium supported for the bacteriophage propagation as well as the bacteriophage produced in high titres equivalent to the result obtained in meat extract broth.

1.2.4.4 Factors influence on bacteriophage production

Bacteriophage infection process is dependent on not only the physicochemical characteristics of bacteriophage genome-encoded functions but also on intracellular resources of the bacterial host. The intracellular resources of the host depend further on the physiological state of bacterial cell which is regulated by for example growth media and temperature (You, Suthers, & Yin (2002)). There have been reports on various factors with regard to; growth phase of bacterial host cell when infection (Sillankorva et al., (2004)), growth media (Rountree (1949); Williams & Rippon (1952); Gross, (1954); Hadas et al., (1997); Sillankorva et al. (2004) and temperature (Ellis & Delbruck (1939); Sillankorva et al. (2004); Muller-Merbach, et al., (2007). In addition, moi is another factor having an effect on bacteriophage infection (Petty et al., (2006). These studies shown that the faster the bacterial host cells grow at the time of bacteriophage infection, the faster the bacteriophage will be produced. This result corresponded to a shorter latent time, faster bacteriophage production, and larger burst size. These studies are examined in detail below.

1.2.4.4.1 Physiological state of bacterial host

1.2.4.4.1.1 Growth phase of bacterial host

Sillankorva et al., (2004) found that the physiological state of bacterial host had an effect on cell lysis. The results showed that the infection of bacterial cells during the exponential growth phase was more effective than that of cells in the stationary or decline stages, as greater rates of cell lysis, greater numbers of bacteriophages produced and the lowest latent period were found. These result showed that bacterial cells in the exponential phase had better potential than cell in stationary and decline phases for large scale culture. Further investigation in terms of the outer membrane profiles showed that cells in the exponential, stationary and decline stages had a similar composition of cell wall proteins. The only exception was a difference in the concentration of the proteins. They concluded that the decline in the efficiency of bacteriophage infection in stationary and decline phases was likely to be due to host quality rather than to the presence or absence of cell wall receptors.

1.2.4.4.1.2 Growth media

Rountree (1949) investigated the propagation of staphylococcal bacteriophages using a buffered glucose broth which was modified from Todd-Hewitt (1932). This found that some of the serological group A bacteriophages (3A, 3B, 3C and 51) multiplied in this medium, others required the addition of tryptophan. The propagation of bacteriophage group B and C could only be achieved in broth enriched with certain factors from the vitamin B complex. There were no explanations as to why these additional substances were required for propagation in broth but not on agar. It was also found that bacteriophage K (serological group D) and bacteriophage W (serological group E) multiplied in Todd & Hewitt broth whereas there was no multiplication of bacteriophages serological group F in this medium.

Williams & Rippon (1952) reported the propagation in liquid culture of staphylococcal bacteriophages (serological group A and B) used for bacteriophage typing. The experimental basis uses the culturing of the appropriate Staphylococci after dilution for 2-3 hr and then inoculated with bacteriophages, added to the culture at 10^3-10^4 particles per ml. The cultures were incubated at 37ºC for 6-9 hr. The media used was; Nutrient broth (NB), modified Todd-Hewitt broth (TH), 0.2% glucose peptone water (GPW) made with 1% Evans peptone, and glucose phosphate peptone water (GPPW) (0.2% glucose, 0.5% K_2HPO_4); to these were added when indicated DL-tryptophan (usually at 0.008 mg/ml) and 1%Yeastrel sterilized by Seitz filtration. It was found that all group A bacteriophages multiplied in one or more of these media, giving final titres at least as high as with agar propagation. The group B bacteriophages multiplied well in GPW but irregularly in NB. Addition of tryptophan had little effect in any of the three media. The addition of Yeastrel was examined only on the group B bacteriophages. It improved the propagation some of them (31, 42C and 52A) in TH broth but had no effect in NB or GPW.

Gross (1954) studied the production of coliphage T2 in *Escherichia coli* strain K12 and found that addition of yeast extract in minimal medium (M9) supported bacteriophage production as well as nutrient broth, even if bacterial host cells were washed or starved, they still remained effective for bacteriophage production and no difference was seen to that of cell without washing or starving. Moreover, addition of enzymatic casein hydrolysate or acid casein hydrolysate plus tryptophan in M9 medium showed similar results to the addition of yeast extract in M9 medium. Furthermore, addition the mixture of amino acids (in L-amino acid form) in M9 medium supported the bacteriophage production.

Hadas et al. (1997) studied factors influencing the adsorption and growth of bacteriophage T4 in *Escherichia coli* B/r. These results found that all parameters changed with the growth rate (μ) of the bacteria which was modified by altering the composition of the media. Adsorption rate was faster at higher growth rates. This was because of increase cell size and hence the total cellular surface area which resulted in the presence of more receptors on the cell envelopes. Bacteriophage development and lysis also depended on the growth rate. Faster bacteriophage

development and lysis was observed in the higher bacterial host growth rates. Due to the use of different carbon sources to change the growth rate, cell size and macromolecular composition of bacterial host cells resulted in larger bacterial host cells which grew faster because of a higher proportion of their mass included the protein-synthesising system (PSS). The burst size increased, whereas the eclipse and latent periods decreased with increasing of growth rate. Similar results were obtained in the study of Rabinovitch et al. (2002).

Sillankorva et al. (2004) studied two media (Nutrient broth media; NBM and Glucose media; GM) widely used for bacterial host growth and bacteriophage infection of *P. fluorescens*, the best medium for growing host bacteria, infection and lysis was NBM. Further investigation on the outer membrane protein profiles of cells grown in both media showed proteins in NBM which were absent in the cells grown in GM. It was suggested that these proteins were cell wall receptors for bacteriophage in the outer membrane of cells and this accounted for the superiority of NBM.

1.2.4.4.1.3 Temperature

Ellis & Delbruck (1939) found that the latent period of bacteriophage infection varied with temperature in the same way as did the division period of the bacteria. As a result, increasing temperature (between 16.5, 25 and 37ºC) resulted in a decreasing latent period. The results also found that the average burst size was independent of the temperature as well as independent of latent period.

Sillankorva et al. (2004) found that the optimal temperature of *P. fluorescens* growth (also the highest growth rate) is the best temperature for bacteriophage infection as well as the lowest latent period and highest rates of cells lysis and phage release. Similar results were found by Muller-Merbach et al. (2007).

1.2.4.4.2 Multiplicity of infection (moi)

Petty et al., (2006) isolated and characterized bacteriophage ΦIF3 which was specific to *Serratia marcescens* strain Db11. Infection at different moi showed that lysis occurred more quickly with a higher moi. Moreover, it was found that infection of Db11 earlier in exponential phase also had the same result; increasing moi caused quicker lysis.

Ellis & Delbruck (1939) reported that the first step in the growth of bacteriophage is adsorption which is defined as the attachment of phage to specific receptors on bacterial cell wall. Bacteriophage cannot multiply unless attached to the bacterial host. Therefore, the rate of attachment under certain conditions may limit the rate of bacteriophage growth. The adsorption rate was found to be first order with respect to the bacteriophage concentration and first order with respect to the bacterial host concentration. Moreover, it was found that for bacterial concentrations greater than 3 x 10^7cfu/ml adsorption was very fast; 70-90% attachment in 10 min. This means that adsorption under proper condition is so rapid and complete.

Furthermore, Delbruck (1940a, 1940b) reported that adsorption rate of bacteriophages depended on the physiological state of the bacterium which may vary with their culture conditions or the growth phase of the bacterial host. The result was found that adsorption rate were much greater in actively growing bacteria than in the non growing bacteria (an overnight culture). This observation was attributed to two possible reasons. First, the size of bacteria changed considerably depending on the growth phase in a given culture medium, with higher growth rates of bacteria which were correlated with increased cell surface area, and an increased cell surface may lead to an increased in adsorption rate on to a given number of bacteria. Second, motile bacteria, like *B. coli*, in actively growing bacteria move rapidly, increasing the chance of bumping into bacteriophage particle. Therefore, the adsorption will be faster when bacteria move. This resulted in faster bacterial host growth, given the better growth of bacteriophage or shorter latent period. Furthermore, under optimum conditions (the bacteria used in logarithmic growth phase and aeration), bacteriophage adsorption at higher bacterial concentration was faster than at lower bacterial concentration which corresponded with the study by Shao & Wang (2008).

Shao & Wang (2008) also conclude that host density had an influence on adsorption process; an environment with high host density, adsorption rate would be high, and vice versa. Bacteriophages with a higher adsorption rate would have a shorter lysis time, and vice versa.

In addition, Wang et al. (1996) studied bacteriophage lysis timing; two factors examined; (1) the host quantity (the density of available hosts), which implied the average time for a bacteriophage to find and infect an uninfected host, and (2) the host quality (the physiological state of the host infected), which influenced the rate by which the bacteriophage progeny were assembled/matured. It was concluded that the both host quantity and host quality had effect on bacteriophage lysis time which strongly influenced the latent period. In conditions of high bacterial host density, it selected for short latent period as well as in the condition of presence a good bacterial host quality also supported a short latent period whereas when bacterial host density is low, the density of bacterial host is more important for determination of the latent period length. In contrast, when bacterial host density is high, the quality of bacterial host is more important for determination of the latent period length. The studies of Abedon (1989) and Abedon et al. (2001); (2003) also supported the study of Wang et al. (1996). Moreover, You et al. (2002) and Fischer, Yoichi, Unno, & Tanji (2004) also found that the faster the bacterial host grows at the time of bacteriophage infection, the faster the bacteriophage will grow. This corresponds to a shorter latent time, faster progeny production rate, and larger burst size.

1.2.5 Bacteriophage Immobilisation

Bacteriophages have been widely used in several application areas (Mattey & Wilkinson (2006); Petty (2006); Schmelcher & Loessner (2008)) for example; detection of bacterial pathogens, bacteriophage display, bacteriophage-based biosorbent (Bennett et al. (1997); Sun, Brovko & Griffiths (2001); Minikh, Tolba, Brovko & Griffiths (2010)) including treatment of antibiotic resistance particularly MRSA (Scott & Mattey (2003)).

Immobilisation of bacteriophages is one of methods used as a technique for such applications. Several methods of immobilisation developed for protein immobilisation have been reviewed by Nakanishi et al. (2008) for instance, physical adsorption, immobilisation on Au surface, streptavidin-mediated immobilization. There have been several reports regarding immobilization techniques on bacteriophage for various applications.

Bennett et al. (1997) studied a method to separate and concentrate *Salmonella* from food materials by using a biosorbent consisting of *Salmonella*-specific bacteriophages passively immobilized on polystyrene surfaces. The method involved the "Sapphire" lytic bacteriophage immobilised onto two different polystyrene surfaces; microplates and dipsticks, by soaking the surfaces with bacteriophage suspensions at a minimum concentration of bacteriophage of $5x10^{10}$ pfu per milliliter, followed by washing in order to remove unbound bacteriophages, and blocking of the remaining adsorption sites with 1% bovine serum albumin (BSA). After incubating the resulting biosorbents with *Salmonella* in mixed cultures of bacteria, the ability of biosorbent for separation *Salmonella* from suspension in the presence of competing Enterobacteriaceae at the same concentration as the target bacteria was assessed either by PCR or by epifluorescence microscopy using acridine orange as dye for labeling the bacterial nucleic acids. The results showed that nine out of eleven *Salmonella* strains gave positive signals, after running PCR the products obtained were analysed by electrophoresis and the bands corresponding to the amplified region of the *Salmonella* genome were observed. The detection limit for this method was at a concentration of 10^5 cfu per milliliter was required to generate a positive signal of PCR detection step and a concentration of 10^7 cfu/ml was

necessary in the initial culture to ensure on quantity of cells captured by the biosorbent. This indicated that efficiency of capture was clearly not sufficient. This was because the orientation of the bacteriophage on the solid phase. Bacteriophages were passively adsorbed to the solid phases which could be immobilised via both head and tail groups. For maximum efficiency, bacteriophages must immobilise via head groups leaving tails free to capture the target to their receptors. They also suggested that a possible solution would be to immobilise actively the head groups of the bacteriophage.

Sun et al. (2001) improved the method of separation and concentration of *Salmonella* from food materials by a specific immobilization of bacteriophages, exploiting the high affinity of biotin to streptavidin to capture the bioluminescence of bacterial *S. enteritidis* cells. In this study, *Salmonella* bacteriophage SJ2 was biotinylated with sulfosuccinimidobiotin, which reacts with primary amines of the bacteriophage head proteins. This resulted in biotinylation of the bacteriophage particles. Following this, biotinylated bacteriophages were coated onto streptavidin-labeled magnetic beads. This bacteriophage-based biosorbent was applied to capture target bacterial cells of *S. enteritidis* and used magnetic beads coated with non biotinylated bacteriophage as a control. The capture efficiency of *S. enteritidis* by the biosorbent was assessed by comparing the relative light output (RLU) of the captured cells to the correlation between RLU and colony forming units of the bioluminescent strain which was also observed to be a linear relationship. As the result, the cells captured by the biosorbent were from 5.4% to 19.3% when an initial culture of 2×10^8cfu/ml decreased to 2×10^6cfu/ml. This showed a significant improvement compared to the passive immobilization method (Bennett et al. (1997)), which represented the capture efficiency less than 1%. However, this system could capture at low numbers of *Salmonella enteritidis* cells in foods. This may be because of the orientation and/or the inactivation of the bacteriophage.

Scott & Mattey (2003) reported the covalent immobilization of bacteriophages onto substrates to use to fight strain-specific bacterial infections as a "bactericide", which was selective killing of bacteria through cell lysis, or as a "bacteriostatic agent" by prohibiting growth of bacteria. The substrate used was any solid material which may

be advantageously activated to allow head-group specific binding of bacteriophages such as nylon and any other polymer with amino or carboxyl surface groups, cellulose or other hydroxyl-containing polymer, polystyrene or other similar polymer, various plastics or microbeads including magnetic particles, biological substances. Moreover, particularly a substrate made of a material regularly used in therapy/medicine. For example, nylon thread for use in surgery; plastics, lint or gauze material for dressing open wounds; microbeads, which can be ingested, adhesives such as cyanoacrylates and/or biological substances such as collagen or hyaluronic acid.

The bacteriophages were immobilised via covalent bonds formed between the bacteriophage coat proteins and the substrate. The bacteriophages were immobilised to the substrate via the head groups by activating the substrate before the addition and coupling of bacteriophage. The method comprising of: (a) activating the substrate so as to enable bacteriophages to bind; and (b) mixing the activated substrate with bacteriophages and a coupling agent to help the bacteriophages binding to the substrate.

The activation of a nylon substrate was achieved by hydrolysis with HCl followed by a wash step of water and an alkali (sodium bicarbonate) to remove the acid. Coupling of bacteriophages to a substrate was as a result of the covalent bonds formation between the coat protein of bacteriophages and the substrate such as through an amino group on a peptide, for example a peptide bond. Coupling agents chosen was dependent on the substrate used as described into 3 difference criterion; (1) Coupling to the substrate nylon or other polymer with amino or carboxy surface groups, the coupling agent was carbodiimide or glutaraldehyde, (2) Coupling to the substrate cellulose or other hydroxyl-containing polymer, the coupling agents were vinylsulfonylethylene ether or triazine (3) Coupling agents to the substrate polythene or other similar polymer, the coupling agents were permanganate oxidation or corona discharge.

According to Gervais et al. (2007) and Tolba et al. (2010) genetic modification was one possibility to improve the orientation of bacteriophage heads in order to enhancing specific binding which were carried out by procedures such as biotin or

cellulose binding domains. This leads to higher infectivity. However, such genetic modification was a time consuming and expensive procedure. Consequently, Cademartiri et al. (2010) studied an alternative method of immobilising bacteriophage by modification of the surface charge of silica from highly anionic to highly cationic and examined the ability of surfaces to immobilised bacteriophage. The modification of surfaces with aminopropyltriethoxy-silane (APTS), led to an increase in the concentration of surface amine groups which in turn lead to an increase in bacteriophage binding. This is because the net negative charge from the bacteriophage head interacts with the positively charged surfaces. The number of infective phage bound to the silica was increased by the increasing surface charge; maximum surface charge correlated with the greatest concentration of adsorbed bacteriophages. The bacteriophages remained infective to the bacterial host while adsorbed on the surface of the silica particles.

Boss & Lieberman (2009) found a bacteriophage binding and orientation method comprising the steps of: providing a long-chained alkyl amine immobilized on a silica-based substrate: binding a nucleic acid complexing agent to the long-chained alkyl amine, wherein the nucleic acid complexing agent is an unsymmetrical cyanine dye configured to penetrate a bacteriophage head and bind to nucleic acid therein; binding a silanated amine to the silica-based substrate, wherein a silane portion of the silanated amine binds to the silica-based substrate and a primary amine portion of the silanated amine is available upon activation for binding to bacteriophage having head and tail ends; activating the primary amine portion of the silanated amine by reaction with a dialdehyde; adding the bacteriophage, wherein the nucleic acid complexing agent binds to the nucleic acid within the bacteriophage head to orient the bacteriophage and the activated primary amine then binds to the bacteriophage head; and reacting any remaining activated primary amine portions of the silanated amine with an alkyl-amine compound.

1.3 Microbial growth kinetics

1.3.1 Batch culture

Batch culture is a closed system in that all the nutrients required for bacterial cell growth and product formation are contained within the vessel at the start of the culture process. The vessel can be in the form of a shake flask or a fermenter (bioreactor). The process is carried out under sterile conditions, being terminated when one or more of the following has been reached: (i) microbial cell growth is stopped because of the depletion of nutrients or the build up of toxic compounds; (ii) after a fixed predetermined time period; (iii) the product concentration desired has been achieved (Macauley-Patrick & Finn, 2008).

1.3.1.1 Growth curve of bacterial cell in batch culture

Bacterial cell grown in batch culture are divided into 6 phases (Figure 6), although any one or several of these phases may be absent: (1) Lag phase, where the cells adapt from their previous to the new condition; there is no growth at this phase. The lag phase in commercial processes can be time consuming and costly and so it is highly desirable to minimise this phase. This can be achieved by using a suitable inoculum. (2) Acceleration phase, the cells begin to divide but the culture is not synchronous. Growth rate increases in this phase. (3) Exponential phase, cell growth gradually increases, the cells grow at a constant and maximum rate. However, the specific growth rate is influenced by the environmental conditions, for instance; the medium complexity, the major carbon and energy source nature, pH and temperature. (4) Deceleration phase, the cell concentration is still increasing, but growth rate decreases. (5) Stationary phase, nutrient depletion and/or the accumulation of inhibitory metabolites or end products have the effect of deceleration cell growth. The rate of cell growth equals that of cell death and specific growth rate approaches zero, eventually. This phase is the maximum population phase. (6) Decline or death phase, the growth rate is negative as to the rate of cell

growth decrease to zero while the rate of cell death increase because of adverse physical conditions and hence the cell concentration reduces (Macauley-Patrick & Finn, 2008; Mavituna & Sinclair, 2008; Monod, 1949; Stanbury, Whitaker, & Hall, 2003).

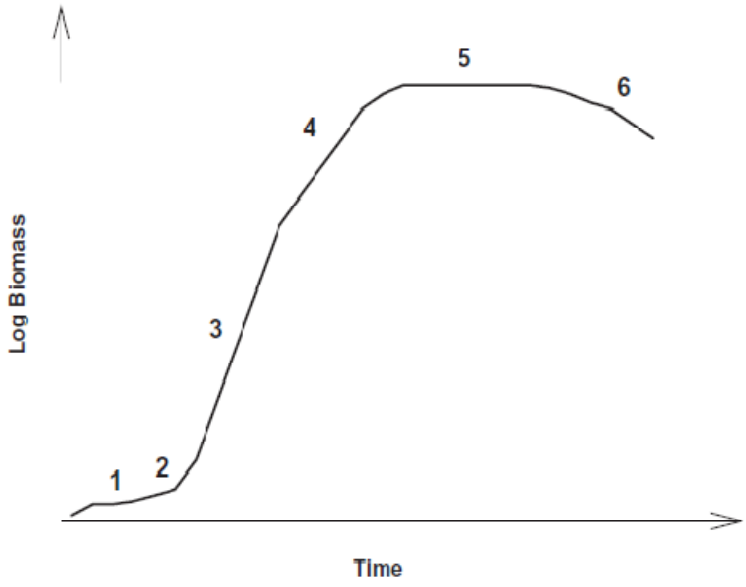


Figure 6 Characteristic growth curve of a microorganism in batch culture. (1) Lag phase; (2) acceleration phase; (3) exponential phase; (4) deceleration phase; (5) stationary phase; (6) decline or death phase

1.3.1.2 Specific growth rate

During the exponential phase, the growth rate of bacterial cells is at its maximum specific growth rate. The growth rate of bacterial cells may be described as below (Mavituna & Sinclair, 2008; Monod, 1949; Stanbury et al., 2003) by the equation:

$$dx/dt = \mu x \qquad 2.1$$

where x is concentration of bacteria cell

t is time, in hours

and μ is the specific growth rate, in hours^{-1}

On integration equation 2.1 gives:

$$x_t = x_0 e^{\mu t} \qquad 2.2$$

where x_o is the original concentration of bacteria cell

x_t is the concentration of bacteria cell after the time interval, t hours

and e is the base of the natural logarithm

On taking natural logarithms, equation 2.2 becomes:

$$ln\ x_t = ln\ x_0 + \mu t \qquad 2.3$$

Using the logarithm base of 10 instead of natural logarithms:

$$\log x_t = \mu t/2.3 + log\ x_0 \qquad 2.4$$

Or by rearranging equation 2.4 we get:

$$\mu = [log\ x_t - log\ x_0]/0.301t \qquad 2.5$$

In additional, in 1942, Monod who first showed that there is a simple relationship between the specific growth rate; and the concentration of a limiting essential growth substrate, μ being proportional to the substrate concentration when this is low but

reaching a limiting saturation value at high substrate concentrations according to the equation 2.6 (Monod, 1949).

$$\mu = \mu_{max} \ [S/ K_s + S] \qquad 2.6$$

where S is the concentration of limiting substrate (gL^{-1})

μ_{max} is the maximum specific growth rate (h^{-1})

K_s is the substrate utilization constant, equal to substrate concentration when μ is half μ_{max}

It follows from equation 2.6 that exponential growth can occur at specific growth rates having any value between zero and μ_{max}, provided the substrate concentration can be held constant at the appropriate value which is a fact of major importance in continuous culture (Herbert et al., 1956; Mavituna & Sinclair, 2008; Monod, 1949; Novick & Szilard, 1950).

The study of Shehata & Marr (1971) corresponded to Monod's theory. When *Escherichia coli* was grown in a glucose, phosphate and tryptophan medium at difference concentrations of the three components, it was found that at high concentrations the specific growth rate was independent of the concentration of nutrient, but at low concentrations the specific growth rate was a strong function of the nutrient concentration.

1.3.1.3 Doubling time

Doubling time, *t*d, is defined as the time required for the cell concentration to be doubled and assuming a constant maximum value of specific growth rate this will be:

$$t_d = \ln 2/ \mu_{max} = 0.693/ \mu_{max} \qquad 2.7$$

1.3.1.4 Advantages of batch culture

• Simplicity of use.

• Operability and reliability: a batch culture is less likely to have instrument failure on short batch runs.

• Fewer possibilities of contamination: all of the materials required for the bioprocess are present in the fermenter and sterilised before starting the run. The only material added (with the exception of the inoculum at the beginning of the process) and removed during the fermentation process are the gas exchange, sterile antifoam and pH control solutions if required (Macauley-Patrick & Finn, 2008).

1.3.1.5 Disadvantages of batch culture

• Toxic metabolites build up is able to restrict bacterial cell growth;

• Batch-to-batch variability;

• Using batch cultures in industrial systems can lead to an increased non-productive period. This is because of down time required for cleaning, re-sterilisation, filling and cooling of equipment (Macauley-Patrick & Finn, 2008).

1.3.2 Continuous culture

Continuous culture is a method of prolonging the specific growth phase of microbial cells in batch culture, while maintaining an environment that has less fluctuation in nutrients, cell concentration or biomass, which is known as *steady state*. The microbial cells are fed with fresh nutrients, and spent medium and cells are removed from the system at the same rate. This ensures that several factors remain constant throughout the process, such as volume of culture, biomass or cell number, product and substrate concentrations, and also the physical parameters such as pH, temperature and dissolved oxygen (Herbert et al., 1956; Macauley-Patrick & Finn, 2008; Mavituna & Sinclair, 2008; Monod, 1949; Stanbury et al., 2003).

Two control techniques of continuous culture are the chemostat and the turbidostat. The most commonly used continuous culture technique, the chemostat, operates based on growth being restricted by the availability of a limiting substrate, while the turbidostat does not use a limiting substrate nutrient but is limited by holding the turbidity constant (Mavituna & Sinclair, 2008; Stanbury et al., 2003).

1.3.2.1 The Chemostat

The feed medium contains an excess of all but one of the nutrients required for growth of the culture. The supply of the nutrient that is not in excess therefore determines growth rate of the bacterial cells. In steady state, the medium flow into the fermenter equals the spent medium and cells flow out. There are some basic relationships in a chemostat as mentioned below.

The dilution rate (D) describes the relationship between the flow of medium into the bioreactor (F) and culture volume within the bioreactor (V):

$$D = F / V \qquad 2.8$$

where D is dilution rate in units per hour (h^{-1})

F is flow rate in units Lh^{-1}

and V is volume in units L

Residence time (τ) is the inverse of dilution rate and is also related to the reactor volume and flow rate:

$$\tau = V/F \qquad 2.9$$

where τ is residence time in units hour (*t*)

Under steady state, the specific growth rate of a culture is set by the dilution rate ($\mu = D$), which means that μ is determined by the rate of flow of nutrient solution to the culture as described below.

At steady state;

$$dx/dt = 0 \qquad 2.10$$

and

$$dS/dt = 0 \qquad 2.11$$

i.e. change in cell numbers or biomass (X) over time (t) is zero, and change in substrate concentration (S) over time (t) is zero, that is, no net accumulation of cell mass or substrate.

$$dx/dt = \text{growth rate} - \text{output rate}$$

$$dx/dt = \mu x - Dx \qquad 2.12$$

Under steady state, the cell concentration remains constant, thus $dx/dt = 0$ and

$$\mu x = Dx \qquad 2.13$$

and

$$\mu = D \qquad 2.14$$

Under steady state, the substrate concentration S_0 is obtained, from the Monod equation:

$$\mu = \mu_{max} \, [S_0 / K_s + S_0] \qquad 2.6$$

or by rearranging equation 2.6 and substituting D for μ, then we get:

$$S_0 = K_s D / \mu_{max} - D \qquad 2.15$$

1.3.2.1.1 Wash out

In the chemostat, when the dilution rate is greater than the maximum specific growth rate (μ_{max}) of the bacterial cell, this results in *washout*. This can be described using the equation:

As $$dx/dt = \mu x - Dx \qquad 2.12$$

When *Dx* is greater than μx, *dx/dt* becomes negative, i.e. the dilution rate, or the rate at which fresh medium is added to and spent medium is removed from the system, exceeds the maximum specific growth rate of the bacterial cell, resulting in a decrease in the number of bacterial cells in the fermenter over time. Therefore, the number of bacterial cells in the fermenter will eventually become zero (Macauley-Patrick & Finn, 2008; Stanbury et al., 2003).

1.3.2.2 The Turbidostat

In a turbidostat, the feed medium contains all nutrients required in excess. Therefore, growth is not substrate limited as in the chemostat, and the bacterial cells are able to grow at its maximum specific growth rate (μ_{max}). The system can be controlled at a cell density desired by monitoring the turbidity which can be achieved by measuring optical density using a spectrophotometer (Macauley-Patrick & Finn, 2008).

1.3.2.3 Advantage of continuous culture

• Productivity and growth rate can be optimised by changing the feed rate during production;

• Longer periods of productivity. In theory, a continuous culture can be operated indefinitely; however, because operation for a long period may leads to mechanical failure, the process have to be stopped occasionally to allow for system maintenance (Macauley-Patrick & Finn, 2008);

• Continuous culture systems have a significant productivity advantage for the production of cell mass or primary products (Shuler & Kargi, 2002).

• Smaller fermenter is required (Metzenberg, 2010)

• Physiological state of cells is uniform (Metzenberg, 2010)

1.3.2.4 Disadvantage of continuous culture

• Contamination can be a major problem in continuous culture, and can lead to the wash out of the organism desired and therefore a loss of product (Macauley-Patrick & Finn, 2008).

• Culture mutation can easily occur in continuous processes (Macauley-Patrick & Finn, 2008; Shuler & Kargi, 2002)

1.4 Growth media

Media used in bioprocesses can be categorized into 3 types based on their composition: synthetic (defined) media, semi-synthetic (semi-defined) media and complex media. Synthetic media contain specific amount of chemical compounds with known chemical compositions. Synthetic media are useful in research and laboratory situations where experimental accuracy is paramount and data interpretation needs to be clear. In general, such media are much more costly than other media types, especially if specific components such as vitamins and growth factors are required, as these ingredients tend to be very expensive when supplied in the pure form. Yields of cells tend to be much lower than those obtained when the same cell line is grown in either semi-synthetic or complex media. Semi-synthetic media contain chemical compounds with known chemical compositions, as mentioned in synthetic media, but include one or more natural compound(s) whose chemical composition is not exactly known, for example, yeast extract. This media are useful in research and laboratory situations. Complex media contain natural compound(s) whose chemical composition is not exactly known, usually of plant or animal such as beet and cane molasses, corn steep liquor, soya bean meals and extracts, whey powders. They are usually relatively cheap (Harvey & McNeil, 2008).

Table 1 shows the composition of bacteria based on macromolecular composition (Harvey & McNeil, 2008).

Table 1 Average macromolecular composition of bacteria.

Macromolecule	%
Protein	55
Carbohydrate	9
Lipid	7
Nucleic acid	23
Ash	6

Source: (Harvey & McNeil, 2008).

Microorganisms require nutrient sources, inorganic ions, specific nutrients and an exogenous source of energy. These nutrients play an important role in cell growth and also biosynthesis of primary and secondary metabolites.

The nutrients required for growth are those which provide the macro-elements; carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, potassium and magnesium (concentration needed greater than 10^{-4}mol L^{-1}) and those which provide the microelements; calcium, manganese, iron, copper, and zinc include vitamins and growth factors (needed concentration lower than 10^{-4}mol L^{-1}) (Ertola, Giulietti, & Castillo, 1995; Shuler & Kargi, 2002).

The main functions related to macro-elements and some microelements are given in Table 2 (Ertola et al., 1995).

Table 2 Functions, elemental composition of microorganisms related to macro- and microelements.

Element	Function or constituent of	Elemental composition of microorganisms
C	C, energy	44-53[a,b,c]
N	Proteins, nucleic acids,	10-14[a]
	Cell wall polymers	7-10[b,c]
P	Nucleic acids,	2.0-3.0[a]
	Phospholipids,	0.8-2.6[b]
	cell wall polymers	0.4-4.5[c]
S	Sulfur amino acids,	0.2-1.0[a]
	biotin, coenzyme A	0.01-0.24[b]
		0.1-0.5[c]
K	RNA, enzyme	1.0-4.5[a,b]
	cofactor, principal cation	0.2-2.5[c]
Mg	Ribosomes, enzyme cofactor	0.1-1.2[a,b,c]
Ca	Bacterial spores, enzyme cofactor	0.01-1.1[a,b]
		0.1-1.4[c]
Fe	Cytochromes, enzyme cofactor	7×10^{-3} –0.9[a,b,c]
Zn	Enzyme cofactor	8×10^{-3} –2.4×10^{-2} [a,b]
Mn	Bacillus spores,	7×10^{-4} –4.8×10^{-2} [a,b]
	enzyme cofactor	

[a] Bacteria, [b] Yeasts, [c] Fungi; **Sources:** Ertola et al, 1995

The aim of any medium design for production purposes is normally emphasized on product formation with the highest yield and productivity and at the minimum cost (Ertola et al., 1995). If cell growth is the primary concern, for instance, production of single cell protein and baker's yeast, the medium should be designed to provide the maximum biomass. The cells must therefore have plenty of carbon and nitrogen sourced as well as other essential nutrients. If the cells run out of nitrogen, growth will cease (Harvey & McNeil, 2008). However, medium design currently trend to avoid extracts of animal origin, particularly bovine sources. This is because these products may lead to potential health risks; from transmission of infection agents such as BSE (Bovine Spongiform Encephalopathy) (Harvey & McNeil, 2008; Merabishvili et al., 2009).

1.5 The Staphylococci

The name *Staphylococcus*, first described by Sir Alexander Ogston, was derived from the Greek nouns staphyle, (meaning bunch of grapes) and coccus (meaning a grain or berry). This organism was found to be the cause of abscesses in man. In 1884 Rosenbach was able to isolate staphylococci and grow them in pure culture (Gotz, Bannerman, & Schleifer, 2006).

1.5.1 Phylogeny

Staphylococcus aureus was formerly classified in the family Micrococcaceae (Kloos & Schleifer, 1986). Currently, this genus is known to belong to the family Staphylococcaceae based on the comparative 16S rRNA sequences. This genus belongs to the Gram-positive bacteria with a low DNA G+C content and are closely related to bacilli and other Gram-positive bacteria with low DNA G+C content such as Enterococci, Streptococci, Lactobacilli and Listeria (Gotz et al., 2006).

1.5.2 General properties

The Staphylococci are non-motile and catalase-positive cocci with a cell diameter of 0.5 - 1.5 µm, occur singly, in pairs, tetrads and irregular grape-like clusters. Resting stages and endospores are not produced. The G+C content of DNA is in the range of 30–39 mol% (Kloos & Schleifer, 1986). The genome is a circular chromosome (approximately 2800 bp), with prophages, plasmids, and transposons. (Lowy, 1998).

They are mesophiles, growing at temperature between 10 and 45ºC (optimum 30-37ºC) and at pH values between 4.2 and 9.3 (optimum 7.0-7.5). Growth is good at NaCl concentration up to 10% and is relatively poor at 15%. They are sensitive to lysis by lysostaphin endopeptidase, which breaks the glycyl-glycine links in the peptide bridge of the peptidoglycan but resistant to lysis by lysozyme (Baird-Parker, 1972; Kloos & Schleifer, 1986).

The metabolism is both respiratory and fermentative, and menaquinones and cytochromes a and b form the electron transport system. Oxygen is the terminal electron acceptor whereas nitrate is used as an electron acceptor under anaerobic conditions. Fermentation of glucose under anaerobic conditions results in the production of mainly lactic acid, whereas under aerobic conditions mainly acetic acid and small amounts of carbon dioxide are produced (Baird-Parker, 1972; Kloos & Schleifer, 1986).

They are facultative anaerobes growing best under aerobic condition. For aerobic growth, they require a medium containing amino acids and growth factors; B group vitamins (Thiamine and nicotinic acid or nicotinamide) (Baird-Parker, 1972; Fildes, Richardson, Knight, & Gladstone, 1936; Kloos & Schleifer, 1986; Knight, 1935, 1937a, 1937b; Mah, Fung, & Morse, 1967; Miller & Fung, 1973; Onoue & Mori, 1997; Porter & Pelczar, 1940; Sober, 1970; Wu & Bergdoll, 1971). For anaerobic growth condition, they also require uracil and a fermentable carbon source (Baird-Parker, 1972; Kloos & Schleifer, 1986).

The major habitats of *S. aureus* include the nasal membranes (anterior nares, nasopharynx) and skin and to a somewhat lesser extent the perineum, gastrointestinal tract and genital tract of warm-blooded animals (Kloos & Schleifer, 1986).

S. aureus is a potential pathogen causing a wide range of infections; furuncles (boils), carbuncles, impetigo, toxic epidermal necrolysis, pneumonia, osteomyelitis, meningitis, endocarditis, mastitis, bacteremia, various abscesses, food poisoning (via enterotoxin), enterocolitis, urogenital infection, toxic shock syndrome (Kloos & Schleifer, 1986).

1.5.3 Growth requirements of staphylococci

Hughes (1932) attempted to develop a simple medium for the cultivation of staphylococci. It was found that the substance present in meat extract was capable of stimulating the growth of staphylococci.

Knight (1935) used autolysed yeast extract (marmite) as growth factor in a gelatin hydrolysate basal medium plus glucose.

Fildes, Richardson, Knight & Gladstone (1936) and Gladstone (1937) reported to develop amino acid basal medium, containing a mixture of 16 amino acids plus glucose, to replace the gelatine hydrolysate basal medium. The chemically-defined medium contained the following 16 amino acids: glycine, alanine, valine, leucine, proline, oxyproline, aspartic acid, glutamic acid, phenylalanine, tyrosine, arginine, histidine, lysine, tryptophane, cystine and methionine. In addition, the medium contained nicotinamide, thiamin (vitamin B1), glucose (0.5%) and inorganic salts (KH_2PO_4, $MgSO_4$. 7H20, $FeSO_4(NH_4)_2SO_4$ $6H_20$).

Knight (1937a, 1937b) reported that the aerobic growth of a strain of *S. aureus* in a chemical define medium, gelatine hydrolysate basal medium plus glucose or amino acid basal medium plus glucose. It was shown that nicotinic acid (or its amide) and aneurin (vitamin B1) together, neither alone being effective, could replace the staphylococcus growth factor obtained from yeast extract as the previous study of Knight (1935), had to be added to either of the chemical define basal medium before the growth of the bacteria could take place.

Porter & Pelczar (1940) reported that the "fastidious" strains of *S. aureus*; X3 and Y2 strains, failed to grow in with neither nicotinic acid nor vitamin B_1 in the basal chemically-define medium of Gladstone (1937). It was found those strains require the growth factor, biotin, in addition to nicotinic acid and vitamin B, in Gladstone's chemically-define medium. However, nutritional requirements for growth of several strains of *S. aureus* were satisfied by the basal chemically-define medium of Gladstone (1937), but their growth were not stimulated by the addition of biotin.

Mah et al (1967) studied the nutritional requirements of *S. aureus* S-6, which was cultured in both synthetic medium and complex medium containing an equivalent amount of protein hydrolysate. Due to protein hydrolysate powder (PHP) medium supported optimal growth of S-6 in the presence of nicotinic acid and thiamine. The synthetic medium was developed based on the constituent 18 amino acids present in PHP. These consisted of glycine, alanine, aspartic acid, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophan, lysine, arginine, histidine, glutamic acid, cystine, methionine, and proline which were obtained from commercial sources. Each was added at a final concentration of 25µg/ml. The amino acids mixture was calculated such that an equivalent amount of amino nitrogen was present in 0.08% PHP. It was found that the 11 amino acids; glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine and histidine, and three vitamins (thiamine, nicotinic acid and biotin) were necessary for the optimal growth of S-6 in the synthetic medium with glucose as a carbon source, under aerobic condition at 37°C. Moreover, it also found that biotin was a growth factor requirement of S-6 when glutamic acid but not glucose was used as a carbon source. The specific growth rate in 0.08 and 0.06% PHP with glucose as carbon source were 1.33 hr^{-1} and 1.26 hr^{-1}, respectively. In the synthetic medium with glucose, the specific growth rate was 0.625 hr^{-1}. In the synthetic medium, all cultures were in the maximal stationary phase within 12 hr of incubation when glucose as a carbon source and 24 hr when MSG as a carbon source. In the PHP medium, cultures were in the maximal stationary phase after 12 hr, regardless of carbon source.

Sober (1970) reported the amino acid requirements of microorganisms for growth. For 21 strains of Staphylococci need these 7 amino acids, which are arginine, aspartic acid, cystine or cysteine, leucine, phenylalanine, proline and valine, for their growth. The strains 2059, 2062 and 2063 need arginine cystine or cysteine, valine and histidine for their growth whereas these amino acids; aspartic acid, glutamic acid, glycine, leucine, methionine, phenylalanine, proline and serine, stimulate their growth. Moreover, these strains 1989 and 2091need arginine, cystine or cysteine and valine for their growth while aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine and threonine stimulate their growth.

Miller & Fung (1973) studied growth of *S. aureus* S-6 cultured in minimal chemically-defined medium containing 18 amino acids that was used by Mah et al (1967), monosodium glutamate as a source of carbon, nitrogen and energy, six inorganic salts; NaCl, $NaHPO_4.7H_20$, $KHPO_4$, $MgSO_4.7H_2O$, NH_4Cl, $FeSO_4.7H_2O$ and four vitamins (thiamine, nicotinic acid, pantothenate and biotin). It was found that arginine, cysteine and phenylalanine were the three amino acids necessary for growth and toxin formation of the bacteria. In addition, proline and valine were required when glucose added as the primary source of energy.

Onoue & Mori (1997) found that the growth of *S. aureus* strains FRI-100, S-6, FRI-361, K858 and T7436 in the chemically-defined medium containing 18 amino acids, inorganic salts; K_2HPO_4, KH_2PO_4, $Na_3citrate.2H_2O$, $MgSO_4$ and $(NH_4)_2SO_4$, four vitamins; thiamine, nicotinic acid, pantothenate and biotin and purines and pyrimidines; adenine, guanine, cytosine, uracil and thymine, required valine for growth whereas arginine and cysteine were required for growth and enterotoxin production.

1.5.4 Cell wall composition

The cell wall, which consists of a thick rigid layer, generally functions in protection and giving shape to a cell. The composition of cell wall of *S. aureus* is similar to that of other Gram-positive bacteria. Mainly, it is made up of multiple layer of peptidoglycan, teichoic acid, and protein A. Firstly, the peptidoglycan is a heteropolymer of repeating units of β-1,4-*N*-acetylglucosamine (NAG) and *N*-acetyl-muramic acid (NAM). A short tetrapeptide units terminating with D-alanine is attached to NAM with 1, 4-β linkage. The interpeptide bridges linking peptidoglycan are type of the penta- and hexaglycine interpeptide bridges (type L-Lys-Gly_{5-6}). Secondly, cell wall teichoic acids are water-soluble polymers containing repeating phosphodiester groups that are covalently linked to peptidoglycan. The cell wall teichoic acid of *Staphylococcus aureus* is a ribitol teichoic acid which is composed of ribitol with either α or β glycosidically linked *N*-acetyl-glucosamine residues. Finally, protein A is an immunoglobulin G (IgG) binding protein. Protein A is covalently bound via its C-terminal end to the pentaglycine interpeptide bridge of peptidoglycans and can be released by treatment with lysostaphin, a glycyl-glycine endopeptidase (Gotz et al., 2006; Kloos & Schleifer, 1986; Madigan & Martinko, 2006; Moat et al., 2002).

Xia, Kohler, & Peschel (2010) have reported that the functions of teichoic acid (TA) polymers in cell envelopes of Staphylococci and most other Gram-positive bacteria can be classified in three major aspects: (i) protection against damaging molecules and stresses from environment, (ii) control activities of enzyme and concentrations of cation in the cell envelope and (iii) binding to surfaces and receptors, particularly wall teichoic acid (WTA) serving as a bacteriophage receptor, which agrees with a study of Chatterjee (1969).

1.5.5 Catabolite regulation in *S. aureus*

In anaerobic growth condition, *S. aureus* metabolises glucose to lactate (73-94%); acetate (4-7%) and traces of pyruvate are also formed, whereas in aerobic growth, acetate and CO_2 are the predominant end products and lactate appears only 5–10% of the glucose carbon (Strasters & Winkler, 1963). In the presence of glucose, glycolysis is enhanced. By contrast, many enzymes of the pentose phosphate (PP) pathway and the tricarboxylic acid (TCA) cycle are suppressed. Moreover, pyruvate oxidation and the cytochrome contents are decreased in glucose grown *S. aureus* cells. This is "glucose-mediated catabolite repression" or "glucose effect" in staphylococci (Gotz et al., 2006).

With the addition of glucose to aerobically grown *S. aureus* cells, glucose is catabolised via glycolytic pathways and the PP pathway to pyruvate and the subsequent oxidation of pyruvate via the TCA cycle. It also found that the specific activity of various PP enzymes of *S. aureus* grown with or without glucose in nutrient broth has no marked differences. Furthermore, the specific activities of two enzymes of the FBP pathway (glyceraldehyde-3-P dehydrogenase and lactate dehydrogenase) are increased in the presence of glucose. However, the specific activities of the TCA cycle enzymes (succinate dehydrogenase and fumarase) are decreased and fumarase activity is not detectable (Gotz et al., 2006). During aerobic growth in the presence and absence of glucose, the oxidation of acetate, succinate and malate by resting *S. aureus* cell suspensions was observed only in the absence of glucose (Collins & Lascelles, 1962; Strasters & Winkler, 1963). Growth of *S. aureus* in nutrient broth with glucose (0.1%) results in a 40% decrease in the cytochrome content compare with that in the absence of glucose (Strasters & Winkler, 1963).

Moreover, Strasters & Winkler (1963) found that when glucose was present in the growth medium the oxidation of amino acids was markedly decreased compared with that in the absence of glucose. Although glutamic acid was still oxidised noticeably other amino acids including glycine, serine, aspartic acid and ornithine were rarely oxidised. These results suggest that staphylococci growing in nutrient broth in the presence of glucose do not use amino acids for energy production. On the other hand,

growth of staphylococci in nutrient broth in the absence of glucose can use amino acids for energy production.

1.5.6 Antibiotic resistant staphylococci

Soon after penicillin was first introduced in 1941, there was a report by Kirby (1944) of a resistant strain of *S. aureus* which produced penicillinase. Methicillin was introduced in 1961 and shortly after *S. aureus* strains developed resistance to methicillin (Chambers, 2001).

Now Methicillin-resistant *S. aureus* (MRSA) is one of the most common causes of hospital-acquired (nosocomial) and community-acquired infection (Chambers, 2001; Lowy, 1998; Rice, 2006)

Resistance to methicillin is caused by the presence of the *mecA* gene. The *mecA* encodes the 78kDa penicillin-binding protein (PBP) 2a. Basically, *β*-lactam antibiotics bind to PBPs in the cell wall, resulting in the failure of peptidoglycan layer synthesis and the bacterial death. However, *β*-lactam antibiotics are unable bind to PBP2a, so that synthesis of the peptidoglycan layer and cell wall synthesis can continue in *mecA* containing strains. The 2.1 kb *mecA* gene is carried on a mobile genetic element, designated the Staphylococcal Cassette Chromosome *mec* (*SCCmec*). To date, five *SCCmec* types (I–V) have been distinguished that differ in size and genetic composition. All *SCCmec* elements carry genes for resistance to *β*-lactam antibiotics and also genes for the regulation of expression of *mecA* (Deurenberg et al., 2007).

Vancomycin is “the typical treatment of last resort” for MRSA infection. Unfortunately, the first case of VRSA (Vancomycin resistant *Staphylococcus aureus*) was reported in 2002. This report describes a clinical isolate of *S. aureus* that is fully resistant to vancomycin. This is because of transference of the *van* resistance genes (*vanA* genes) from *Enterococcus faecalis* to *S. aureus* in a patient. Transfer of *van* resistance genes from enterococci to *S.aureus by* conjugation had been predicted, having been first demonstrated in vitro (Gotz et al., 2006).

CHAPTER 2
MATERIALS AND MATHODS

2.1. Microorganisms

The bacterial strain used for this research was *Staphylococcus aureus* type strain, 8588 (Kindly provided by Janice Spencer, University of Strathclyde).

The bacteriophage used for the research was bacteriophage K (Rees and Fry, 1981).

2.2. Media and solutions for bacterial cell culture and bacteriophage methods

2.2.1. Media

The bacteriological media employed during this research project were purchased from Oxoid Ltd, England, UK (yeast extract, tryptone and agar); BDH Laboratory Supplies, England, UK (glucose); Sigma-Aldrich Inc, USA (peptone, corn steep liquor, NaCl and NH_4Cl); Sigma-Aldrich Chemie GmbH, Germany (soy peptone, casein hydrolysate, $NaHPO_4$ and KH_2PO_4); Sigma-Aldrich, Japan ($CaCl_2$); Sigma-Aldrich Co. Ltd, UK ($MgSO_4$);

Culture media were prepared as described below. The media were sterilized at 121ºC under pressure using a Prestige Medical Omega Media autoclave (Model 220140, England, UK). After autoclaving, the agar media were cooled to 45ºC by placing in a Griffin water bath (Model 100 series, Griffin &George Ltd., UK) at 45C. 20-25 ml of the agar media were poured into sterile Petri dishes and allow to settle and solidify. The broth media were dispensed before autoclaving into any flask sizes used at any volume of media designed.

2.2.1.1. **Luria media** (Luria & Burrous, 1957)

Luria broth; LB, for the preparation of inoculum, experiments of infection/lysis and experiments of adsorption. Luria Agar (LA) was employed for sub-culturing new cultures, cell count, bacteriophage stock preparation by plate lysis and plaque assay method. These media contained; tryptone 10 g, yeast extract 5 g, and $NaCl_2$ 10 g. The components were suspended in 1L distilled water with the addition of agar (15 g) for Luria agar. The pH was adjusted to 7.5 with 4M NaOH (1 ml into 1L of the medium).

2.2.1.2. Modified minimal media (modified M9) (Sambrook & Russell, 2001)

Modified Minimal Media (modified M9) was used for bacterial host growth experiments, bacteriophage stock preparation in liquid culture, adsorption and activity of immobilised bacteriophage, experiments on bacteriophage production in fermenters both in batch culture and in continuous culture.

The medium was prepared as follows. Stock solution of M9 salts consisted of Na_2HPO_4, 68 g; KH_2PO_4, 30 g and NaCl, 5 g, were added in 1 litre of the solution. M9 medium was prepared by adding 10 ml of M9 salt solution, 1 ml of 1 M $MgSO_4$, nitrogen source (NH_4Cl or yeast extract); (at appropriate level according to the experiment plan as shown in each Table), to 1 litre with distilled water. The pH was adjusted to 7.0-7.5 with HCl or NaOH. After autoclaving and cooling, 1 ml of sterile 100 mM $CaCl_2$ was added under sterile conditions as well as addition of carbon source (glucose) at the appropriate level according to the experiment plan as shown in each Table.

2.2.1.3. Top agar

Top Agar was used as an agar overlay for plating bacteriophage, for instance plaque assay method, for bacteriophage stock preparation by plate lysis. This medium was prepared as LB with the exception that 0.66% agar was used instead of 1.5% agar.

2.2.2. Solutions

2.2.2.1. Phosphate Buffer Saline (PBS)

Phosphate Buffer Saline (PBS) (Oxoid, England, UK) was used as the solution for the maintenance of bacterial cell and bacteriophages for example for serial dilutions of bacterial cell cultures and bacteriophage suspension. This solution was prepared by adding 10 pellets of PBS tablets (Oxoid) to 1L distilled water and sterilized by autoclaving. This solution contained NaCl 8.0 g; KCl 0.2 g; Na_2HPO_4 1.15 g and KH_2PO_4 0.2 g in 1 L of the solution.

2.2.2.2. Storage Medium; SM

Storage Medium; SM, was used as a solution for the maintenance of bacteriophages for example for bacteriophage stock preparation by plate lysis, for doing serial dilution of bacteriophage suspension. SM was prepared as follows: Solution for 1M Tris.Cl (pH 7.5) of Trizma HCl (Sigma-Aldrich Inc., USA) 31.52 g and 1M NaOH (Fisher Scientific Ltd., UK) 22 ml, made up to 200 ml of the solution. To prepare 1 litre of the SM solution, it contained 1M Tris.Cl (pH 7.5) 50 ml; NaCl 5.8 g; $MgSO_4$ 0.98 g and Gelatin (Sigma-Aldrich Inc., USA) 0.1 g of the solution were added up to 1L of the solution with distilled water as well as autoclaving the solution before use.

2.3. Culture maintenance

2.3.1. Seed maintenance of *S. aureus*

A stock culture of *S. aureus* 8588 was stored using Microbank^{TM} Cryo beads (PL.160; Prolab,Wirral, UK) at -20ºC. Fresh cultures were revived on a monthly basis and sub-cultured onto Luria agar plates weekly.

2.3.2. Inoculum preparation

A single colony from a S. *aureus* 8588 seed culture plate was selected and transferred into a 250 ml Erlenmeyer flask containing 100 ml sterile LB medium. Then the flask was incubated in a Stuart orbital incubator (SI50, Bibby Sterilin) at 37°C, 180 rpm for 18 hours. After this time, the culture flask was removed and stored at 4°C until used as starter culture or inoculums.

2.4. Bacterial host growth measurements

2.4.1. Absorbance

The absorbance of a growing bacterial culture was measured with the spectrophotometer at a wavelength of 650 nm. 1 ml of bacterial cultures were transferred into a cuvette and measured against distilled water as a blank.

2.4.2. Viable cell count method

A 100 µl of a growing bacterial culture was transferred and spread, using a sterile glass rod spreader, onto L-agar plates. The plates were then incubated at 37°C overnight. After that the plates were examined for colony forming units per millilitre (cfu/ml).

Bacterial cultures were serially diluted in sterile PBS prior to plating out to give a suitable number of colonies for counting.

2.5. Statistical analysis

These data were analysed by ANOVA using SPSS 13.0 for windows. Then, post hoc testing using Duncan's multiple range test (DMRT) were performed to see which data were significantly different. In all the analysis performed the confidence interval used were 95%.

2.6. Bacteriophage methods

2.6.1. Plaque assay

LB top agar (0.66% agar)

LB top agar was melted by heating in a microwave and was stored in the water bath at 47°C to prevent agar from solidifying until needed.

Plating bacteria preparation

A single colony of *Staphylococcus aureus* 8588 from stock culture was picked and inoculated in 100 ml of LB contained in 250 ml Erlenmeyer flask. The culture flask was then incubated at 37°C overnight (18 hours). After removing from the incubator, the culture was stored at 4°C until used.

Tenfold serial dilution preparation

Tenfold serial dilution of bacteriophage stock solution were prepared (in SM or PBS). Each dilution was mixed by vortex gently.

Method

0.1 ml of an overnight fresh culture of bacteria was dispensed into a series of sterile tubes and then 0.1 ml of each dilution of bactriophage stock was added. After that, 4 ml of molten top agar was added. The contents of the tube were mixed and poured onto the agar plate. The plate was swirled gently. Repeat this step with the remaining dilution of the bacteriophage stock solution.

All plates were allowed to set at room temperature before incubating the inverted plates at 37°C overnight. After incubating overnight, the plates were removed from the incubator and the number of plaque were counted and calculated the plaque forming unit per millilitre (pfu/ml) was calculated.

2.6.2. Bacteriophage stock preparation

2.6.2.1. Bacteriophage stock preparation by plate lysis

Preparation of infected culture for plating: an overnight culture of bacteria was mixed with bacteriophage stock solution in a ratio of 3:4.5. The infected culture was incubated at 37ºC for 10 minutes

After incubating, 0.2 ml of the infected culture was transferred into the first universal tube and 4 ml of molten top agar was added. The contents of the tube were mixed gently before pouring onto a labeled agar plate, without delay. The plate was swirled gently and allowed to set. This was repeated until the total volume of the infected culture was transferred onto separate plates.

The plates were then incubated in a Gallenkamp incubator (HI-150, England) without inversion at 37°C overnight. After removing these plates from the incubator, 5 ml of SM was added and plates were stored at 4°C for 3-4 hours. Every half an hour they were swirled gently. SM containing bacteriophages from each plate was then transferred, using a sterile pipette, into sterile (screw cap) centrifuge tubes and the plates were discarded. Bacterial debris was removed by centrifugation at 4,000 rpm for 10 minutes. The supernatant containing bacteriophages was filtered through

a 0.22 µm filter (Nalgene, USA). Bacteriophage stock solution normally was stored at 4°C.

The concentration of the bacteriohages was measured as describe in 2.6.1 Plaque assay.

2.6.2.2. Bacteriophage stock preparation by liquid culture

Preparation of an overnight culture of bacteria. A single colony of *Staphylococcus aureus* 8588 from stock culture was picked and inoculated in 100 ml of LB contained in 250 ml Erlenmeyer flask. The culture flask was then incubated at 37°C overnight (18 hours). After removing from the incubator, the culture was stored at 4°C until used.

The fresh overnight bacteria culture was inoculated into a 500 ml Erlenmeyer flask containing 200 ml modified M9 medium with 2% inoculum size used. Then the flask culture was incubated in the orbital incubator at 180 rpm 37°C until the absorbance 650 nm of the culture reached at 0.3, which was equivalent to $1.0x10^8$ cells/ml. Bacteriophage stock was then added with a ratio of 1:5 between bacteriophage and bacteria. The co-cultures were then incubated in the incubator shaker at 180 rpm 37°C. The cultures were monitored until completely lysis (3-4 hours). Then the lysate, which contained bacteria cell debris, was transferred to centrifuge tubes. The bacteria cell debris was removed by centrifugation at 4,000 rpm for 10 minutes. After that the supernatant was filtered through the 0.22 µm filter unit. The bacteriophage stock was stored at 4 °C until needed.

Measurement of the concentration of bacteriophages is described in 2.6.1 Plaque assay.

2.7. Preparation of bacterial host culture for experiments on bacteriophage

Generally bacterial host culture for use in experiments on bacteriophage should be in its exponential phase growth.

A 5 ml of fresh overnight bacteria culture was inoculated into a 250 ml Erlenmeyer flask containing 45 ml of LB medium. The culture flask was then incubated in the orbital incubator speed 180 rpm at 37°C. The bacterial culture growth was determined by measuring absorbance using the Shimadzu UV spectrophotometer at a wavelength of 650 nm until the absorbance of the culture reached 1.0. The cell concentration of the culture was calculated by assuming that 1.0 absorbance = 5.0×10^8 cful/ml, determine from a calibrate curve (Appendix, Figure A.1).

2.8. Bacteriophage Immobilization

2.8.1. Bacteriophage immobilization by the corona discharge method

Nylon membrane strips preparation

Nylon membrane strips were prepared in the size of 1cm×5cm.

Corona treatment

A nylon membrane strip was treated at speed of 5 cm/min by the corona discharge irradiation in 2 passages, on a corona discharge machine (Sherman Treater, The Corona Treatment People, Thame, Oxon, England, UK).

Bacteriophage immobilization

The strip treated was immediately transfer into bacteriophage solution and incubated at room temperature for an hour, shake gently frequently. After that the strip was washed to get rid of unbound bacteriophages as the methods describe in 2.8.2.

2.8.2. Washing and checking of unbound bacteriophages on the bacteriophage immobilization strip

After immobilization the strip was washed to remove unbound bacteriophage followed the washing method as mentioned in 2.8.2.1. Follow by checking for the presence of free bacteriophage as the method described in 2.8.2.2. Repeat the washing step until checking of unbound bacteriophage shown negative result.

2.8.2.1. Washing of free bacteriophages on the bacteriophage immobilization strips

The strip was rinsed with 10 ml of sterile PBS each side and then was transferred into a Petri dish containing 20 ml of sterile PBS. Repeat this wash step.

For the last wash step, the strip was checked for the presence of free bacteriophage as the method describe in 2.8.2.2

2.8.2.2. Checking for the presence of free bacteriophages on the bacteriophage immobilization strip

One millilitre of sterile PBS was rinsed both side of the strip and the PBS rinsed was collected to carry out a plaque assay to check for free bacteriophage presence.

Positive result: plaque presence or unbound bacteriophage presence. Then the strip should be carried out the washing step as described in 2.8.2.1

Negative result: plaque absence or unbound bacteriophage absence. This means the strip is no free bacteriophage and should be immobilized properly. To confirm this, the strip was checked as described in 2.8.3.

2.8.3. Confirmation of bacteriophage immobilization on nylon membrane strips

After the immobilized bacteriophage strip was washed completely which there was no unbounded bacteriophage on the strip. For the next step, the strip was checked to confirm that the bacteriophages were immobilized properly on the strip as described below.

The 1cm x 1cm immobilized bacteriophage strip was transferred into a universal tube containing 5 ml of LB and 100 µl of plating bacterial host. Then the tube was incubated in a incubator shaker 18 rpm 37°C overnight (or at least 3-4 hours) to allow bacterial host multiply, infect and lyse. After that the culture was serially diluted and carried out on plaque assay to determine presence of plaque.

Positive result : plaque presence at any serial dilution. This meant the strip was immobilized properly and ready to use for the next step.

Negative result : plaque absence at any serial dilution. This meant the strip was no immobilized bacteriophage and not ready to use for the next step.

The properly immobilized bacteriophage strips were prepared in the size of 1cm x 1cm and then they were stored in SM at 4ºC until used.

2.9. Bacteriophage production

2.9.1. Fermenters

The fermenter used in this study were a SGi fermenter (Model 7F/L, Setric Genie Industrial, Toulouse, France) for bacteriophage infection and lysis in batch and continuous culture and a BioFlo 110 fermenter (Model M1273-0054, New Brunswick Scientific Co. Inc., Edison, New Jersy, USA) for bacterial host culture in batch culture.

2.9.1.1. The SGi fermenter

The SGi fermenter comprised:

1) A round bottom borosilicate glass vessel had a total volume of 2 l with a working volume of 0.6–1.5 l. The vessel's internal diameter was 12 cm and height was 20 cm.

2) An agitation system comprising a variable speed 36 wall motor with a flexible universal coupling between motor and drive shaft, a drive shaft with a height adjustable Rushton turbine impeller with 4 blades and 2 baffles.

3) A stainless steel top plate fixed to glass vessel using a flange and a series of bolts and nuts. The top plate was provided with entry port for a cooling baffle, a heating baffle, and air inlet and air outlet, a temperature probe, (a pH electrode, a dissolved oxygen electrode and an inoculation tube).

4) Support chassis, including an air flow meter, cooling water solenoid and the air inlet and outlet filters.

5) An electronic control unit including a power supply module and an agitation module.

Temperature within the vessel was monitored via a stainless steel temperature sensor connected to the electronic control unit for recording the process temperature. The culture vessel contents were heated with a temperature probe and cooling was achieved via a closed cold water system. The temperature set point for all fermentation was 37°C (±0.5) unless otherwise stated.

Air supply to the system from a pump connected to the air inlet via pressure tubing. Inlet air and outlet air flow through 0.3 µm Whatman membrane filters which were steam sterilized (autoclaved at 121°C 20 min) when the fermenter sterilization. The flow rate was controlled by a rotameter at 0.6 dm^3min^{-1} (1 volume of air per volume of culture per minute (1vvm).

pH, dissolved O_2 were off, not controlled, not monitored.

Foam control, polypropylene glycol 2025 was used as an antifoam agent. The addition of 0.1 ml of the antifoam agent per 1.0 litre of medium prior to sterilisation was sufficient to control foaming during the fermentation.

2.9.1.2. The BioFlo 110 fermenter

The BioFlo 110 fermenter comprised:

1) A round bottom borosilicate glass vessel had a total volume of 14 L with a working volume of 4–10.5 L. The vessel's diameter was 29 cm and height was 61 cm.

2) A stainless steel head plate fixed to glass vessel using a flange and a series of 6 bolts and nuts. The head plate was provided with ports for probes and various accessories; a temperature probe, a pH electrode, a dissolved oxygen electrode, air inlet and air outlet, and an inoculation and harvest tube.

3) Agitation was provided by two six flat bladed Rushton turbines driven by an electric motor with adjustable speed. Agitation was varied between 50-1200 rpm.

4) Aeration was provided by a stainless steel ring sparger, which was firmly premounted into the head plate, located directly below the lower turbine. The sparger was 5mm in diameter and had 7×1mm diameter equally spaced holes around the ring.

5) Temperature was controlled by using heat blanket.

6) A primary control unit (PCU) is an automatic system for controlling the function of the BioFlo 110 fermenter. The PCU monitored and controlled temperature, pH, stirrer speed, airflow including a power supply module. The signals were fed to the fermenter via a plug connection and process data were indicated on the monitor display. For this work a stirrer speed, temperature and airflow were employed.

2.9.2. Fermenter sterilisation

The fermenter vessel and the medium were sterilised by autoclaving for 20 min at 121 °C.

In continuous experiment, various accessory parts, such as supply lines for feeding in/out including inoculating tubing were connected to the top plate by means of piercing needles and housing fitting. They were previously sterilised in an autoclave before mounting to the port at the top plate. The accessory parts were connected after sterilisation of the culture vessel prior to inoculation.

2.9.3. Bacteriophage infection and lysis in shake flask culture

The fresh overnight bacteria culture was inoculated into a 500 ml Erlenmeyer flask containing 200 ml modified M9 medium, 0.5% yeast extract, with 2% inoculums size used. Then the flask culture was incubated in the Stuart orbital incubator at 180 rpm 37°C until the $OD_{650\ nm}$ of the culture reached at any point as planned. After that the bacteriophage stock was added with the ratio between bacteriophage and bacteria at any point as planned. The co-cultures were then incubated continually in the incubator shaker at 180 rpm 37°C. The cultures were monitored for complete lysis. The number of bacteriophage was determined by plaque assay.

2.9.4. Bacteriophage infection and lysis in batch culture

In batch culture experiments, operating parameters were as follows; temperature 37°C, agitation 200 rpm, aeration 1vvm, initial pH 7.0, working volume 0.75 L (for bacterial host culture) and 0.6 L (for bacteriophage infection and lysis).

The fresh overnight of bacterial culture was incubated into 0.75 L modified M9 medium (0.5% yeast extract) by 2% inoculums size used. The optical density of the culture at OD_{650nm} was measured to estimate growth of bacterial host culture. Bacteriophage infection with at an moi; at any point as planned, was performed at an OD_{650nm} of that; at any point as planned, after reducing the working volume to 0.6 L and decreasing the agitation to 80 rpm (after bacteriophage added). The cultures were monitored for complete lysis. The number of bacteriophage was determined by plaque assay.

2.9.5. Bacteriophage production in continuous culture

2.9.5.1. Bacteriophage lysate in batch culture

In batch culture experiments, operating parameters were as follows; temperature 37°C, agitation 200 rpm, aeration 1vvm, initial pH 7.0, working volume 0.75 L (for bacterial host culture) and 0.6 L (for bacteriophage infection and lysis).

The fresh overnight of bacterial culture was incubated into 0.75 L modified M9 medium (0.5% yeast extract) by 2% inoculums size used. The optical density of the culture at OD_{650nm} was measured to estimate growth of bacterial host culture. Bacteriophage infection with an moi of 2 was performed at an OD_{650nm} of 0.5, after reducing the working volume to 0.6 L and decreasing the agitation to 80 rpm (before bacteriophage added). The number of bacteriophage was determined by plaque assay.

2.9.5.2. Bacteriophage continuous culture

In continuous culture experiment, operating parameters were as follows; temperature 37°C, agitation 80 rpm, aeration 1vvm, initial pH 7.0, working volume 0.6 L, dilution rate (D) between 0.50 -2.61 hr^{-1}.

Peristaltic pumps (Watson-Marlow 101, England, UK) were used to supply bacterial host culture in its stationary growth phase as described below in 2.9.5.2.1, which was prepared every 12 hr for feeding for 12 hr, and remove the mixed culture from the vessel at the same flow rate. The culture volume was 0.6 L and the dilution rate was altered by changing the pump running speed as data shown Table 3. Continuous culture was achieved via a fixed volume system where a feed was continuously pumped in up to a volume of 0.6 L and another pump continuously removed excess liquid via a tube placed at a fixed height.

2.9.5.2.1. Bacterial host culture in its stationary growth phase preparation for feeding in continuous culture

Bacterial host culture in its stationary growth phase was prepared in the BioFlo 110 fermenter in batch culture. The operating parameters were as follows; temperature 37°C, agitation 200 rpm and aeration 1vvm.

The bacterial host culture was prepared in the fermenter vessel containing modified M9 medium (0.5% yeast extract) by using 2% inoculum size for 12 hr. Then the bacterial host culture was withdrawn and collected into empty sterile aspirator for feeding in bacteriophage production in continuous culture.

Table 3 Bacteriophage production in continuous culture parameters

Residence Time (T) (hr)	Time (min)	Working Volume (V) (L)	Feed Rate (L/hr)	Dilution Rate (hr^{-1})
2.00	120	0.6	0.300	0.50
1.50	90	0.6	0.400	0.67
1.00	60	0.6	0.600	1.00
0.67	40	0.6	0.900	1.50
0.38	23	0.6	1.565	2.61

Bacteriophage complete lysis was prepared from the method as described above prior to supplying bacterial host culture into the vessel culture. The continuous culture was periodically sampled to determine the concentration of bacteriophage by counting the bacteriophage particles in a unit of particle per milliliter (particles/ml)

using a NanoSight (Model LM10, NanoSight Ltd., Salisbury, UK) every 12 hr until the number of bacteriophage particles (particles/ml) was in a steady state exception at dilution rate 2.61 hr^{-1} sampling at 0.5, 1, 3, 5 and 8 hr.

2.9.5.3. The NanoSight

The NanoSight (Model LM10, NanoSight Ltd., Salisbury, England, UK) (Figure 7 and Figure 8) was used to count bacteriophage particles in samples.

The instruments comprise of a microscope which is installed with Marlin digital camera and NanoSight camera adapter, an LM10 sample unit and are installed connection with a computer control unit which runs the Nanoparticle Tracking Analysis (NTA) software.

The LM10 sample unit uses single-mode laser diode (<40mW, 635 nm) to illuminate particles in liquid suspension which are introduced and held within the unit.

The NTA software is a method of visualising and analysing particles in liquids that relates the rate of Brownian motion to particle size from videos captured. The rate of movement is related only to the viscosity of the liquid, the temperature and size of the particle and is not influenced by particle density or refractive index. This is carried out for all particles in the laser scattering volume to produce a particle size distribution using the Stokes-Einstein equation. The software used to analyze videos captured using the instrument, giving a particle size distribution and particle count.

Figure 7 The NanoSight machine. **Source**: www:\nanosight.com

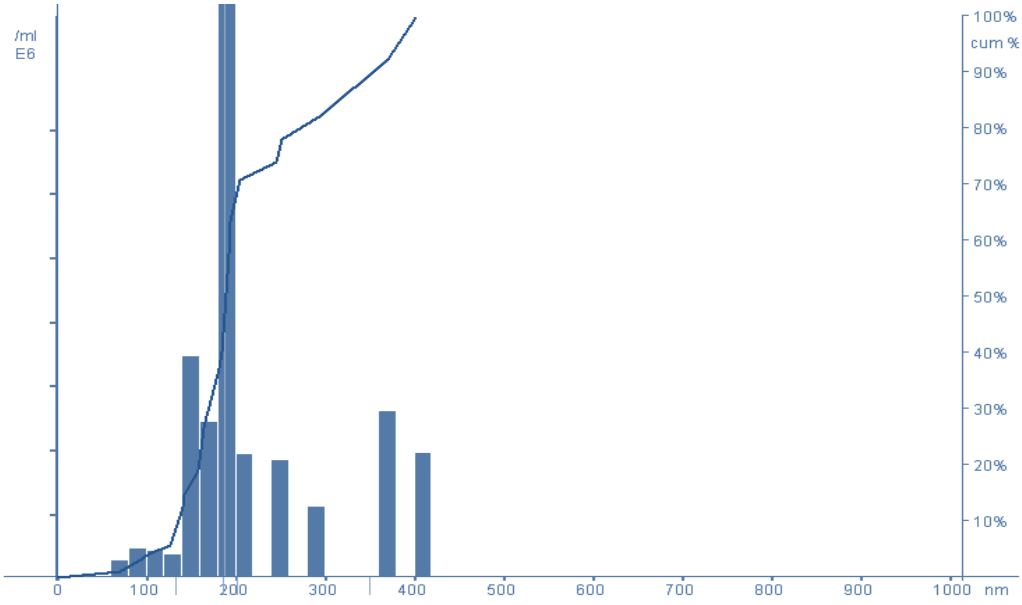


Figure 8 Bacteriophage particle sizes and concentrations plotted by the NanoSight.

CHAPTER 3
RESULTS AND DISCUSSION

First bacterial host growth and medium optimisation was investigated. The growth of the bacterial host with different medium components using shake flask culture under aerobic conditions and the Rapid Automated Bacterial Impedance Technique (RABIT) (static conditions) was studied with factorial experimental design. Examination of the effects of physical factors (temperature and initial pH of the medium) on the growth of the bacterial host was also made. Secondly, infection and lysis was investigated which involved the study of the factors influencing bacteriophage growth. Thirdly, bacteriophage immobilisation was examined in an investigation of a further contribution on a wound dressing for using treatment of bacterial infections including MRSA. Finally, bacteriophage production in true continuous culture was used for the production of bacteriophages. In this method continuous culture of bacteria as feedstock could be linked to continuous bacteriophages production.

3.1. Growth of the bacterial host cells and medium optimization

Bacterial host cells play an important role in the production of bacteriophages. Bacterial culture is a necessary precursor to bacteriophage production. *S. aureus* 8588, was used as a host bacterium as an example of pathogenic bacteria that will be used in commercial bacteriophages production. It is a facultative anaerobic bacterium which can be grown both in aerobic and anaerobic conditions. Initial experiments on bacterial host culture in both shake flask culture and RABIT method were carried out by varying medium components and concentrations and using factorial experimental design to minimise the number of experiments required for optimisation.

Minimal medium (M9) was chosen as basal medium for initial experiments. This is because it is a cost effective medium. M9 was modified by addition glucose, NH_4Cl and yeast extract. Glucose is as carbon and energy sources for growth of many

bacteria, NH_4Cl provides a source of nitrogen, while yeast extract also provides both nitrogen and carbon source for growth of *S. aureus*. Na_2HPO_4 and KH_2PO_4 play a role as a buffer against pH of the medium change due to metabolism. Moreover, Na_2HPO_4 and KH_2PO_4 supply the phosphorus atoms needed for nucleic acid synthesis as well as being needed for the functioning of many proteins. MgSO4 supplies the sulphur atoms needed to synthesize proteins whereas the magnesium atoms required in a variety of enzymatic reactions, including DNA replication.

3.1.1. Effect of culture conditions and medium components on growth of the bacterial host cells

In the process of medium optimisation it is necessary to know the effect of changing experiment conditions and the addition of different medium components on the growth of the bacteria. An experiment using factorial design allows the examination simultaneously of the effects of multiple independent variables and their degree of interaction. A factorial experiment was used to investigate two or more factors simultaneously in the same study. The treatments can be formed by combining the levels of the different factors. The advantages of factorial experiment design over one factor-at-a-time experiments is that it is highly efficient because every observation provides information about all the factors in the same study. Another important advantage, interactions between the factors can also be detected. An interaction provides the information regarding the relationship between the effects of different factors or the variation among the difference between means for different levels of one factor over different levels of the other factor (Montgomery, 2005; Snedecor & Cochran, 1980). Where many factors are involved and a full factorial design would be too large a fractional factorial design may be employed. In the experiments described here the number of factors is low and full factorial design is possible.

3.1.1.1. Effect of culture conditions and medium components with a two-level three-factor (2^3) factorial experiment design

Both the RABIT method and shake flask culture were carried out with three medium components; glucose, NH_4Cl and yeast extract, by using a two-level three-factor (2^3) experiment design (Davies, 1992) as shown in Table 4 as well as two different conditions; static and aerobic condition.

Table 4 Components of modified M9 media used for study on growth of bacterial host both in static and in aerobic condition in 2^3 experiment design.

Medium	Medium Components (g/l)		
	Glucose	NH_4Cl	Yeast extract
A	1.0	0.5	0.1
B	15.0	0.5	0.1
C	1.0	10.0	0.1
D	15.0	10.0	0.1
E	1.0	0.5	2.0
F	15.0	0.5	2.0
G	1.0	10.0	2.0
H	15.0	10.0	2.0

3.1.1.1.1. Growth of the bacterial host in static condition by using a RABIT (Rapid Automated Bacterial Impedance Technique) method

Growth of *S. aureus* 8588 in culture media in static condition were determined by using the RABIT method. Growth of *S. aureus* in various media was monitored by following conductance change values with time.

The RABIT method was developed by Don Whitley Scientific Ltd. Shipley, England, UK. This is a method to assessment microbial/bacterial growth by using an indirect impedance technique. In the indirect impedance technique, the CO_2 produced during bacterial growth reacts with potassium hydroxide (KOH) solution resulting in a reproducible change in conductance of the KOH. The direction of conductance change is negative. Growth takes place in media in a small tube located above the electrodes which are immersed in the KOH, the whole assembly being isolated within an outer tube to prevent the escape of CO_2. The indirect impedance method avoids the complications of direct conductance methods where the electrodes are placed directly in the growth medium and metabolites taken up or excreted by the bacteria affect the result in a complex and often unpredictable manner.

The advantages of the RABIT method are that it can be used for all CO_2-producing microorganisms, independent of their metabolism and the culture media. It is also a convenient and rapid method to monitor the growth of microorganisms without using basic microbial technique, for example plating count, which takes longer in determining and/or monitoring growth. (Dezenclos, Ascon-Cabrera, Ascon, Lebeault, & Pauss, 1994; Owens, Thomas, Thompson, & Timmerman, 1989).

RABIT cells preparation and cell culture method

The KOH solution was prepared by mixing equal volumes of molten 1% agar and 0.5% KOH solution. Then 0.75 ml of the mixture was added to each RABIT electrode tube, so that the tops of the electrodes protruded above the level of the solidified KOH agar. The tubes were tightly stoppered after cooling the agar and allowed to stabilize at room temperature before use.

Culture media were dispensed in 4.75 ml volumes in sterile, 12×75 mm glass tubes and inoculated with 0.25 ml of fresh overnight bacterial culture. The media were varied in composition as shown Table 4. The inoculated glass tubes were then placed inside the RABIT tubes. They rested on top of the protruding electrodes. The tube was promptly re-stoppered, placed in the RABIT incubator block, allowed to stabilize and reach the incubation temperature of 37°C then monitored for 24 h. Growth was monitored by conductance changes recorded at 6 min intervals and the magnitude of response (µS) and the maximum rate of change (µS/h) determined.

All tests were performed in 4 replicates and the mean results for the maximum conductance change and the rate of change of conductance determined.

Results from conductance changes in KOH agar due to adsorption of CO_2 produced by growth of *S. aureus* in different components of the medium as see in the Table 4 shown in Figure 9, indicates that conductance changes decreased dramatically in medium F and H, gradually decreased in medium D and B. Similar trends occurred in medium C, G and E, decreased slowly, whereas the conductance change in medium A was a very slightly decrease.

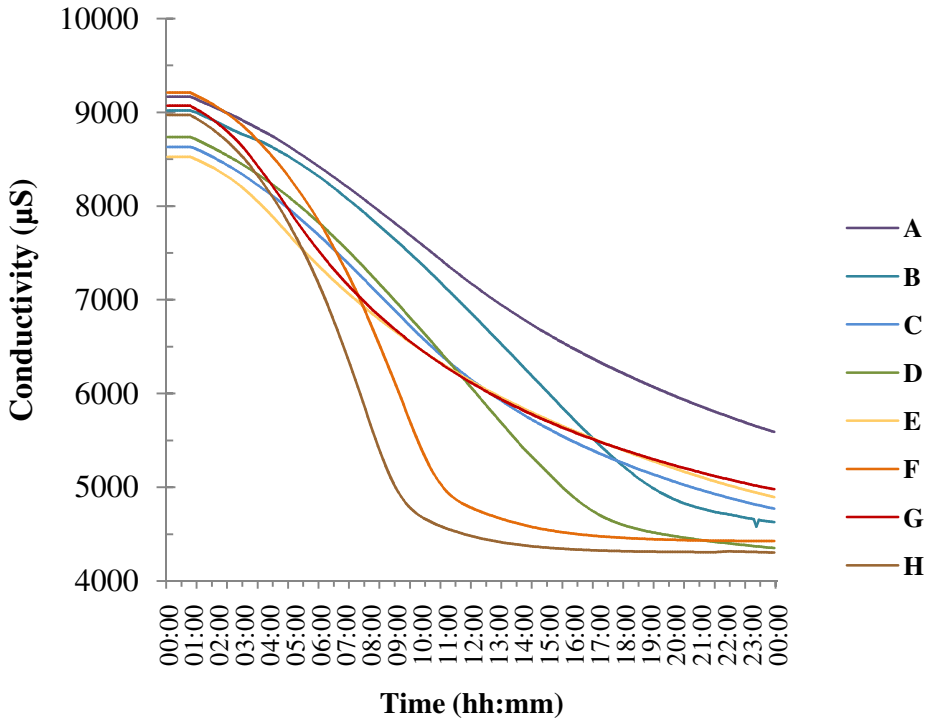


Figure 9 Conductance changes due to growth of *S. aureus* 8588 in modified M9 media at different medium components , as shown in the Table 4, culture in RABIT method in 2^3 experiment design.

Figure 10 shows cc
different media in th
was nearly identical a
E and A, respectively

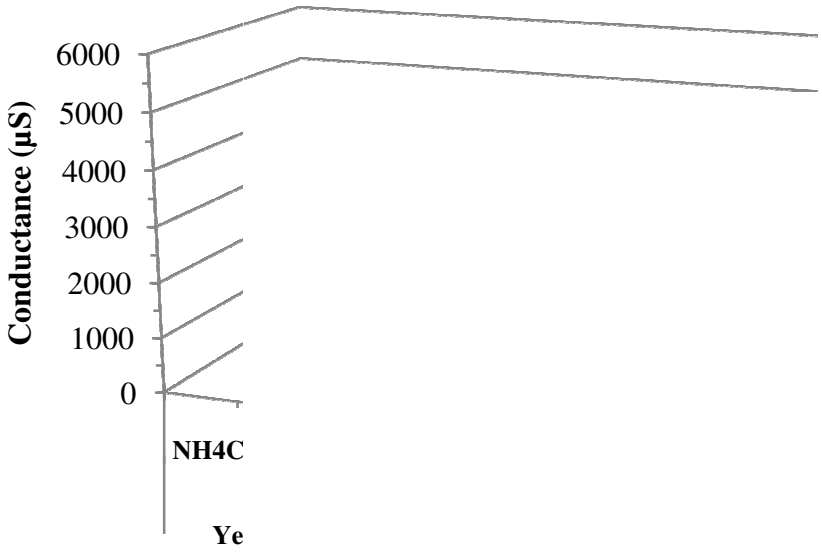


Figure 10 Conductar
media differ
RABIT metl

As data analysis by ANOVA shows Table 5, the main effect of glucose and yeast extract were highly significant (all have very small *P*-value) as well as the main effect of NH_4Cl was also significant. The interaction between glucose and NH_4Cl was also highly significant; thus there was strong interaction between glucose and NH_4Cl.

Table 5 Analysis of variance for conductance change values due to growth of *S. aureus* 8588 in modified M9 media in the RABIT method in 2^3 experiment designs.

Source of variation	***Sum of Squares***	***df***	***Mean Square***	***F_0***	***p-value***
Glucose	4721664.50	1	4721664.50	107.98	0.000
NH_4Cl	195312.50	1	195312.50	4.47	0.045
Yeast extract	480200.00	1	480200.00	10.98	0.003
Glucose*NH_4Cl	399171.13	1	399171.13	9.13	0.006
Glucose*Yeast extract	68265.13	1	68265.13	1.56	0.224
NH_4Cl*Yeast	5460.13	1	5460.13	0.13	0.727
Glucose*NH_4Cl*Yeast extract	46208.00	1	46208.00	1.06	0.314
Error	1049443.50	24	43726.81		
Total	571764970.00	32			

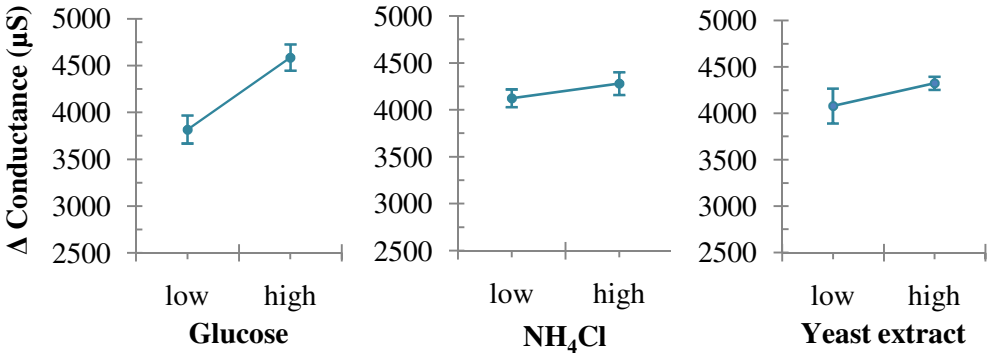
Figure 11 shows plots of main effect (a) and interaction (b) for conductance change due to growth of *S. aureus* in M9 medium in the RABIT method whereas Table 6 and Table 7 show means of conductance change for main effect and for the interaction between glucose and NH_4Cl on growth of *S. aureus* in M9 medium in the RABIT method.

Figure 11 (a) and Table 6 show means of conductance change for main effect on growth of *S. aureus* in M9 medium in the RABIT method. There was a significant different on effect of glucose on conductance, a different between over all means of 4585.31 and 3817.06, showing that at high level of glucose gave higher conductance value than at low level of glucose. This result means that a high level of glucose provides better growth of *S. aureus* than a low level of glucose. The similar result occurred both on effect of NH_4Cl and yeast extract on conductance. Effects of NH_4Cl, a different between over all means of 4279.31 and 4123.06, showing that at high level of NH_4Cl gave higher conductance value than at low level of NH_4Cl. This result means that a high level of NH_4Cl provides better growth of *S. aureus* than a low level of NH_4Cl. The effect of yeast extract shows a difference between over all means of 4323.69and 4078.69, showing that at high level of yeast extract gave higher conductance value than at low level of yeast extract. This result means that a high level of yeast extract provides better growth of *S. aureus* than a low level of yeast extract. Thus, all 3 main factor effects were positive, and if only these 3 main effects would be considered, all 3 factors at the high level to provide better growth.

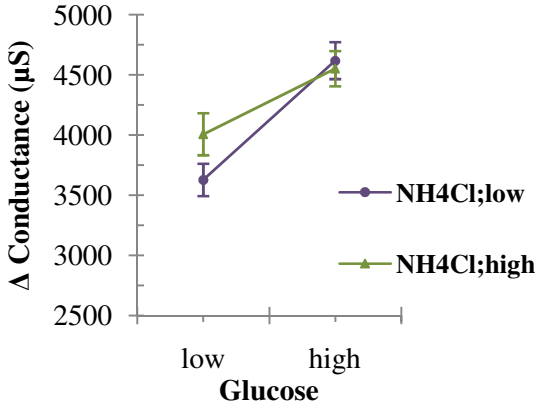
The interaction between glucose and NH_4Cl was plotted in Figure 11 (b). There was interaction between glucose and NH_4Cl which means that at any level of glucose, NH_4Cl had an effect on conductance change as well as at any level of NH_4Cl, glucose had an effect on conductance change. The means of conductance were shown in Table 7, indicates that at the low level of glucose, NH_4Cl had an effect on conductance changes, a different between over means of 4006.88 and 3627.25, showing that at high level of NH_4Cl gave higher conductance than at low level of NH_4Cl. This means that at low level of glucose, high level of NH_4Cl gave better growth than at low level of NH_4Cl. Furthermore, at high level of glucose there was no significantly different between over means of 4618.88 and 4551.75, showing that at high level of glucose, whether at high or at low level of NH_4Cl was not significant different. This means that at high level of glucose, level of NH_4Cl had no effect on conductance change which also means that there was no effect on the growth. However, at high level of glucose, at both high and low level of NH_4Cl, which conductance means of 4618.88 and 4551.75, gave higher conductance values than at low level of glucose at any level of NH_4Cl (means of 4006.88 and 3627.25) and there

were a significantly different among them (Table 7). This means that at high level of glucose, at any level of NH_4Cl also provides better growth of *S. aureus* than at low level of glucose at any level of NH_4Cl.

Thus, growth of *S. aureus* in static condition using the RABIT method in modified M9 media with 3 different medium components, which were glucose, NH_4Cl and yeast extract, was found that addition of only glucose at high level gave the best result as well as addition of glucose at high level with at low level of NH_4Cl gave another choice for growing this bacterium in static condition.



(a)`Main effect plots



(b)`Interaction plot

Figure 11 Main effect plots and interaction plot for conductance change due to growth of *S. aureus* 8588 in modified M9 media in the RABIT method in 2^3 experiment design.

Table 6 Means of conductance change for main effect on growth of *S. aureus* 8588 in modified M9 media in the RABIT method in 2^3 experiment design.[1]

	Levels	
Medium Components	Low	High
Glucose	3817.06±149 [b]	4585.31±140[a]
NH_4Cl	4123.06±95 [b]	4279.31±120[a]
Yeast extract	4078.69±188[b]	4323.69±70[a]

[a-b]Values within a row with different superscripts differ significantly ($P \leq 0.05$).

[1] Values represent the mean ± SD of 4 replications.

Table 7 Means of conductance change for the interaction between glucose and NH_4Cl on growth of *S. aureus* 8588 in modified M9 media in the RABIT method in 2^3 experiment design. [1]

Glucose/	NH_4Cl / Levels	
Levels	Low	High
Low	3627.25±134[c]	4006.88±175[b]
High	4618.88±153[a]	4551.75±147[a]

[a-c]Values the table above with different superscripts differ significantly ($P \leq 0.05$).

[1] Values represent the mean ± SD of 4 replications.

3.1.1.1.2. Growth of the bacterial host in aerobic condition by using shake flask culture method

Shake flask culture experiments were used to investigate the optimum conditions prior to studies in the fermenter. Shake flask culture is easy to manipulate, cost effective and allow large numbers of experiments (Macauley-Patrick, 2002).

Bacterial host cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml of sterile M9 medium varied in medium composition as shown Table 4 with 0.1% inoculum size. The cultures were then transferred and incubated to a orbital shaker (New Brunswick Scientific, C25KC Classic series, New Jersey) at speed 180 rpm 37°C for 24 hours. Bacterial growth was determined by measuring absorbance using a PU 8720 UV/VIS scanning Spectrophotometer at a wavelength of 650 nm. The bacterial cell numbers, which were presented as colony forming unit per millilitre (cfu/ml), were determined by calibration with the standard growth curve. Triplicate cultures were done.

In Figure 12**,** showing viability of *S. aureus* 8588 in different media in shake flask culture, it can be seen that viability of *S. aureus* in medium E, F, G and H, which were at high level of yeast extract, were better than that of in A, B, C and D which were at low level of yeast extract.

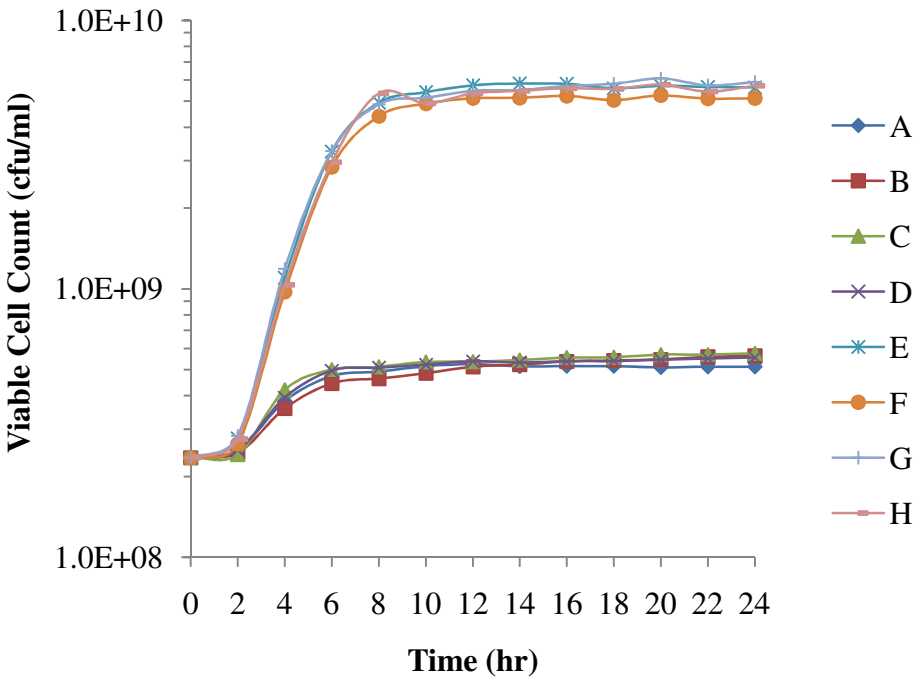


Figure 12 Viability of *S. aureus* 8588 in modified M9 media at various concentrations of medium components, as shown in the Table 4, in 2^3 experiment design.

Figure 13 shows maximum growth rate values of *S. aureus* 8588 in different media in shake flask culture. The growth rate of *S. aureus* in media at high level of yeast extract whether high/low of glucose or high/low of NH_4Cl gave better growth rate than that of at low level of yeast extract whether high/low of glucose or high/low of NH_4Cl.

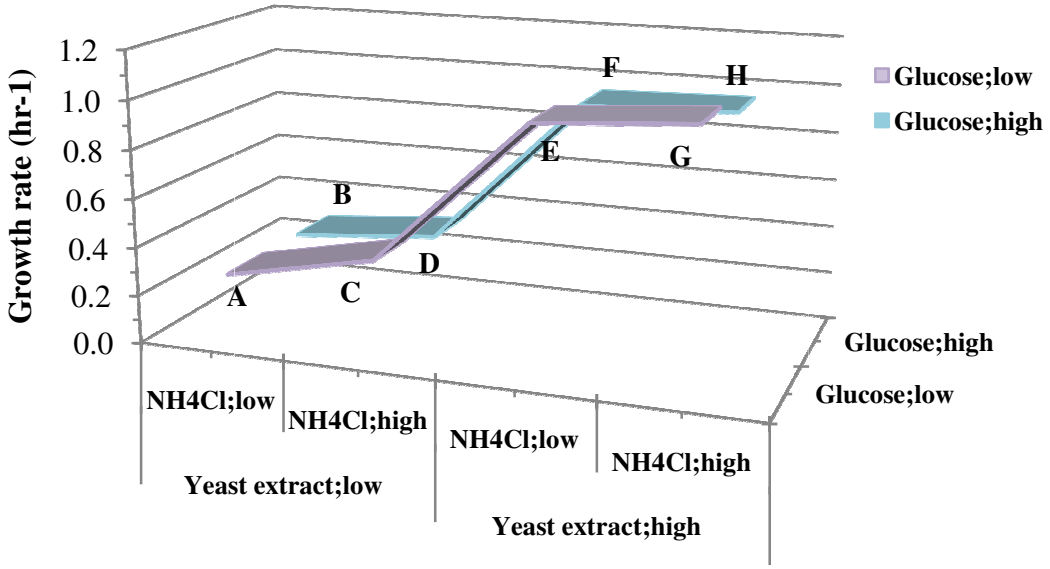


Figure 13 Maximum growth rate values of *S. aureus* 8588 in modified M9 media at various concentrations of medium components, as shown in the Table 4, in shake flask culture in 2^3 experiment design

As data analysis of maximum growth rate values by ANOVA shows Table 8, all the 3 main factors, glucose, NH_4Cl and yeast extract, were highly significant. There was no interaction.

Table 8 Analysis of variance for growth rate values of *S. aureus* 8588 in modified M9 media in shake flask culture in 2^3 experiment design.

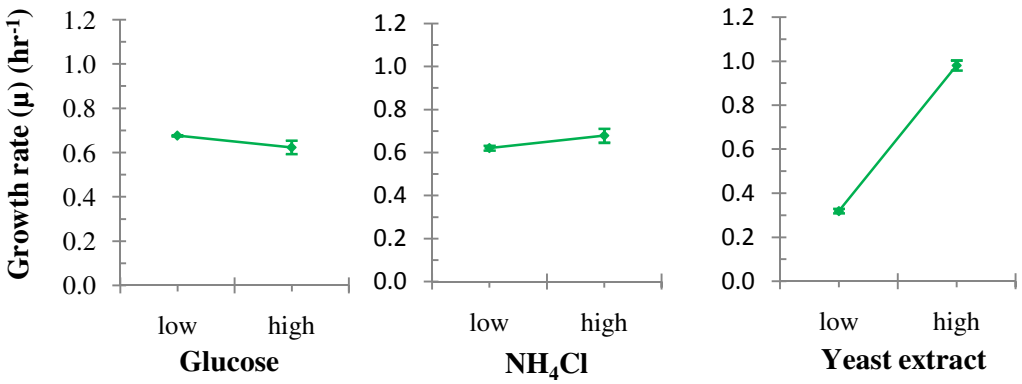
Source of Variation	***Sum of Squares***	***df***	***Mean Square***	***F_0***	***p-value***
Glucose	0.016	1	0.016	13.054	0.002
NH_4Cl	0.020	1	0.020	15.884	0.001
Yeast extract	2.624	1	2.624	2080.300	0.000
Glucose*NH_4Cl	0.004	1	0.004	2.869	0.110
Glucose*Yeast extract	0.001	1	0.001	1.163	0.297
NH_4Cl*Yeast extract	0.005	1	0.005	4.270	0.055
Glucose*NH_4Cl*Yeast extract	0.001	1	0.001	0.594	0.452
Error	0.020	16	0.001		
Total	12.814	24			

Figure 14 shows plots of main effect (a) for growth of *S. aureus* in modified M9 media in shake flask culture whereas Table 9 shows means of growth rate for main effect on growth of *S. aureus* in modified M9 media in shake flask culture.

Figure 14 (a) and Table 9 show means of growth rate for main effect on growth of *S. aureus* in modified M9 media in shake flask culture. There was a significant difference in the effect of glucose on growth rate, a different between over all means of 0.676 and 0.623, showing that low levels of glucose gave higher growth rates than at high levels of glucose. By contrast, the effects of NH_4Cl, a difference between over all means of 0.621and 0.678, showed that high levels of NH_4Cl gave higher

growth rates than low levels of NH_4Cl. Similar result were found with the effect of yeast extract on growth rates with high levels of yeast extract giving higher growth rate than low levels of yeast extract.

For growth of *S. aureus* in aerobic condition using shake flask culture in modified M9 media with 3 different medium components it was found that the addition of only yeast extract at high level gave the best result.



(a)`Main effect plots

Figure 14 Main effect plots and interaction plot for growth rates of *S. aureus* 8588 in M9 media shake flask culture in 2^3 experiment design.

Table 9 Growth rate of *S. aureus* 8588 in shake flask culture in 2^3 experiment design.[1]

Medium Components	Levels	
	Low	High
Glucose	0.676±0.003[a]	0.623±0.030[b]
NH_4Cl	0.621±0.011 [b]	0.678±0.032[a]
Yeast extract	0.319±0.010[b]	0.980±0.023[a]

[a-b]Values within a row with different superscripts differ significantly ($P \leq 0.05$).

[1] Values represent the mean ± SD of 3 replications.

Effect of growth conditions

It is clear that *S. aureus* is able to grow both in anaerobic condition and aerobic condition. The results showed that the best growth of *S. aureus* in static condition was by the addition of glucose at high levels with or without NH_4Cl whereas in aerobic condition it was only the addition of yeast extract at high level (2 g/l) that enhanced growth. However, growth under aerobic condition was greater (Baird-Parker, 1972) because aerobic growth has a higher efficiency in generating ATP from the energy sources than anaerobic growth.

Effect of glucose under anaerobic/ aerobic condition

As the result show in static condition, the effect of glucose on the growth of bacteria is highly significant. This suggests that glucose is utilised for growth in anaerobic conditions the condition. This is supported by the studies of Strasters and Winkler

(1963) which found that in anaerobic growth condition, *S. aureus* metabolises glucose to lactate (73-94%); acetate (4-7%) and traces of pyruvate are also formed. By contrast, in shake flask culture, effect of glucose on the growth of bacteria is still highly significant, but the effect of glucose is negative. Under aerobic growth condition in the presence of glucose, glycolysis is enhanced but by contrast many enzymes of the tricarboxylic acid (TCA) cycle are suppressed, for example succinate dehydrogenase is decreased and fumarase activity is not detectable. Moreover, pyruvate oxidation and the cytochrome content are decreased. This is the "glucose-mediated catabolite repression" or "glucose effect" in staphylococci (Collins & Lascelles, 1962; Gotz et al., 2006; Strasters & Winkler, 1963).

Effect of yeast extract in aerobic condition

In shake flask culture, effect of yeast extract at high level (2 g/l) gave the best growth of the bacteria. Yeast extract is derived from an aqueous extract of autolysis brewer's yeast. It consists of amino acids, peptides, nucleotides, vitamins; especially B complexes, growth factors and it has a high content of carbohydrates (Chae, Joo, & In, 2001; Harvey & McNeil, 2008; Lindan & Work, 1951). The results corresponded to Knight (1935), Fildes, Richardson, Knight, & Gladstone (1936), Gladstone (1937), Knight (1937a, 1937b), Porter & Pelczar (1940), Mah *et al.* (1967), Sober (1970), Wu & Bergdoll (1971), Baird-Parker (1972), Miller & Fung (1973), Kloos & Schleifer (1986) and Onoue & Mori (1997). They found that *S. aureus* requires a medium containing amino acids and growth factors; B group vitamins (Thiamine and nicotinic acid or nicotinamide) for aerobic growth.

The aim of any medium design for production purposes is normally centred on product formation with the highest yield and productivity and at the minimum cost (Ertola et al., 1995). In order to achieve this objective for the production of *S aureus*, yeast extract is one of a number of possible complex nitrogenous medium components.

3.1.1.2. Effect of medium components (continued)

A further experiment of *S. aureus* 8588 grown in shake flask culture was carried out to attempt to confirm the previous experiment.

3.1.1.2.1. A two-level two-factor (2^2) factorial experiment design

Shake flask culture was selected for medium optimization on growth of *S. aureus* 8588. Two of medium components; glucose and yeast extract, were observed by using a two-level two-factor (2^2) factorial experiment design (Davies, 1992) as shown in Table 10.

Table 10 Components of modified M9 media used for study on growth of bacterial host cells in shake flask culture in 2^2 experiment design.

Medium	Medium Components (g/l)	
	Glucose	Yeast extract
I	1.0	1.0
J	1.0	21.0
K	8.0	1.0
L	8.0	21.0

Bacterial host cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml of sterile M9 medium with medium components as shown Table 10 with 0.1% inoculum size. The cultures were then transferred and incubated to the orbital shaker at 180 rpm 37°C for 24 hours. Bacterial growth was determined by measuring absorbance at 650 nm. The bacterial cell numbers, presented in cfu/ml, were determined by reference to the standard growth curve. Triplicate cultures were done.

Results for the growth of *S. aureus* shown in Figure 15, indicate that the viability of *S. aureus* in media J and L, which were at high level of yeast extract, was better than that in medium I and K, which were at low level of yeast extract.

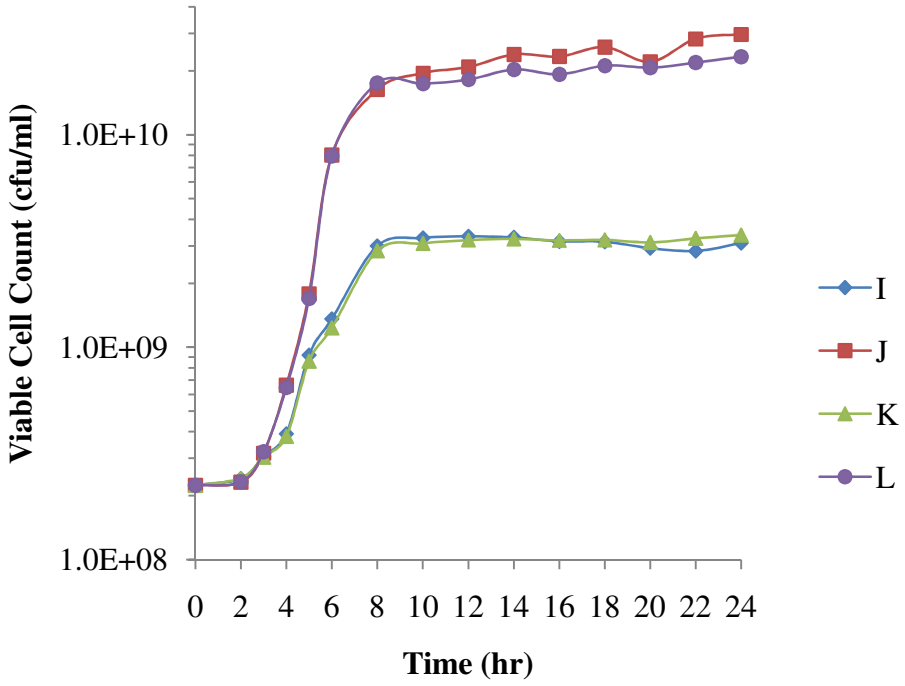


Figure 15 Viability of *S. aureus* 8588 in modified M9 media at various concentrations of medium components, as shown in Table 10, in 2^2 experiment design.

Figure 16 shows maximum growth rate values of *S. aureus* 8588 in different media in shake flask culture. The growth rate of *S. aureus* in media at high level of yeast extract whether high/low of glucose, which were media J and L, gave better growth rate than that of at low level of yeast extract whether high/low of glucose which were media I and K.

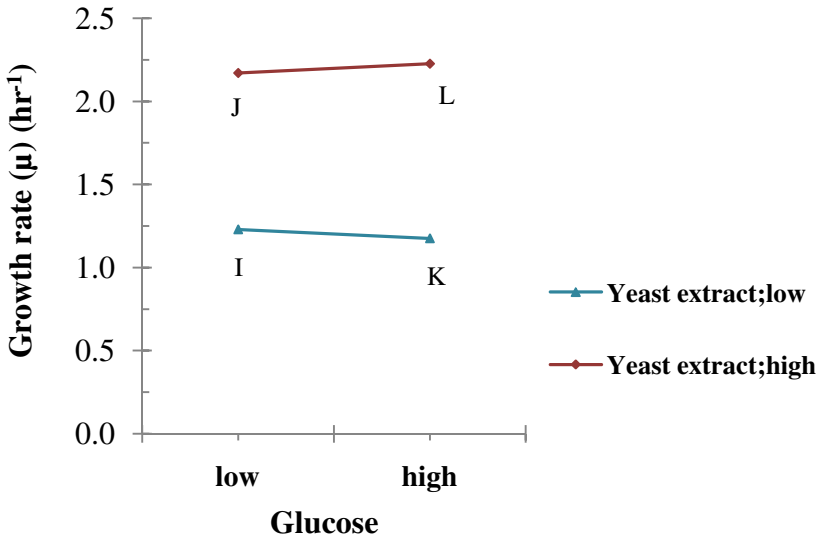


Figure 16 Maximum growth rates of *S. aureus* 8588 in modified M9 media at various concentrations of medium components, as shown in Table 10, in 2^2 experiment design.

As data analysis of maximum growth rate values by ANOVA show Table 11, yeast extract was the only main factor that was highly significant. An interaction between glucose and yeast extract was also significant.

Table 11 Analysis of variance for growth rate values of *S. aureus* 8588 in modified M9 media in shake flask culture in 2^2 experiment design.

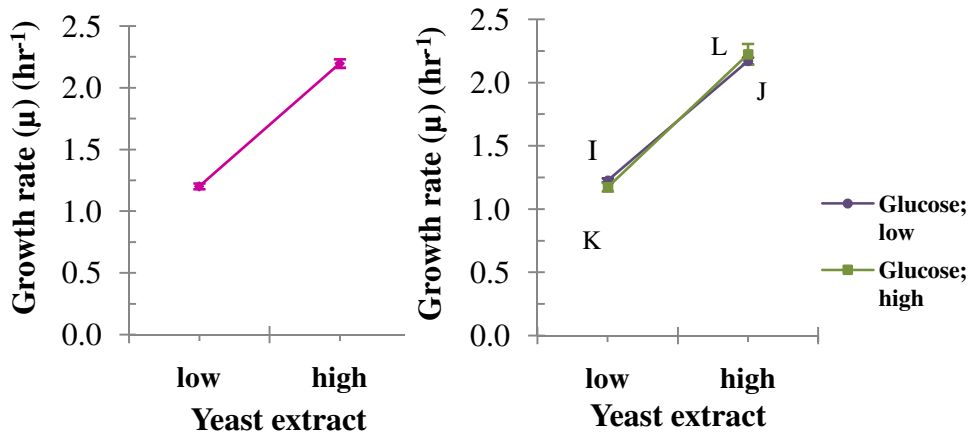
Source of Variation	***Sum of Squares***	***df***	***Mean Square***	***F_0***	***p-value***
Glucose	1.230E-07	1	1.230E-07	0.000	0.994
Yeast extract	3.973	1	3.973	1838.509	0.000
Glucose*Yeast extract	0.012	1	0.012	5.537	0.039
Error	0.026	12	0.002		
Total	50.271	16			

Figure 17 shows plot of main effect (a) and interaction (b) for growth of *S. aureus* in modified M9 media in shake flask culture in 2^2 experiment design whereas Table 12 and Table 13 show means of growth rate for main effect and the interaction between glucose and yeast extract on growth of *S. aureus* in modified M9 media in shake flask culture in 2^2 experiment design.

Figure 17 (a) and Table 12 show means of growth rates for the main effect on growth of *S. aureus* in modified M9 media in shake flask culture. There was a highly significant different on effect of yeast extract on growth rate, a different between over all means of 1.202 and 2.199, showing that high level of yeast extract gave higher growth rate than at low level of yeast extract.

The interaction between glucose and yeast extract was plotted in Figure 17 (b). There was interaction between glucose and yeast extract which means that at any level of

glucose, yeast extract had an effect on growth rate as well as at any level of yeast extract, glucose had an effect on growth rate. The means of growth rate are shown in Table 13, and indicate that at the low levels of yeast extract there was no significant difference between the means of 1.229 and 1.175. This means that at the low level of yeast extract the addition of glucose whether at low or high level had no effect on the growth of this bacterium. Furthermore, at high levels of yeast extract there was no significant difference between the means of 2.172 and 2.226. This means that at high level of yeast extract, addition of glucose whether at low or high level had no effect on growth of the bacterium. However, at high level of yeast extract, both at high and low level of glucose gave higher growth rate than at low level of yeast extract at any level of glucose and these were significantly different (Table 13).



(a) Main effect plot **(b) Interaction plot**

Figure 17 Main effect plot and interaction between glucose and yeast extract plot for growth of *S. aureus* 8588 in modified M9 media in shake flask culture in 2^2 experiment design.

Table 12 Growth rate of *S. aureus* 8588 in shake flask culture in 2^2 experiment design.[1]

	Levels	
Medium Components	Low	High
Yeast extract	1.202±0.023[b]	2.199±0.035[a]

[a-b] Values within a row with different superscripts differ significantly (P≤0.05).

[1] Values represent the mean ± SD of 4 replications.

Table 13 Growth rate of *S. aureus* 8588 in shake flask culture in 2^2 experiment design.[1]

Yeast extract/	Glucose/ Levels	
Levels	Low	High
Low	1.229±0.015[b]	1.175±0.033[b]
High	2.172±0.026[a]	2.226±0.081[a]

[a-c] Values the table above with different superscripts differ significantly (P≤0.05).

[1] Values represent the mean ± SD of 4 replications.

3.1.1.2.2. One factor experiment design

As previous experiment (section 3.1.1.2.1) showed that aerobic growth of *S. aureus* 8588 was dependent on amino acids or peptides from yeast extract, and that glucose was not metabolised. A further experiment with a commercial useful amino acid sources was designed.

Six different organic sources, providing both carbon and nitrogen in the media, were considered with varied concentrations.

The organic nitrogen sources used were yeast extract, tryptone, peptone, soy peptone, corn steep liquor and casein hydrolysate.

The organic nitrogen source concentrations used were 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l.

The method described as the following; bacterial host cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml of sterile M9 medium with medium compositions as above with 0.1% inoculum size. The cultures were then transferred and incubated to the orbital shaker at speed 180 rpm 37°C for 24 hours. Bacterial growth was determined by measuring absorbance at 650 nm. The bacterial cell numbers were determined with the standard growth curve. Triplicate cultures were done.

The growth of *S. aureus* 8588 in shake flask culture in M9 media with 6 different sources; yeast extract, tryptone, peptone, soy peptone, corn steep liquor and casein hydrolysate, and at 9 different concentrations (0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l) were carried out.

Results from the growth of *S. aureus* in media containing different sources of amino acid are shown in Figure 18-23.

From Figure 18, showing viability of *S. aureus* in shake flask culture at various concentrations of yeast extract, it can be seen that at lower concentrations of yeast extract the numbers of cell whether remained constant or dropped slightly before rising 2 hour after inoculation whereas at higher concentrations of yeast extract (50, 100 and 150 g/l) the lag phases were longer and took at least 4 hour before increase in cell numbers occurred. The log phase took around 4-8 hr at concentrations of 0.1, 1, 2 and 5 g/l and around 12 hr at concentrations of 10, 20, 50, 100 and 150 g/l. After that all trends remained constant with the highest numbers of bacterial cell in the stationary phase. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 3.28×10^{8}, 1.48×10^{9}, 2.95×10^{9}, 7.45×10^{9}, 1.83×10^{10}, 2.52×10^{10}, 3.27×10^{10}, 3.44×10^{10} and 3.17×10^{10}, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 2, 5, 12, 17, 22, 23 and 21 times higher than that at concentration of 1 g/l. Concentration of 50, 100 and 150 g/l showed the same number of cells..

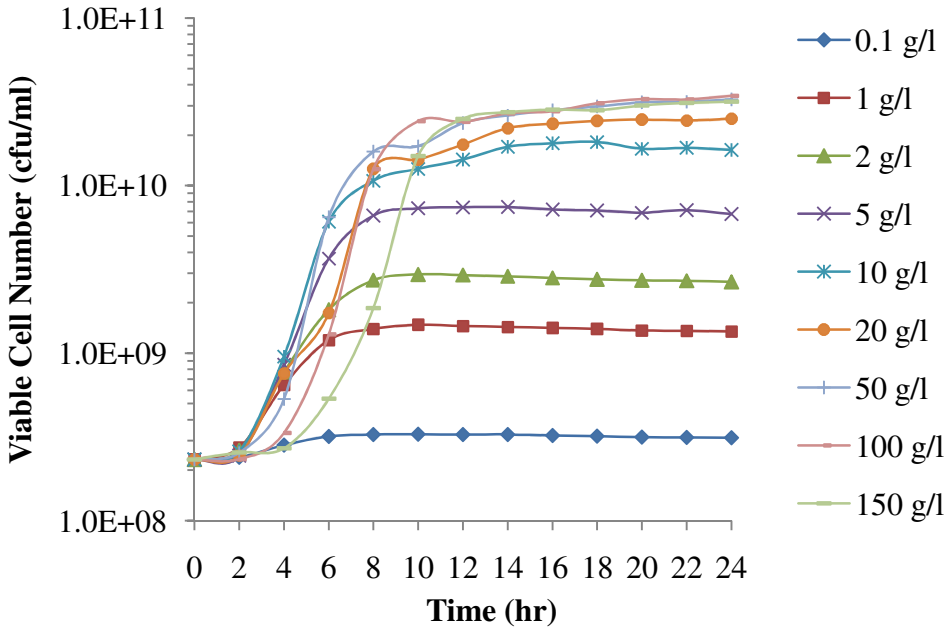


Figure 18 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of yeast extract

Figure 19 shows the viability of *S. aureus* in shake flask culture at various concentrations of tryptone. The lag phase period with tryptone showed a similar pattern to the lag phase periods of yeast extract, higher concentrations took longer to commence growth than lower concentrations. The log phase took around 8-10 hr at low concentrations (1, 2 and 5 g/l) and more than 12 hr at the concentrations of 10, 20, 50, 100 and 150 g/l. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 2.86×10^{8}, 9.48×10^{8}, 1.39×10^{9}, 4.02×10^{9}, 1.20×10^{10}, 2.12×10^{10}, 2.79×10^{10}, 3.33×10^{10} and 3.26×10^{10}, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 1.5, 4, 13, 22, 29, 35 and 34 times higher than that at concentration of 1 g/l. The trend was for increased growth with increased concentrations of tryptone, but with diminishing returns in terms of cell yield, for example increasing the concentration three times from 50 to 150g/l had little effect on cell numbers.

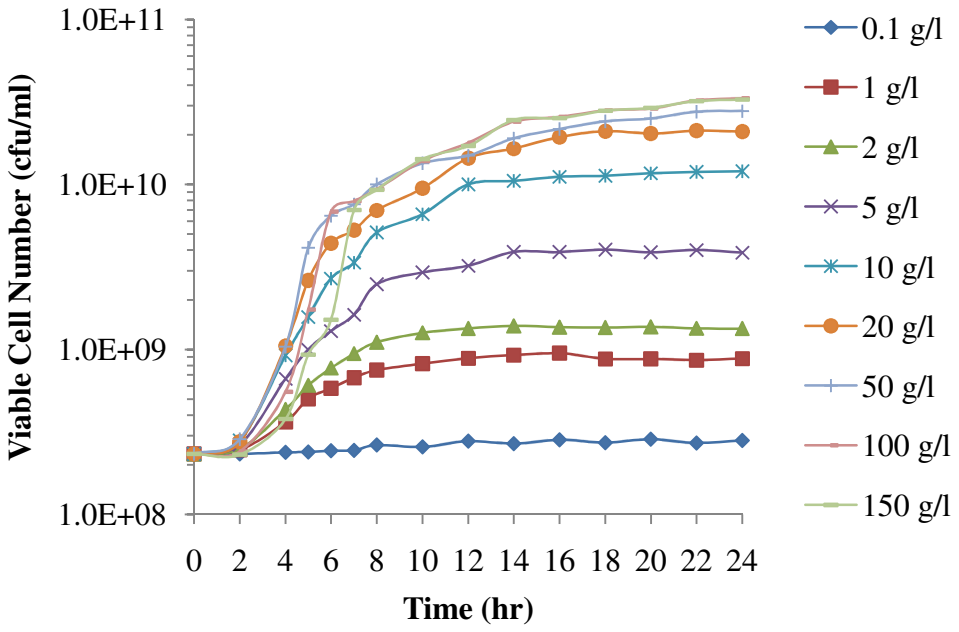


Figure 19 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of tryptone

Figure 20 shows viability of *S. aureus* in peptone medium. It can be seen that lag phase period of peptone shows a similar pattern to that preciously described. The log phase growth was similarly concentration dependent with a longer lag phase followed by a steeper and longer lag phase. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 2.75×10^{8}, 6.11×10^{8}, 8.66×10^{8}, 1.59×10^{9}, 3.02×10^{9}, 6.76×10^{9}, 2.47×10^{10}, 3.22×10^{10} and 3.16×10^{10}, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 1.4, 3, 5, 11, 40, 53 and 52 times higher than that at concentration of 1 g/l showing a decreasing efficiency of substrate to biomass conversion with increasing substrate concentration.

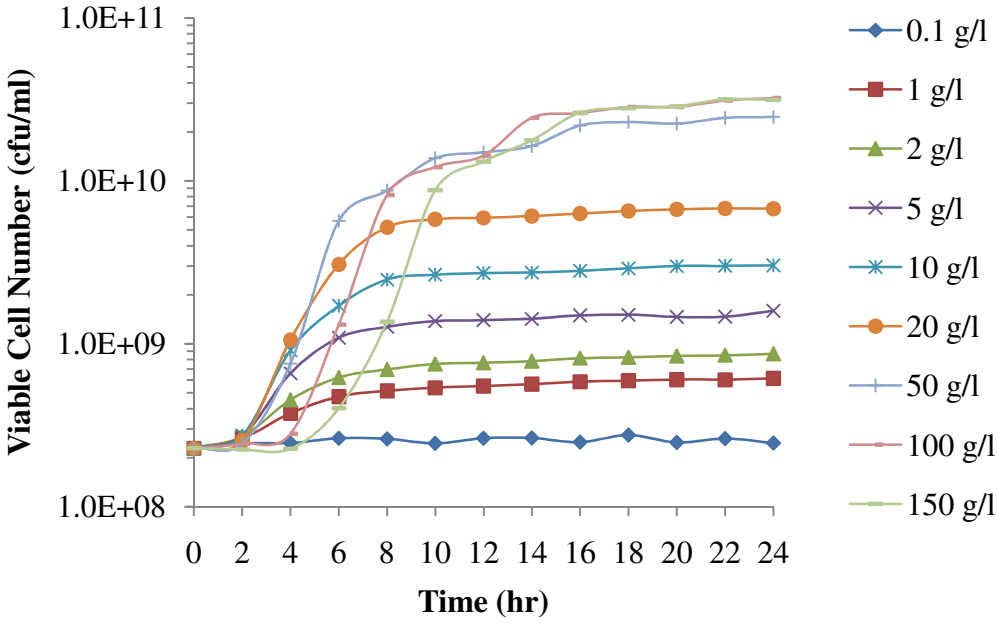


Figure 20 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of peptone

Figure 21, the viability of *S. aureus* in soy peptone, it can be seen that lag phase period of soy peptone showed a similar pattern to the lag phase of yeast extract, at the higher concentrations it took longer than at lower concentrations. The log phase similarly took around 8-10 hr at the concentrations of 1, 2, 5, 10 and 20 g/l and more than 12 hr at the concentrations of 50, 100 and 150 g/l. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 2.85×10^{8}, 9.56×10^{8}, 1.69×10^{9}, 4.86×10^{9}, 1.01×10^{10}, 1.96×10^{10}, 3.66×10^{10}, 4.87×10^{10} and 2.41×10^{10}, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 1.8, 5, 11, 21, 38, 51 and 25 times higher than that at concentration of 1 g/l.

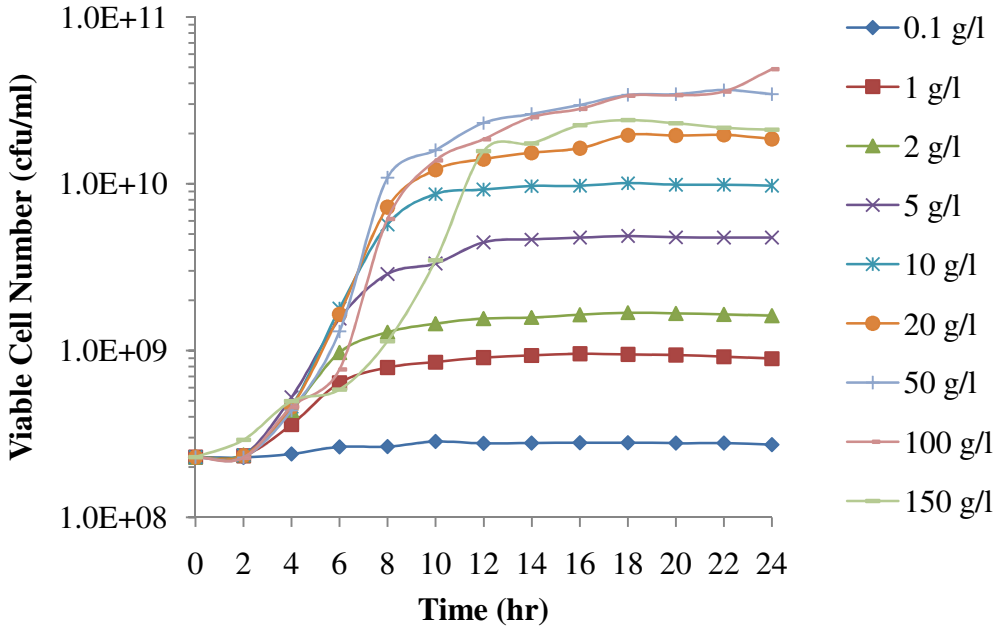


Figure 21 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of soy peptone

Figure 22 shows the viability of *S. aureus* in shake flask culture at various concentration of corn steep liquor. Here it can be seen that after inoculation at all concentrations the numbers of bacterial cell dropped. At concentration 0.1, 100 and 150 g/l, the numbers of cell slightly dropped and remained under the inoculation point, which means that there was no cell growth. A concentration of 50 g/l showed the better growth than that of 100 and 150 g/l. However at concentration of 1, 2, 5, 10 and 20 showed the better results than at concentration of 0.1, 50, 100 and 150 g/l. A concentration of 5 g/l gave the best growth and was also identical with 10 g/l followed by concentrations of 2, 20 and 1g/l, respectively. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 2.54×10^8, 5.44×10^8, 7.94×10^8, 1.28×10^9, 1.13×10^9, 7.32×10^8, 5.33×10^8, 2.34×10^8 and 2.90×10^8, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 1.5, 2.3, 2.1, 1.3, 1, 0.4 and 0.5 times higher than that at concentration of 1 g/l. Overall, all concentration of corn steep liquor gave less growth than other amino acid sources and the results suggest that inhibitory substances are present giving lower growth at higher substrate concentrations.

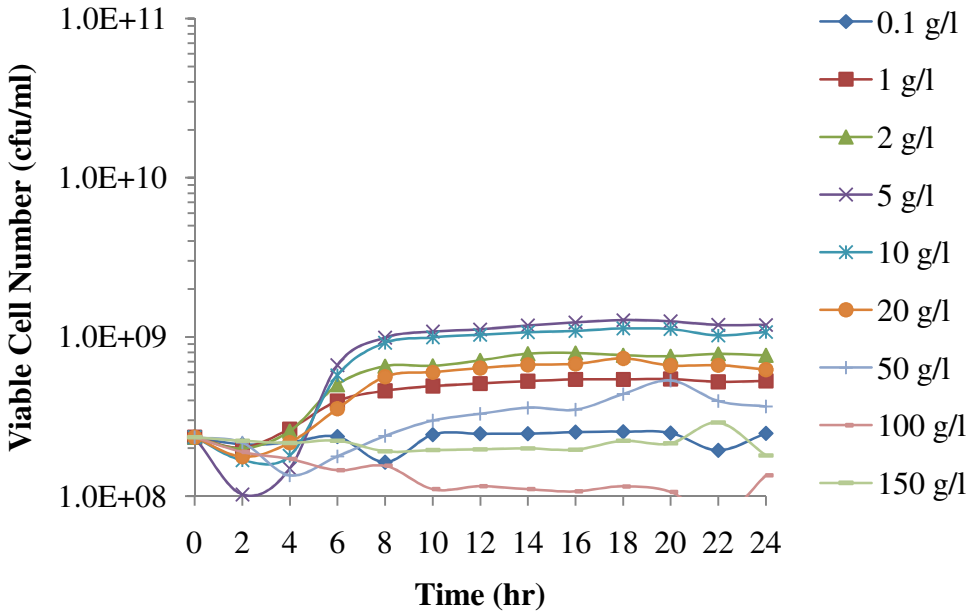


Figure 22 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of corn steep liquor.

Figure 23, shows the viability of *S. aureus* with casein hydrolysate. The curves are complex with the highest cell numbers being reached with a concentration of 20g/l but the viability shows discontinuities. Higher concentrations gave lower cell numbers with increasing concentration, indicating the presence of inhibitory substances. Concentrations lower than 20 g/l showed an increase in viability with increasing concentrations but again indicated the sequential use of sifferent components. The log phases of each concentration gradually increased to reach at their stationary phases and took around 10 hr at the concentrations of 1 and 2 g/l and 14 hr at the concentrations of 5 g/l and 16 hr at the concentrations of 10 and 20 g/l. The highest bacterial cell numbers at concentration of 0.1, 1, 2, 5, 10, 20, 50, 100 and 150 g/l were 2.67×10^{8}, 9.85×10^{8}, 2.09×10^{9}, 4.75×10^{9}, 1.03×10^{10}, 1.72×10^{10}, 1.02×10^{9}, 6.58×10^{8} and 4.33×10^{8}, respectively. The highest bacterial cell numbers at concentration of 2, 5, 10, 20, 50, 100 and 150 g/l were approximately 2, 5, 10, 17, 1, 0.7 and 0.4 times higher than that at concentration of 1 g/l.

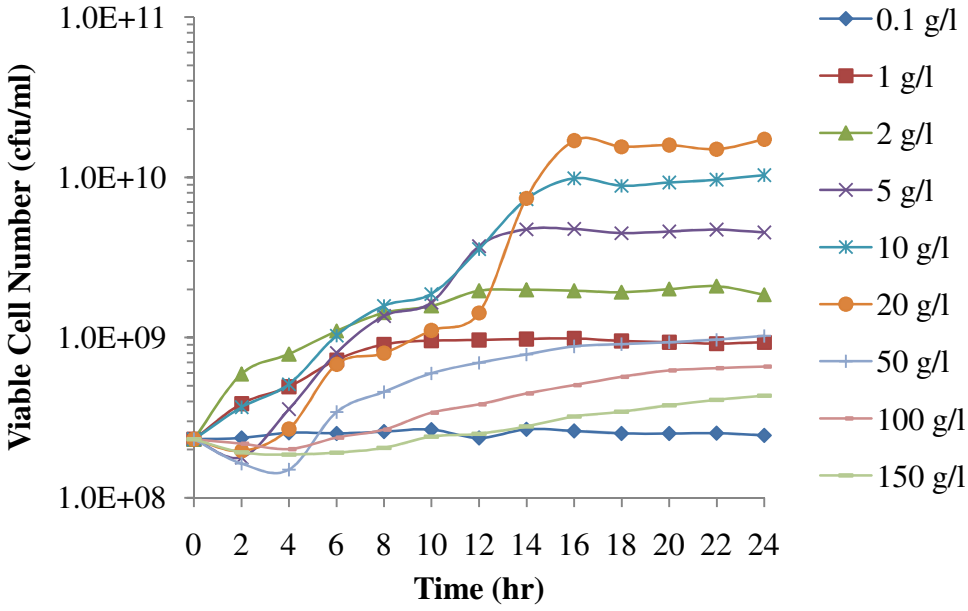


Figure 23 Viability of *S. aureus* 8588 culture in modified M9 media containing various concentrations of casein hydrolysate.

All sources of amino acids were able to support the growth of the bacteria. However, the sources contain amino acids with different qualitative and quantitative content; in yeast extract reported by Chae et al.(2001) and Lindan & Work (1951), in tryptone reported by Clausen, Gildberg, & Raa (1985), in peptone by (Loginova, Manuilova, & Tolstikov (1974), in soy peptone by Marshall (2008), in corn steep liquor by Cardinal & Hedrick (xxxx), in casein hydrolysate by Pedroche et al. (2004) and Gea, Bai, Yuan, & Zhang (1996).

It was also found that for any amino acid source at the lower concentrations were likely to reach stationary phase quicker than at the higher concentrations. This is because at the lower concentrations the substrates were exhausted earlier than at the higher concentrations. In the other hand, at higher concentrations the log phase of growth tended to increase. This is because bacteria cells was likely to grow better when more substrate availability and give a higher growth rate than at lower concentration during period of logarithm phase. This is correspond to the fact that growth rate is proportional to the substrate concentration when this is low but reaching a limiting saturation value at high substrate concentrations according to the equation 2.6 of Monod's (Monod, 1949).

As Figure 24**,** the plot of concentration of amino acid sources against the maximum growth rate of the bacteria, shows similar trends occurred in yeast extract, tryptone, peptone and soy peptone. At low concentrations, 0.1-10 g/l, the specific growth rate is a function of the substrate concentration but at high concentrations, from 20 g/l onwards, the specific growth rate is independent of the concentration of nutrient. At concentration of yeast extract, peptone and soy peptone 50 g/l gave the highest of specific growth rates but the growth rates decreased at concentrations of 100 and 150 g/l. For the utilisation of corn steep liquor the specific growth rate is a function of the substrate concentration at low concentration, at 1.0-10 g/l and at 10 g/l gave the highest growth rate. However, the growth rates dropped dramatically at 20 g/l and slightly dropped at 50, 100 and 150 g/l. For casein hydrolysate, the specific growth rate is a function of the substrate concentration at concentration of 1.0-5 g/l and then fluctuated at 10-20 g/l. At concentration of 20 g/l gave the highest growth rate. The growth rates dropped gradually at concentration of 50, 100 and 150 g/l.

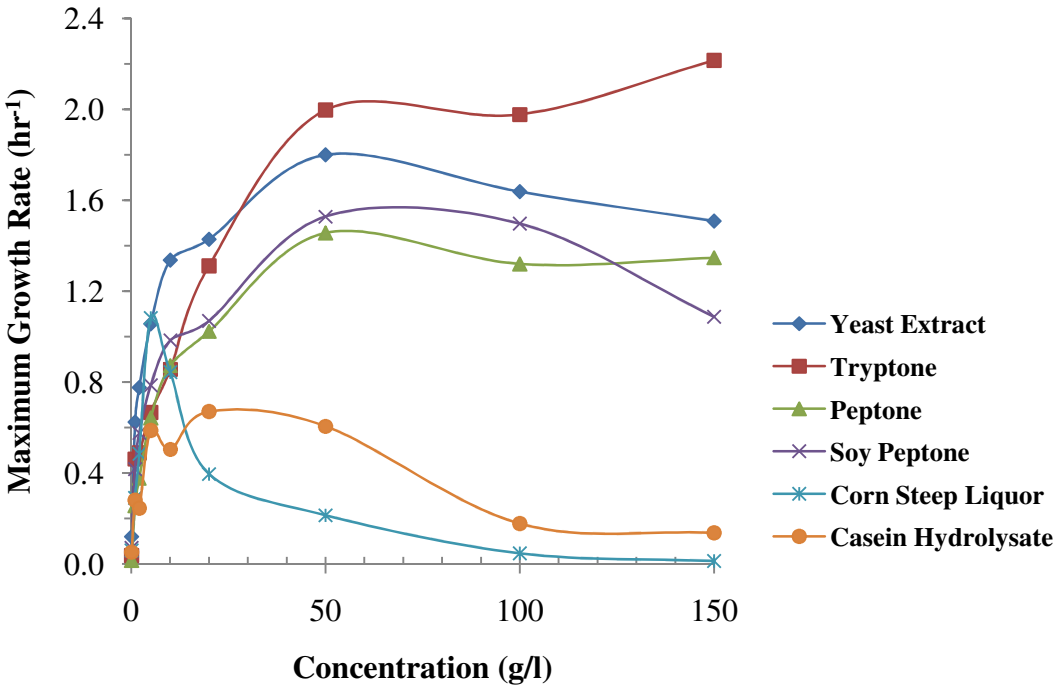


Figure 24 Maximum growth rates of *S. aureus* 8588 culture in modified M9 media containing various concentrations of six differnt organic substances.

However, as can be seen in Figure 24**,** the plot of concentration of amino acid source against the maximum growth rate of the bacteria shows that at low concentrations the specific growth rate is a function of the substrate concentration but at high concentrations the specific growth rate is independent of the concentration of nutrient as previously demonstrated by Monod (1942). This is because in the metabolic process an enzyme solution has a fixed number of active sites to which substrates can bind. At high concentrations of substrate, all active sites may be bound by substrates, as a result the enzymes are saturated. Another reason, this is due to high substrate concentrations lead to inhibition in the enzymatic reactions known as substrate inhibition (Shuler & Kargi, 2002)

Yeast extract was chosen for further investigations; investigation of physical factors on the growth effect, infection and lysis and bacteriophage production in continuous culture. This is because as Figure 16 shows in comparison yeast extract gave better slope of maximum growth rate value than others. This means that the bacteria culture in the medium containing yeast extract grow quicker, particularly in a range of a limiting saturation concentration of amino acid source, than other sources. Moreover, this medium also avoids extracts of animal origin, particularly bovine sources which present risk from transmission of infectious agents such as BSE (Harvey & McNeil, 2008; Merabishvili et al., 2009). Furthermore, to produce maximum bacteriophages needs a large quantity of bacteria host cells. If cell growth is the primary concern, the medium should be designed to provide the maximum biomass. The cells must therefore have plenty of carbon and nitrogen sourced as well as other essential nutrients (Harvey & McNeil, 2008) which could obtain from yeast extract. Another aspect, 5% of yeast extract was considered to be used further due to a reason of cost effective. To consideration, the values of x times of the highest bacterial cell number compare to that of 1 g/l of yeast extract, maximum growth rates and generation times of the bacterial culture in the media containing 2, 5 and 10 g/l of yeast extract are shown in Table 14.

Table 14 The x times of the highest cell numbers compare to that of 1 g/l yeast extract, maximum growth rates and generation times of the bacteria culture in the media containing yeast extract in shake flask culture.

Yeast extract (g/l)	x times of the highest cell numbers compare to that of 1 g/l yeast extract	Maximum growth rate (hr^{-1})	Generation time (min)
1	1	0.624	96
2	2	0.776	77
5	5	1.056	57
10	12	1.336	45

As shown in Table 14, interestingly, there is not much difference of generation time, which is time requires for cell division, between 5 and 10% yeast extract. So addition of 5% yeast extract should be better than addition of 10% yeast extract in aspect of economic.

3.1.2. Effect of physical factors on growth of the bacterial host

In order to study the effect of physical factors (temperature and the initial pH of media) on the growth of *S. aureus* 8588 in shake flask cultures in 500 ml Erlenmeyer flask containing 200 ml of modified M9 medium with 0.5% yeast extract were carried out.

3.1.2.1. Effect of the incubating temperature on growth of the bacterial host

The effect of incubating temperature on growth of *S. aureus*, shake flask culture in 500 ml Erlenmeyer flask containing 200 ml of modified M9 medium with 0.5% yeast extract were carried out at 4 different temperatures; 25, 30, 37 and 42ºC.

Bacterial host cultures were prepared in 500 ml Erlenmeyer flasks containing 200 ml of modified M9 medium (0.5% yeast extract) with a 2% inoculum size. The cultures were then transferred and incubated in an orbital shaker (New Brunswick Scientific, C25KC Classic series, New Jersey) at 180 rpm at temperatures of 25, 30, 37 and 42°C, for 12 hours. Bacterial growth was determined by measuring absorbance at 650 nm. The bacterial cell numbers were determined with the standard growth curve. Triplicate cultures were used for all the various temperatures.

The results show that the viability of *S. aureus* at temperature of 37ºC and 42ºC were identical and better than that of at temperature 30ºC or 25ºC (Figure 25).

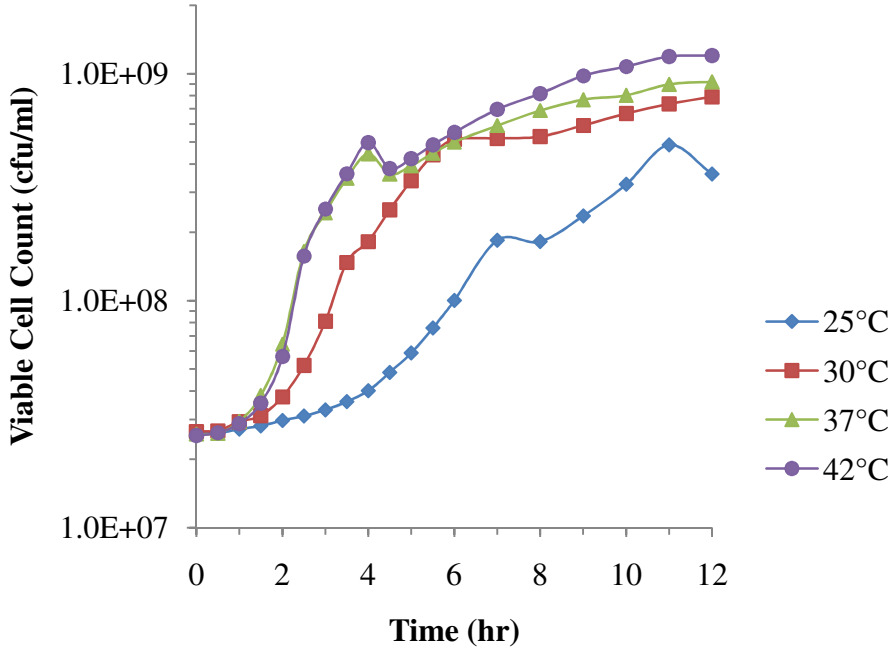


Figure 25 Viability of *S. aureus* 8588 in modified M9 media containing 0.5% yeast extract at various incubating temperatures

Analysis of the maximum growth rates values by ANOVA (shown in Table 15) show there was a highly significant difference. This means that temperature had an effect on growth of the bacteria as shown in Figure 26.

Table 15 Analysis of variance for growth rate values of *S. aureus* 8588 in modified M9 media in shake flask culture at different temperature.

Source of variation	***Sum of Squares***	***df***	***Mean Square***	F_0	***p-value***
Temperature	7.875	3	2.625	214.975	0.000
Error	0.098	8	0.012		
Total	59.507	12			

Figure 26 shows the maximum growth rates of the bacteria incubating at any temperature, found that there were significant difference among treatments. The maximum growth rates observed at incubating temperature 42ºC was slightly higher than that at 37°C but was not significantly different from that at 37ºC, but was significantly different from that at temperature 30ºC and 25ºC. Thus, 37ºC should be chosen for the best growth of the bacteria rather than at 42°C. In economic terms, the lower the growth temperature the better but this must be balanced against the growth rate. Moreover, Sillankorva et al. (2004) and Muller-Merbach et al. (2007) found that the optimal temperature of growth (also the highest growth rate) is the best temperature for bacteriophage infection, the lowest latent period and highest rates of cells lysis and phage release.

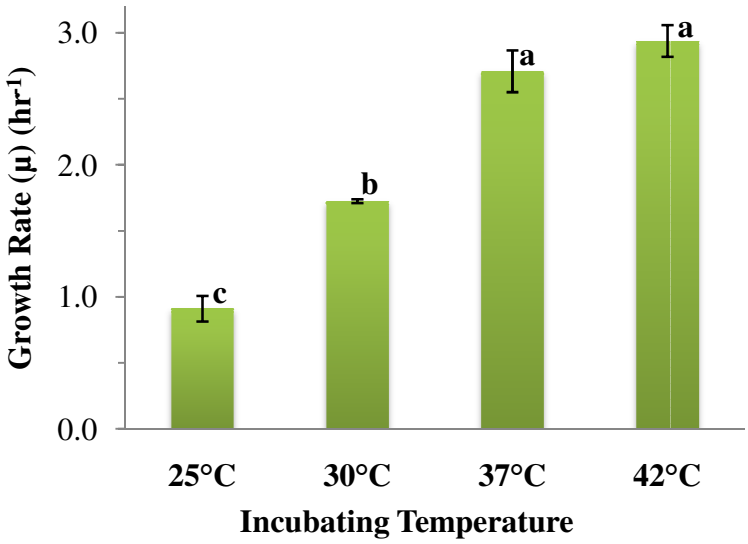


Figure 26 Maximum growth rates of *S. aureus* 8588 in modified M9 media containing 0.5% yeast extract at various incubating temperature

3.1.2.2. Effect of the initial pH of medium on growth of the bacterial host

The effect of initial pH on the growth of *S. aureus* in shake flask culture in 500 ml Erlenmeyer flask containing 200 ml of modified M9 medium with 0.5 % yeast extract were examined at 4 different initial pH values 5, 6, 7 and 8 as method mentioned below.

Bacterial host cultures were prepared in 500 ml Erlenmeyer flasks containing 200 ml of modified M9 medium (0.5% yeast extract), which were adjusted the initial pH values of 5.0, 6.0, 7.0 and 8.0, with a 2% inoculum size. The cultures were then transferred and incubated in the orbital shaker at 180 rpm, 37°C, for 12 hours. Bacterial growth was determined by measuring absorbance at 650 nm. The bacterial cell numbers were determined with the standard growth curve. Triplicate cultures were used for all the various temperatures.

Results of the viability curves of *S. aureus* are summarised in Figure 27 and indicate that the growth of *S. aureus* is virtually identical regardless of the initial pH.

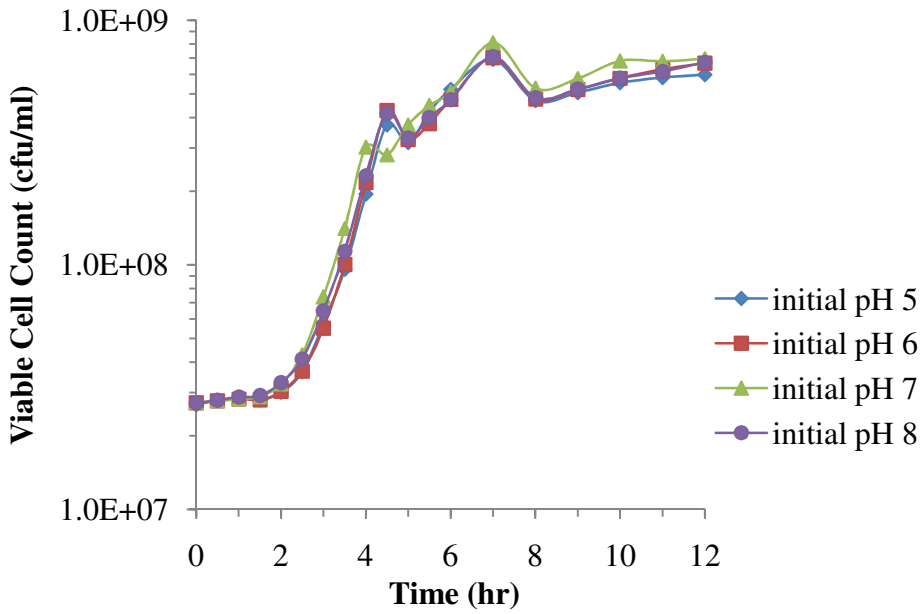


Figure 27 Viability of *S. aureus* 8588 in modified M9 media; 0.5% yeast extract at different initial pH of media

As data analysis of maximum growth rate values by ANOVA shown in Table 16, there was no significant different. This means that initial pH of media had no an effect on growth of the bacteria as shown in Figure 27.

Table 16 Analysis of variance for growth rate values of *S. aureus* 8588 in modified M9 media in shake flask culture at different initial pH.

Source of variation	***Sum of Squares***	***df***	***Mean Square***	***F_0***	***p-value***
initial pH value	0.107	3	0.036	1.004	0.440
Error	0.285	8	0.036		
Total	52.754	12			

Figure 28 shows the maximum growth rates of the bacteria incubated at the different initial pH values. The maximum growth rates observed at initial pH of media 7 was slightly high but was not significantly different to that of initial pH of media at 8, 6, and 5. This means that initial pH is not important, pH change during growth buffered by amino acid in the medium. Thus, it is unnecessary to concern on pH. Moreover, it also found that the initial pH of the medium prepared was around pH 7 without pH adjustment.

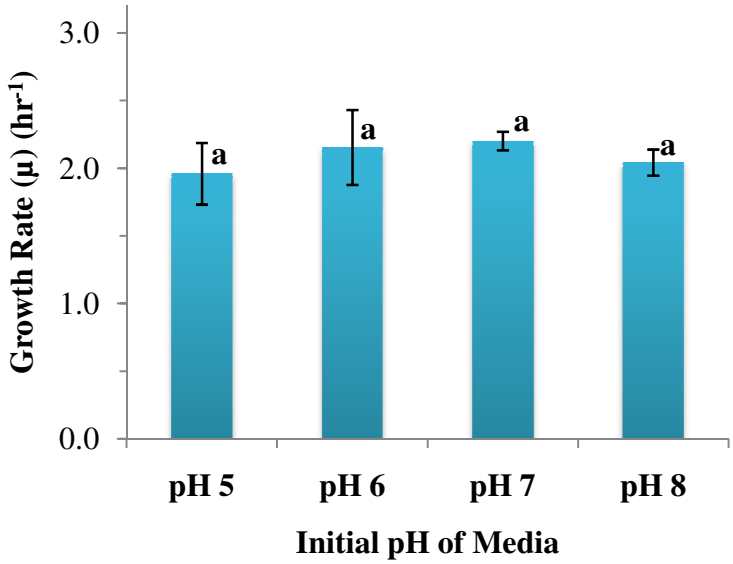


Figure 28 Maximum growth rates of *S. aureus* 8588 in modified M9 media containing 0.5% yeast extract at any initial pH of media

3.2 Infection and Lysis

Two major factors influencing infection and lysis are moi and temperature.

Two experiments were performed, first an experiment to investigate the influence of incubation temperature and multiplicity of infection (moi) on infection and lysis with results shown in Figure 29-34 and a second experiment to investigate influence of the latter factor on infection and lysis as shown in Figure 35.

The effect of host growth rate or medium composition on infection was not examined at this stage as continuous culture (see later) is the only reasonable method of altering growth rate and maintaining a constant growth rate for sufficient time to measure the effect or lysis.

3.2.1 Effect of incubating temperature and multiplicity of infection (moi) on bacteriophage infection

Infection was carried out at 6 different incubation temperatures: 15, 20, 25, 30, 35 and 37ºC. At any incubation temperature, infection was performed at 5 different moi; 0.01, 0.02, 0.1, 0.2 and 1.0, as described below.

Bacteriophages and bacterial host cells, which were in exponential growth phase, were mixed at various moi and dispensed into a 96 well plate with 200 µl of the mix culture per well and 5 replicates, were carried out. An uninfected bacterial culture acted as a no bacteriophage control. The 96 well plate of mix cultures were then immediately measured absorbance by using a plate reader (iEMS LabSystem, Finland) at a wavelength of 720 nm. After that the mix cultures were incubated in the Stuart orbital incubator speed 250 rpm at any temperature until complete lysis. Every an hour, the mix culture were measured. The curves were plotted between absorbance and time at any temperature incubated.

The mixed culture of bacteriophages and bacteria host at different moi's and at different incubation temperatures was measured by absorbance until completely lysis was achieved (results are shown in Figure 29-34).

Figure 29 shows infection at 37°C any different moi. Infection with bacteriophage at an moi of 0.01 and 0.02 produced a slightly reduction of the absorbance's compare to control. This is because the number of bacteria lysed in the first burst was too small a fraction of the total bacteria used in the experiment to be measured as a change in absorbance. The bacteriophages released in the first burst were free to infect more bacteria. These bacteriophages then infected and multiplied within the newly infected bacteria. This process was repeated until all the growing and uninfected bacteria in the medium were infected with the bacteriophages, eventually achieving complete lysis which was shown by the reduction the absorbance to constant levels. After the absorbance reached a maximum at absorbances of 0.703 and 0.625 at 2.5 and 2 hr for infection at moi of 0.01 and 0.02, respectively, there was a reduction of the absorbance and lysis was observed. Lysis was complete by 8 hr for both moi of 0.01 and 0.02. With infection at an moi of 0.1 and 0.2, there was a gradually decrease of the absorbance compared to the control. After the absorbance reached a maximum at 0.377 and 0.287 at 1 hr for both infection at 0.1 and 0.2, respectively, reduction of the absorbance and completely lysis achieved at around 4 and 3 hr for infections at moi of 0.1 and 0.2, respectively. Infection at an moi of 1 had an immediate effect on the absorbance with a very slightly increase in the absorbance to 0.112 for 1 hr post infection, followed by rapid and complete lysis within 2 hr.

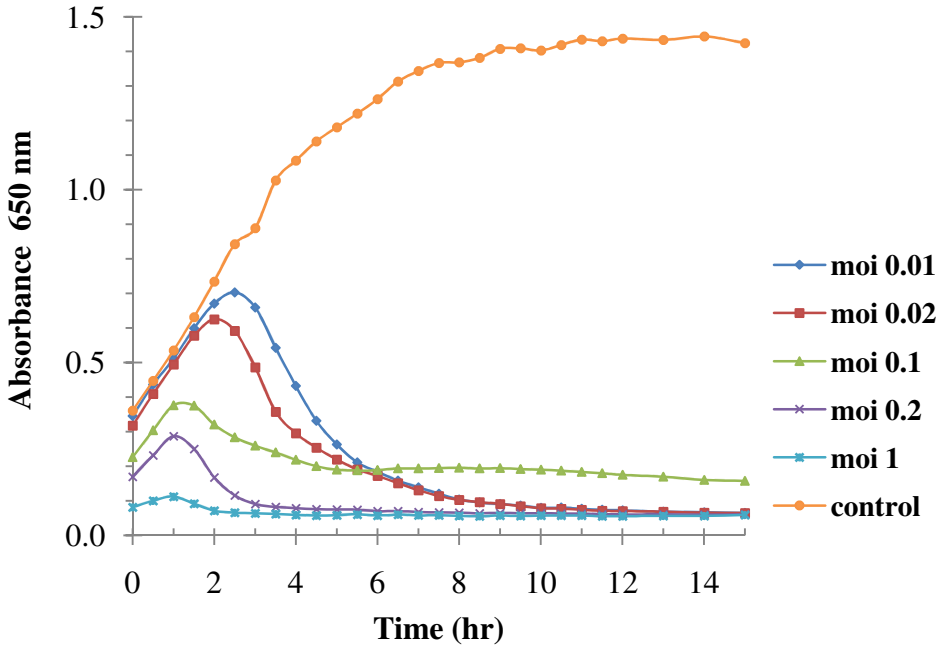


Figure 29 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 37°C, the bacteriophage added at t = 0 hr.

Figure 30 shows infection at 35°C different moi. Similar trends occurred as for infections at 37°C. Infection at moi of 0.01 and 0.02 produced a slightly reduction of the absorbance compared to the control. After the absorbance reached a peak of 0.990 and 0.871 at 3 and 2.5 hr for infection at moi of 0.01 and 0.02 respectively there reductions of the absorbance and lysis was observed at around 8 hr for both moi of 0.01 and 0.02. For infection at an moi of 0.1 and 0.2 there was a gradually decrease of the absorbance compared to the control. After the absorbance reached peaks at absorbance of 0.653 and 0.559 (at 1.5 hr for both of infection) at 0.1 and 0.2, respectively, lysis was around 5 hr for both at moi of 0.1 and 0.2. Infection at an moi of 1 had an immediate effect on the absorbance with a very slightly increase in the absorbance to 0.423 for 0.5 hr post infection, followed by rapid and complete lysis within 3 hr. However with infection at moi of 0.02, 0.1, 0.2 and 1, there were increases in absorbance after complete lysis suggesting bacterial growth possibly due to resistant bacteria in the culture.

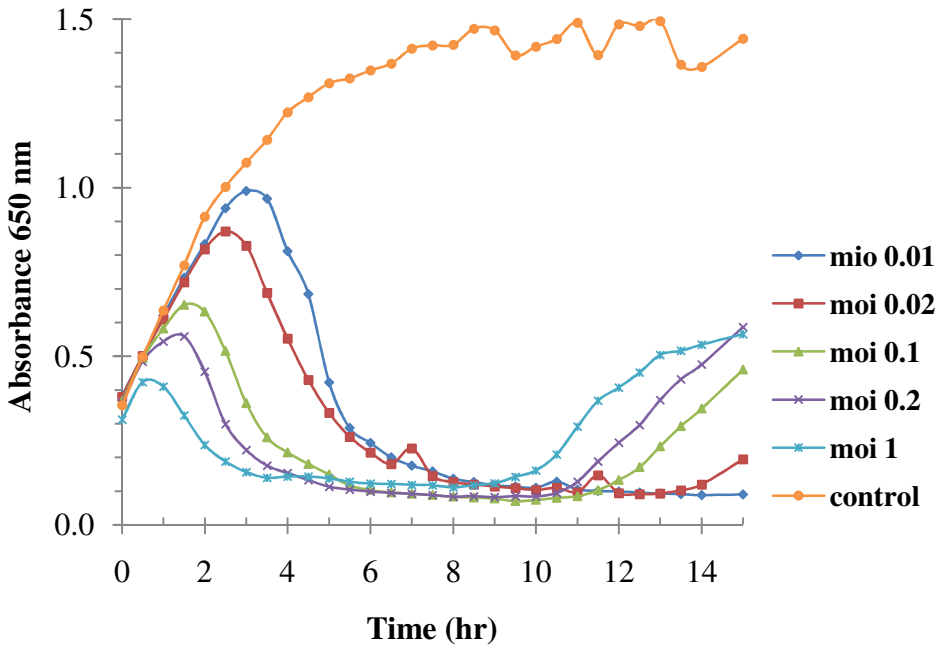


Figure 30 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 35°C, the bacteriophage added at t = 0 hr.

Figure 31 shows infection at 30°C at different moi. Similar trends occurred as in the infections at 37°C. Infection at moi of 0.01 and 0.02 produced a slightly reduction of the absorbance compared to control. After an absorbance maximum of 0.793 and 0.763 at 3.5 and 3 hr for infection at moi of 0.01 and 0.02 respectively lysis was complete at around 9 and 8 hr for at moi of 0.01 and 0.02 respectively. Infection at moi of 0.1 and 0.2 showed peaks at absorbance of 0.648 and 0.595 (at 2 hr for both infection) and lysis at around 5 hr. Infection at an moi of 1 had an immediate effect on the absorbance with a very slightly increase in the absorbance to 0.474 for 1.5 hr post infection, followed by rapid and complete lysis within 3.5 hr.

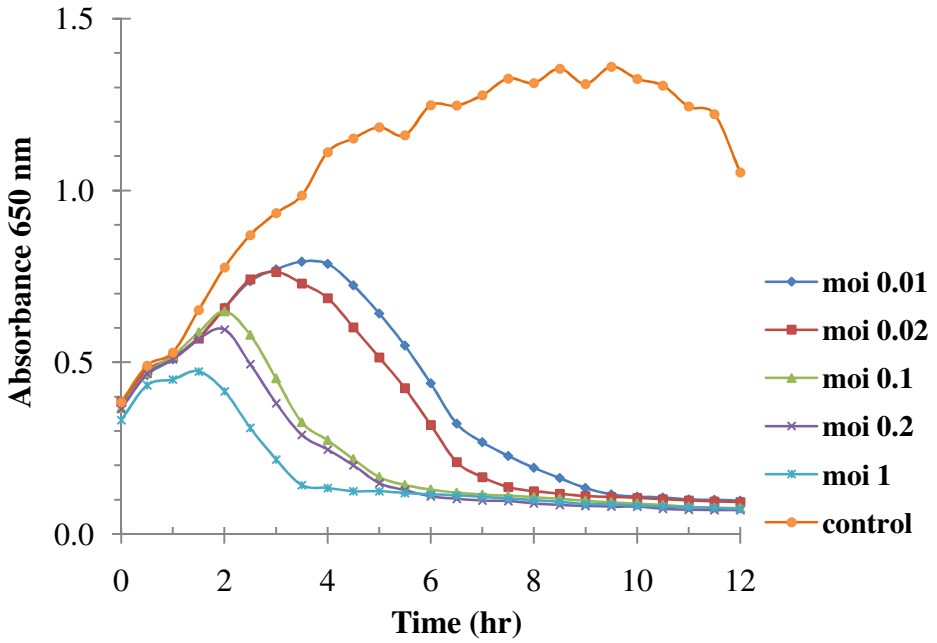


Figure 31 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 30°C, the bacteriophage added at t = 0 hr.

Figure 32 shows infection at 25°C any. Similar trends occurred again, but with a longer time for complete lysis.

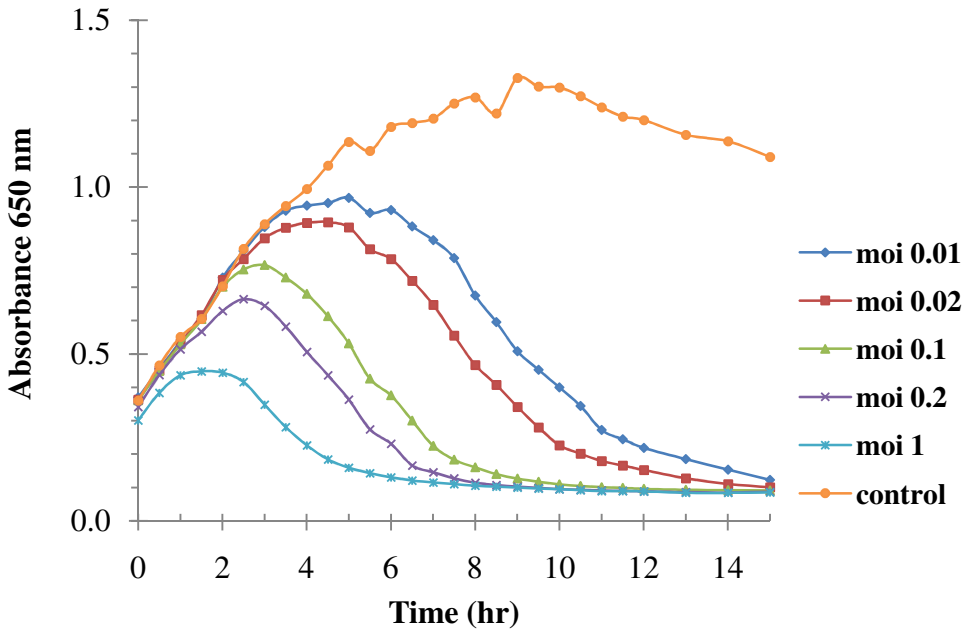


Figure 32 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 25°C, the bacteriophage added at t = 0 hr.

Figure 33 shows infection at 20°C. Similar trends occurred but both the peak values and complete lysis were even further delayed.

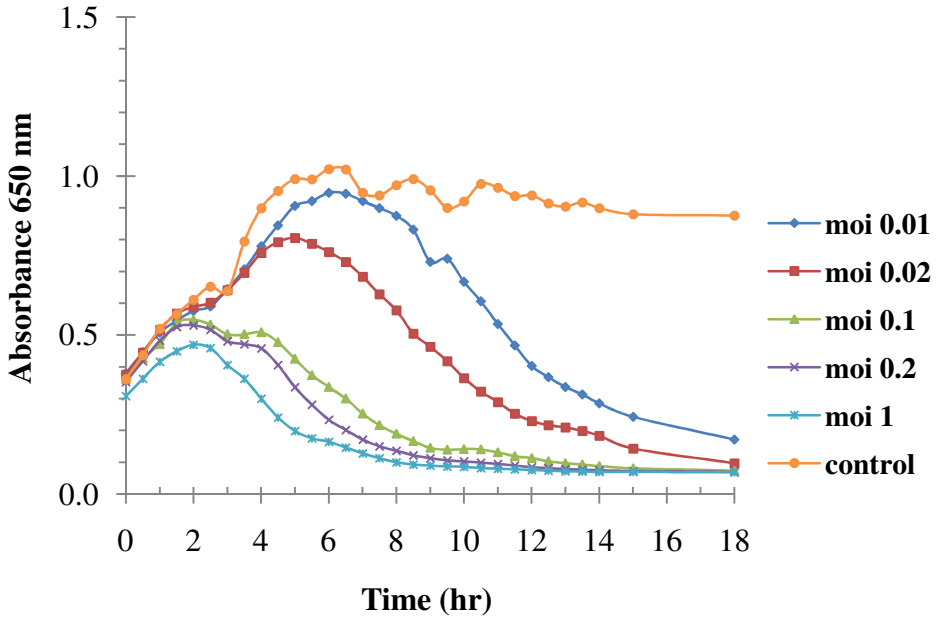


Figure 33 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 20°C, the bacteriophage added at t = 0 hr.

Figure 34 shows infection at 15°C. Infection at moi except 0.1, 0.2 and 1 showed little effect in the duration of the experiment with was little reduction of the absorbance compared to the control. Infection at moi of 0.1 and 0.2 produced a similar trends with a slight reduction compared to the control. The absorbance increased to about 0.8 with fluctuations until 10-18 hr after which a slight decline was seen but not complete lysis within 24 hr. Infection at an moi of 1 had an immediate effect on the absorbance with a very slightly increase in the absorbance to 0.474 for 1.5 hr post infection, Infection at moi of 1 absorbance reached a maximum at absorbance of 0.670 at 8.5 hr of infection, then lysis followed which was complete around 24 hr post infection.

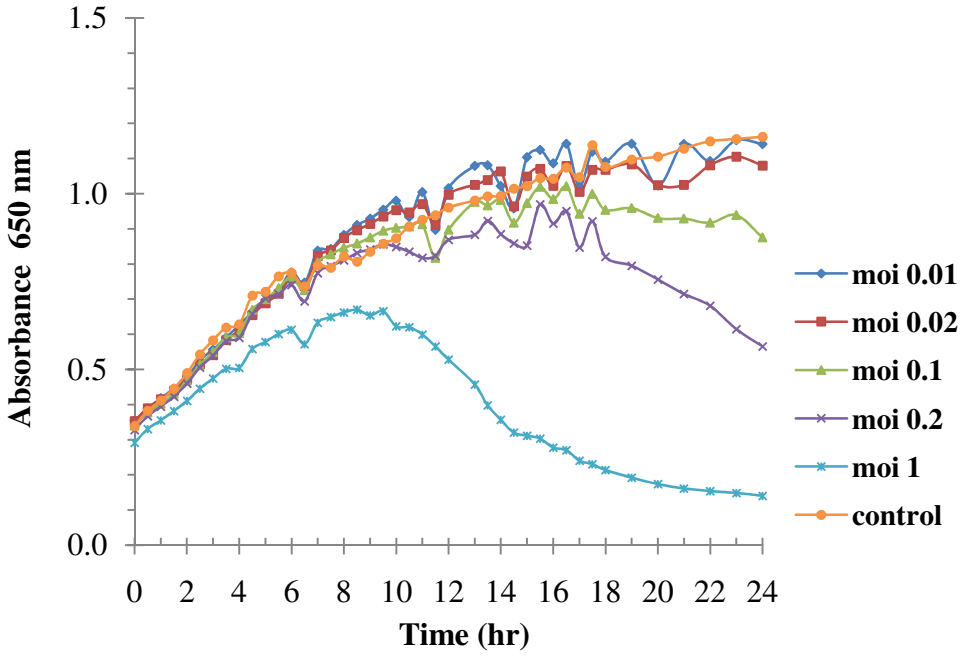


Figure 34 Bacteriophage infections at various multiplicity of infections (moi) incubating at 250 rpm 15°C, the bacteriophage added at t = 0 hr.

As the results showed that after infection at any incubation temperature or at any moi, the absorbance increased to reach a peak. After declining, the absorbance remained constant when complete lysis had been achieved. Similar trends occurred at any incubation temperature, except for the time to achieve complete lysis. At all incubation temperatures, infection at moi 1.0 had the shortest period of time for complete lysis followed by 0.2, 0.1, 0.02 and 0.01, respectively. These results obtained illustrate that complete lysis occurs more quickly with a higher moi. This is because a higher moi provides the higher number of bacterial host cells initially infected and then releases the higher number of new progeny in the first burst to infect more new host bacteria in second round and so on when compare to a lower moi. This result is similar to that reported by Petty et al. (2006) who found that infection at different moi showed that lysis occurred more quickly with a higher moi.

Considering the results of infection at different incubation temperatures, it was found that infection at 37ºC, at any level of moi, was virtually identical with that at 35ºC, and that complete lysis was more rapid than infections at 30, 25, 20 and 15ºC, respectively. The results obtained demonstrate that temperature had an influence on bacteriophage growth. Infection at higher incubation temperature, at which is not higher than the optimum temperature for bacterial host growth, seem to be the optimum for bacteriophage infection and lysis. This corresponds to the study of Ellis & Delbruck (1939) found that the latent period of bacteriophage infection varied with temperature in the same way as did the division period of the bacteria. Furthermore, increasing temperature (between 16.5, 25 and 37º) resulted in a decreasing latent period. The similar results were found by Sillankorva et al., (2004) and Muller-Merbach et al (2007).

Thus infection at higher incubation temperatures (at optimal growth) and at higher moi was likely to achieve complete lysis more quickly than infection at lower incubation temperature and at lower moi. The magnitude of the temperature effect increases with decreasing temperature so that at 15°C significant lysis will not occur unless an moi of 1 or more is used. This may simply reflect the doubling time which is about 2 hours above 30°C but rises to about 14 hours at 15°C.

3.2.2 Adsorption at different concentrations of the bacterial host cell

The effect of the concentration of bacterial host cells on bacteriophage adsorption was investigated. The experiments were carried out by adsorption at different concentrations of bacterial host cell incubating at room temperature and sampling at 5 min intervals. Different concentrations of bacterial host cell were achieved by changing the final volume of suspension culture of bacterial host and bacteriophage, which means that the relative number of bacterial host cell and bacteriophage remained constant. By changing the final volume of the suspension culture, the concentration of bacterial host cell was changed. The experiments were done at 3 different concentrations of bacterial host cell and bacteriophages; 0.1, 1 and 10, with the same ratio bacteriophage and bacteria, as in the method described below and the result shown in Figure 35

[A] Adsorption at 1 time concentration [1x of volume of whole contents]

A 40 ml of bacterial host in its exponential growth phase was infected with the bacteriophage at density 10^3 pfu/ml. The same amount of bacteriophage was added to an LB-only control. Samples (100 µl) were removed every 5 minutes for a total of 25 minutes and added to 900 µl PBS. Then they were mixed and immediately filtered through the 0.22 µm filter before washing as described below [D].

[B] Adsorption at 10 time more concentration [10x less volume of whole contents]

A 40 ml of bacterial host in its exponential growth phase was centrifuged at 4,000 rpm for 10 minutes. Then the pellet was re-suspended in 4 ml of fresh LB medium. The same amount of the bacteriophage was added, as used at adsorption 1 time concentration, and mixed. The same amount of bacteriophage was added to an LB-only control. Samples (100 µl) were removed every 5 minutes for a total of 25 minutes and added to 900 µl of PBS. Then they were mixed and immediately filtered through the 0.22 µm filter before washing as described below [D].

[C] Adsorption at 10 time less concentration [10x more volume of whole contents]

A 40 ml of bacterial host in its exponential growth phase was added up to 400 ml with fresh LB medium. The same amount of the bacteriophage was added, as used at adsorption 1 time concentration, and mixed. The same amount of bacteriophage was added to an LB-only control. Samples (1,000 µl) were removed every 5 minutes for a total of 25 minutes. Then they were immediately filtered through the 0.22 µm filter before washing as described below [D].

[D] Washing of the filter on adsorption of free bacteriophage experiments

After filtering the filters were washed with 10 ml of PBS for 5 times to remove bacteriophage particles on the filters. Then the filters on which remained the infected bacteria was transferred onto an agar plate and 4 ml of molten top agar, with 100 µl of plating bacteria added, poured to cover the filters on the agar plate. The plates were swirled gently and allowed to set at room temperature before incubating without inversion at 37°C overnight. After removing the plates from the incubator, the number of "plaques" were counted and calculated in pfu/ml. These plaques represented an infected bacterium, not a bacteriophages as in the conventional plaque assay.

The number of infected bacteria was determined by the plaque assay. As shown in Figure 35, for all 3 concentrations of the bacterial host cell and the bacteriophages, the number of infected bacteria increased gradually and reached a maximum at 15 min after infection. The number of infected bacteria remained constant at 10 times less and 1 time concentration of the mixture but at 10 times more concentration of the mixture, the number of plaques decreased after 15 minutes. At 10 times more concentration of the mixture, the numbers of plaques at any time were higher than that of 1 time and 10 times less concentration of the mixture. This study illustrates that at higher concentrations of bacterial host cells, bacteriophages had higher chance to adsorb to uninfected host cells than at lower concentration of bacterial host cells. This result corresponds to studies of Delbruck (1940a, 1940b), Ellis & Delbruck (1939) and Shao & Wang (2008).

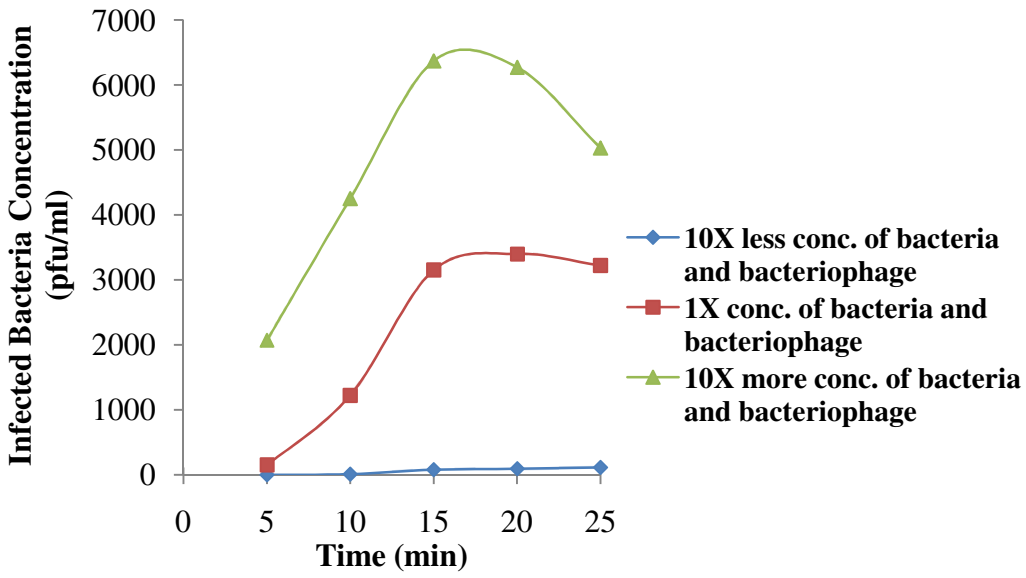


Figure 35 Infected bacteria concentration at various densities of the bacterial host and the bacteriophage inoculum with constant ratio of bacteria to bacteriophages (see section 3.2.2 *[A], [B]* and *[C]*).

Delbruck (1940a, 1940b) found that under optimum conditions (the bacteria used in logarithmic growth phase and aeration), bacteriophage adsorption at higher bacterial concentration was faster than at lower bacterial concentration which corresponded with the study by Shao & Wang (2008). Furthermore, Ellis & Delbruck (1939) also found that the adsorption rate was found to be first order with respect to the bacteriophage concentration and first order with respect to the bacterial host concentration. This study concluded that bacterial host density had an influence on adsorption step. The circumstance with high density of bacterial host cell, adsorption rate would be high, and vice versa. Moreover, it was found that for bacterial concentrations greater than 3×10^{7}cfu/ml adsorption was very fast; 70-90% attachment in 10 min. This means that adsorption under proper condition is rapid and complete.

As the results show, the number of bacteria with adsorbed bacteriophages increased rapidly within 15 minutes after infection and remained constant afterward. This may be because the number of free bacteriophages and the number of uninfected bacteria decrease, then decreasing the chance of bacteriophage and bacterial host cells meeting. The result suggests that it is unnecessary to incubate for longer than 15 min for period of adsorption step.

This study concludes that concentration of bacterial host had effect on bacteriophage adsorption in an environment with high concentration of bacterial host cells, adsorption rate would be high, and vice versa. This is because at a high concentration of bacteria, bacteriophages have highly chance to meet uninfected host cells and vice versa. Moreover, it also illustrate that adsorption step occurs very quickly so it is unnecessary to incubate for long time period. Another possible way increasing adsorption rate whether by decreasing a volume of the mixture of bacteria and bacteriophage in adsorption step or using as high as possible concentration of bacteriophage stock solution or decreasing a volume of the bacterial cell culture by centrifuging and re-suspending in a small volume before bacteriophage stock added, followed by dilution of the mixture to a large volume.

3.3. Bacteriophage Immobilisation

A method for bacteriophage immobilisation was examined by the corona discharge (as described in 2.8) using bacteriophage stock solution prepared by plate lysis. This section was a preliminary study for a further contribution on a wound dressing for using treatment of bacterial infections including MRSA.

A nylon membrane strip of 1cm × 5cm was treated with corona discharge to create reactive site on the surface of the nylon membrane strip. Then the nylon strip was immediately soaked in bacteriophage stock solution (7×10^7pfu/ml); which was prepared by plate lysis (as described in 2.6.2.1), to immobilise bacteriophages via covalent bonds. Then the immobilised bacteriophage nylon strip was washed remove unbound bacteriophages (section 2.8.2) and checked to confirm bacteriophage immobilisation (section 2.8.3). Prior to proceeding to further experiments the immobilised bacteriophage nylon strip was cut into 1cm × 1cm squares which were stored in SM at 4ºC until used.

3.3.1. Infection of bacteriophage immobilisation

To investigate infection by bacteriophages immobilised on nylon membrane strips was carried out as the method described below.

The immobilized bacteriophage strips (size of 1cm × 5cm) were prepared in the size of 1cm × 1cm and were stored in SM at 4°C until used

Two ml of bacteria in its exponential growth phase were dispensed into a series of universal tubes. Then the immobilized bacteriophage strips (1cm × 1cm) were put into the tubes at the same time. The contents were mixed and incubated at room temperature. Every 5 minutes for a total of 25 minutes, was taken and carried out on method as described below.

One ml of the mixed culture was removed and determined the number of bacteriophages by using plaque assay (code 1.1 of Table 17). Another 1 ml of the

mixed was further incubated for 30 minutes before determining the number of bacteriophages by using plaque assay (code 1.2 of Table 17).

The strip was washed with 5 ml of PBS twice and the washed PBS was collected for later testing. The strip was transferred into a new universal tube containing 1 ml LB medium. The contents were incubated at 37°C for 40 minutes to allow the infected bacteria lyse. After that the strip was removed and the culture (1 ml) was carried out on determination the number of bacteriophages by using plaque assay (code 2.1 of Table 17).

The washed PBS, which was collected (10 ml), was carried out as the following method. One ml of the washed PBS was determined the number of bacteriophages by using plaque assay (code 3.1 of Table 17). The rest of washed PBS was further incubated at 37°C for 40 minutes shaking to allow lysis and after that 1 ml of it was determined the number of bacteriophages by using plaque assay (code 3.2 of Table 17).

The immobilised bacteriophage strips (1cm × 1cm) were incubated with a bacterial host cell suspension culture in the exponential phase (2 ml). The immobilised bacteriophage strips were removed at 5 min interval and three outcomes for bacterial infection were considered, (1) that the infected bacteria fell off the strip after infection (code 1.1 and 1.2 of Table 17), (2) that the infected bacteria remained attached to the strip via the bacteriophages binding (code 2.1 of Table 17) and (3) that infected bacteria were washed off the strip during the PBS washing (code 3.1 and 3.2 of Table 17). The results are shown in Table 17.

Table 17 Infected bacteria by immobilised bacteriophages on the nylon membrane strips.

Code	***Condition***	***Plaque number (pfu/ml)***				
		Time (min)				
		5	10	15	20	25
	Suspension					
1.1	infected and fell off the strip	1	11	16	13	22
1.2	infected and fell off the strip further incubating 30 min	7	11	14	16	34
	The strip					
2.1	infected and stuck on the strip further incubating 40 min	300	80	160	30	380
	PBS washing					
3.1	infected and washed off the strip further incubating 40 min	20	20	20	0	340
3.2	the suspension from 3.1 further incubated for 30 min	95	38	627	38	760

Bacterial host cells infected by the immobilised bacteriophages which fell off the immobilised bacteriophage strip (Table 17 code 1.1 and 1.2) showed the number of "bacterial plaques" (pfu/ml) increased as function of time. Similar results were obtained when the rest (1ml) of the suspension culture was further incubated. The presumption was that the figures from the further incubation should be at least 20-30 times higher than the figures shown in code 1.1 because, after incubating for 30 min at 37ºC and 180 rpm, infected bacterial host cells should be lysed and released progeny (1 bacterium from 1.1 should give 20-30 progeny as average burst size), however this was not the result This may be because only partial lysis occurred after

further incubating or an individual burst size of each bacteriophage is lower than an average burst size.

Secondly, bacterial host cells infected by immobilised bacteriophages were still stuck on the immobilised bacteriophage strip (Table 17 code 2.1). After incubating for 40 min at 37ºC and 180 rpm, the number of bacteriophages fluctuated between 80 and 380 with no time trend. This means that there was no relationship between the numbers of bacterial host cell infected from the immobilised bacteriophage on the strip and incubating time duration.

Thirdly, bacterial host cells infected but which were washed off the immobilised bacteriophage strip with PBS (Table 17 code 3.1 and 3.2) showed that after adding an equal volume of double strength LB medium to the collected PBS washings and incubating for 40 min (37ºC, 180 rpm), the number of bacteriophage remained constant for 20 minutes then increased at 25 min of incubating period. Meanwhile the rest of the content was further incubated and the result shown that the numbers of bacteriophage fluctuated but showed some increase with time. In both cases, there was a poor relationship between the numbers of bacterial host cell infected from the immobilised bacteriophage on the strip and incubating time duration.

However, overall the results showed that the more time the immobilised bacteriophage strip was exposed to the suspension culture of bacterial host cells, the more bacterial host cells became infected (Figure 36). It also showed that some bacteria become infected by immobilised bacteriophages but either break the bacteriophages adhesion bond or the bond to the immobilisation substrate, while far more (10 to 20 times) are retained on the surface of the immobilised strip by the strength of the bacteriophage adhesion bond. Relatively vigorous washing with PBS can remove some (10-20%) of these “bound bacteria”. This raises the possibility that bacteria bound by bacteriophage adhesion to the strip may block the access to potentially infective bacteriophages on the strip.

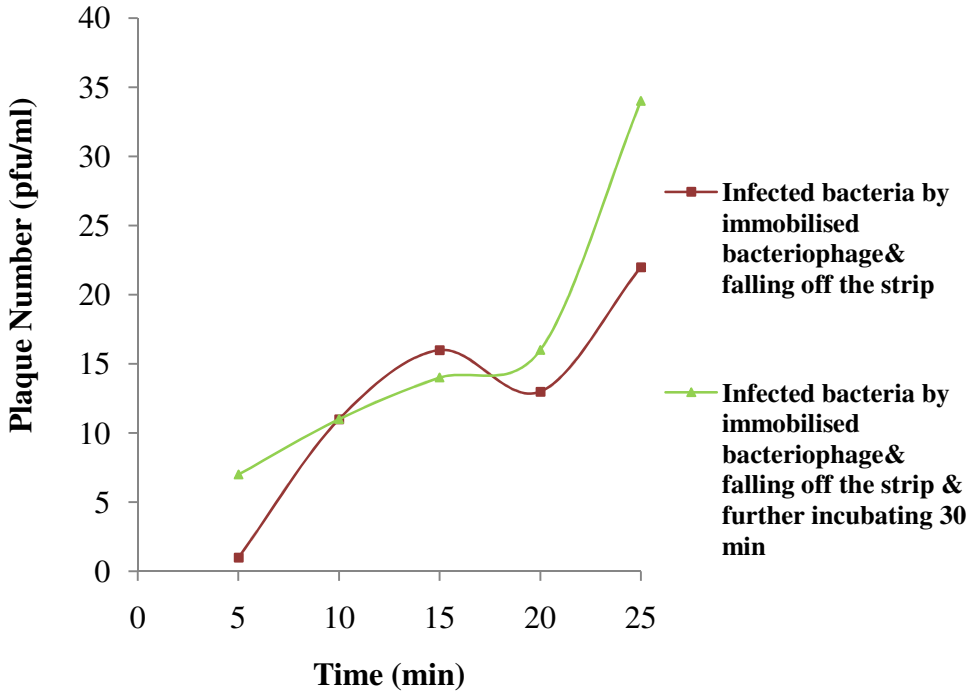


Figure 36 Infected bacteria by immobilised bacteriophage on the nylon membrane strip; size 1cm × 1cm.

The results illustrate that immobilised bacteriophages on the nylon membrane was successful by using the corona discharge. The corona discharge creates reactive sites (covalent bond) on the nylon membrane to bind the head of bacteriophages and made the tail end of bacteriophages are available for the bacteria to attach and so on. Furthermore, it is apparent that immobilised bacteriophage on the nylon strip can infect the bacteria as free bacteriophages. Increasing chance for bacteriophages and the bacteria meeting by long time incubation would be suggested. However, as the result implies that the bacteria bound by bacteriophage adhesion to the nylon strip may lead to blocking the access to potentially infective bacteriophages on the nylon strip. This is an unavoidable appearance. This can be solved by modifying/selecting for new material which would be increasing surface area for bacteriophage immobilisation.

In addition, the result shows that bonding between immobilised bacteriophages and the infected bacteria was quite weak. This because the infected bacteria either falling off the nylon strip after infection (as Table 17 code 1.1 and 1.2) or washing off the strip (as Table 17 code 3.1 and 3.2). This is due to bonding between the bacteriophage and the bacterium is a hydrogen bond which is easier to crack down compare to covalent bond between bacteriophage and nylon strip surface.

In conclusion, the bacteriophages were immobilised on surface of the nylon membrane strip by corona discharge method. All three possibilities to confirm immobilisation of the bacteriophage on the nylon strip were; (1) the infected bacteria falling off the strip, (2) the infected bacteria remaining attach to the strip via the bacteriophages binding and (3) the infected bacteria being washed off the strip during the PBS washing.

3.3.2. Determination activity of bacteriophage immobilisation.

To determine if the binding of bacteria to immobilised bacteriophages blocked the binding of potentially active, neighbouring, bacteriophages and experiment was carried out as detail described below. The immobilised bacteriophage strips (1cm × 1cm) were incubated with bacterial host cell suspension culture in its exponential growth phase (2 ml). After the immobilised bacteriophage strip had been incubated for 20 min, (a "cycle"), it was removed briefly washed and the total number of bacteriophages produced from the immobilised strip were determined (Figure 37). The strips were again exposed to the bacterial host culture and the cycle repeated.

Method: 2 ml of bacterial host in its exponential growth phase was transferred into a series of universal tubes. The 1cm × 1cm immobilized bacteriophage strips was added, mixed briefly and incubated at room temperature for 20 minutes for a cycle. Before removing the strip out, the tubes was shaken vigorously and then each 1 ml of mixed culture was transferred into new universal tubes. Then they were determined the number of bacteriophages by using plaque assay.

The strip was washed to get rid of infected/ uninfected bacteria stuck on it.

The strip was washed with 10 ml sterile PBS, twice and was transferred into a universal tube containing 5 ml sterile PBS. The contents were incubated at 37°C for 40 minutes without shaking to allow infected bacteria lyse. Then the tube was shaken vigorously before removing the strip out. After that the strip was washed with 10 ml sterile PBS, 4 times. Then the strip was ready for determining activity of bacteriophage immobilization in the next cycles.

This method was repeated for another 4 cycles or until no immobilized bacteriophage was left on the strip.

As the result shown in Figure 37 indicate, infection of the immobilised bacteriophage strip at each cycle dropped slightly at first 4 cycles and significantly decreased at the 5th cycle. The number was much less at the 5th cycle. For 5 cycles, the total number of bacteriophages immobilised on the nylon membrane strip was (at least) 108 bacteriophages

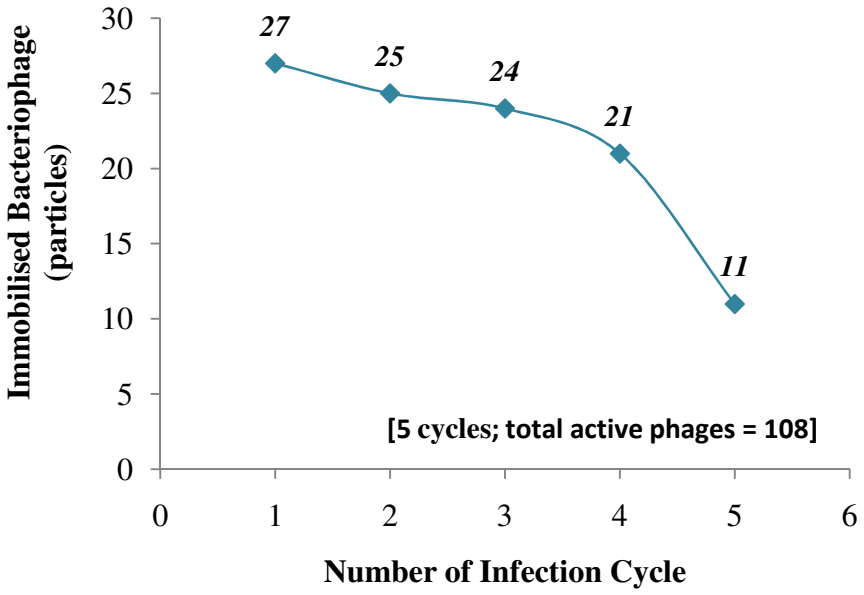


Figure 37 Active bacteriophages present on the immobilised bacteriophage on nylon membrane strip; (size 1cm × 1cm)

This illustrates that the bacteriophages were immobilised properly on the nylon membrane strip and were able to infect the target bacteria as free bacteriophages. Thus, this result shows an excellent sign and the potential on the further contribution, especially, a wound dressing product, by using the corona discharge as a tool for immobilisation of bacteriophage on surface membrane.

3.4. Bacteriophage Production

3.4.1. Bacteriophage infection and lysis in shake flask culture

According to previous results in (3.2.1), 37ºC was the optimum temperature for bacteriophage infection. Initial experiments on bacteriophage infection and lysis in shake flask culture were carried out in 500 ml Erlenmeyer flask containing 200 ml of modified M9 medium supplemented with 0.5% yeast extract at 4 different moi; 0.01, 0.02, 0.1 and 0.2, and three different bacterial concentrations; infection of the bacterial host cells was followed spectroscopically at $OD_{650\ nm}$, The results are shown in Table 18.

Table 18 Condition for bacteriophage infection and lysis in shake flask culture

Initial Absorbance	Multiplicity of Infection (moi)	Condition of Lysis		Plaque Count (pfu/ml)
		Complete	Not Complete	
1.0	0.01		✓	ND
	0.02		✓	ND
	0.1		✓	ND
	0.2		✓	ND
0.5	0.2	✓		3.96E+09
0.3	0.2	✓		3.33E+09

ND: not determined;

✓: result found

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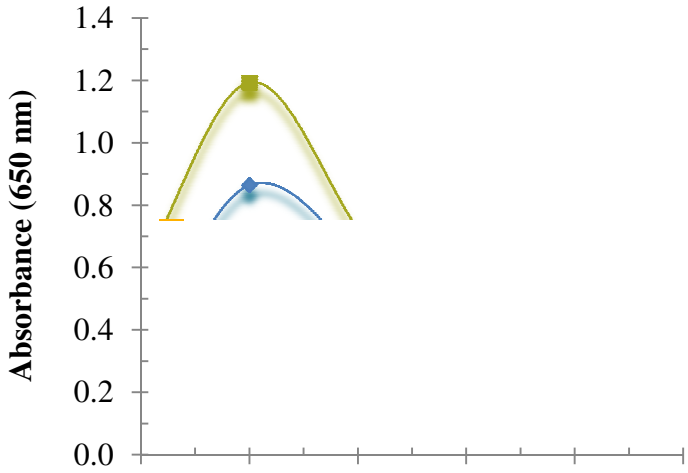

Figure 38 Absorban
S. aureus c
the bacteri

In a further experiment, bacteriophage production in shake flask culture was investigated with a bacterial host cell concentration of 0.3 ($OD_{650\ nm}$) with an moi of 0.2 in 500 ml Erlenmeyer flask containing 200 ml of modified M9 medium supplemented with 0.5% yeast extract. Infection of the bacteria at an absorbance of 0.3 was carried out instead of using the absorbance of 0.5. The results are shown in Figure 39.

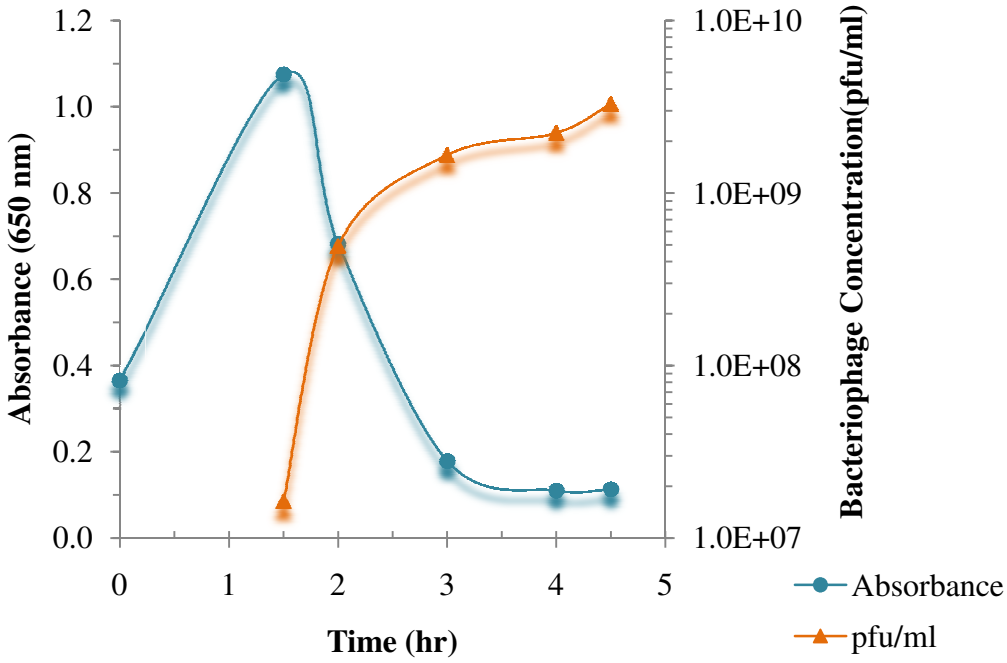

Figure 39 Bacteriophage infection and lysis in shake flask culture; absorbance of cells measurement after bacteriophage K infection of *S. aureus* cells when the bacteriophage added at the bacteria absorbance of 0.3.

After infection the absorbance of the culture reached a peak and went down within 3 hr with the concentration of bacteriophages increasing rapidly, and remaining high after complete lysis (3 hr) with the concentration of bacteriophage 1.65×10^{9} pfu/ml.

The results showed that state of bacterial host had an effect on infection and lysis. Even, the bacterial host cells in exponential phase, early, mid and late exponential phase, showed different results, in particularly when the scale up from 96 well plate to the shake flask was considered. As the result showed there was no complete lysis when infection with the bacterial cell density at absorbance of 1 (late exponential phase). This may because the bacterial host cells grew quicker in a bigger flask and passed through stationary phase which was in the state cannot be infected. So, changing state of the cells to earlier state of exponential phase would assist to achieve complete lysis. This result corresponds to the study by Sillankorva et al. (2004) found that the infection of bacterial cells during the exponential growth phase was more effective than that of cells in the stationary or decline stages, as greater rates of cell lysis, greater numbers of bacteriophages produced and the lowest latent period were found.

3.4.2. Bacteriophage infection and lysis in the fermenter in batch culture

A further experiment in the fermenter in batch culture at an moi of 0.2 was carried out.

Bacteriophage infection and lysis in the fermenter in batch culture was investigated in the 1.5L SGi fermenter with 600 ml working volume of modified M9 medium supplemented with 0.5% yeast extract. An initial experiment at an moi of 0.2 with at different bacterial host cell concentrations (0.3 and 0.5 $OD_{650\ nm}$) were examined. The result shown in Table 19, there was not complete lysis. This may be because an moi of 0.2 was not an optimum ratio to lyse the bacteria host cells when multiplying in the fermenter. This is because the bacterial host cells would grow faster in the fermenter than in the shake flask culture because of better aeration conditions in the fermenter.

In a further experiment infections of bacterial host cells at concentration of 0.3 and 0.5 ($OD_{650\ nm}$) with an moi of 2.0 and an infection at a concentration of 0.3 ($OD_{650\ nm}$) with an moi of 8.0 were investigated, also shown in Table 19. In all cases complete lysis occurred. The concentrations of bacteriophages from each case were similar. This result showed that infection when scaling up may need a larger amount of bacteriophage to achieve complete lysis.

Table 19 Bacteriophage infection and lysis in the SGi fermenter in batch culture

Absorbance of Infection	Multiplicity of Infection (moi)	Condition of Lysis		Plaque Count (pfu/ml)
		Complete	Not Complete	
0.3	0.2		✓	ND
	2.0	✓		1.67E+09
	8.0	✓		1.33E+09
0.5	0.2		✓	ND
	2.0	✓		1.84E+09

ND: not determined;

✓: result found

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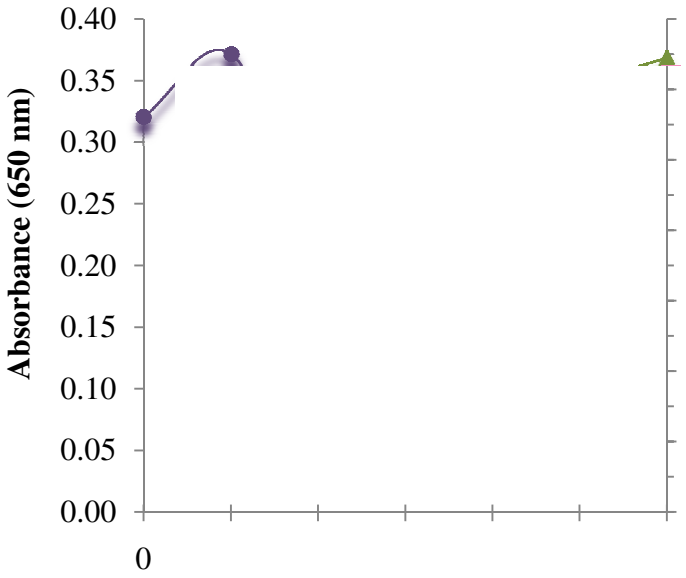

Figure 40 Bacteriop
absorbance
aureus cells

According to the result in section 3.4.1 and 3.4.2 showed that the scaling up of the shake flask culture to the fermenter culture was not proportional. So, in order to achieve complete lysis, it was necessary to provide higher moi. These results indicate that the larger volume of the co-culture, the higher moi need to achieve complete lysis. This may be because a larger volume flask/vessel provides a better mixing. This leads to enhancing a better growth of the bacterial host cell compare to a smaller flask.

Moreover, the bacteriophage density obtained when infected with a higher moi seems to be equivalent to an infection with lower moi (section 3.4.2 Table 19). This may due to the fact that bacteriophage production is dependent on the bacterial host cells which are in a state to support process of bacteriophage growth. It is known that bacteriophages are obligate parasites and only are able to multiply using their host machinery. For maximum bacteriophage replication, maximum bacterial density is needed. However, at early infection a high moi would be require, therefore, to balance the cost of running this process infection at a later stage (yet still in a growing culture) would be the most efficient approach. It was found that the higher moi, the less time required achieving lysis (data not shown). Thus, to achieve as high as possible bacteriophage production, infection at the appropriate time is vital. The optimal time of infection to harvest the maximum bacteriophage titre is while the host bacteria population is still growing but at a high density, just preceding the stationary phase. In conclusion, the culture lysate having higher bacteriophage density could be obtained from denser culture of bacterial host, but that a dramatic drop in yield occurred if infection was delayed until the bacterial host cells approaching in their stationary state of growth (Sergeant & Yeo, 1966).

Overall, things to consider achieving as high as possible bacteriophages;

1) at appropriate time to infection (bacteriophage added)

2) at denser culture of bacterial host as possibly

3) at optimal moi added

3.4.3. Bacteriophage production in continuous culture

Although the concept of continuous production of microorganisms, bacterial, fungal and yeasts, is not new, the use of true continuous culture to produce bacteriophage biomass has not previously been reported. Previous studies on "continuous culture" of bacteriophages have been designed to elucidate the developmental relationship between host bacteria and the bacteriophages and were really co-cultures (De Haan et al., 1955; Mizoguchi et al., 2003; Schwienhorst et al., 1996). The overall concept of this project was to produce bacteria in continuous culture from a basic medium and to use the output stream to feed a bacteriophages continuous culture. As the continuous culture of bacteria is well established it is not relevant here.

The production of bacteriophage in continuous culture was carried out as described in 2.9.4. The bacterial host cell culture was initially used in its stationary phase and was pumped into a bacteriophage lysate in the fermenter with a working volume 0.6 L, and was removed from the vessel at the same flow rate. Feeding bacterial host cell culture in its stationary phase was used to provide a constant concentration of bacterial host cell at any particular dilution rate. In these experiments, yeast extract was the sole limiting substrate.

3.4.3.1. Bacteriophage continuous culture

Bacteriophage continuous culture experiment was operated at 5 different dilution rates as shown in Table 3 (section 2.9.4.2) to study the effect of bacterial growth on lytic bacteriophage activity and bacteriophage production. The bacteriophage continuous culture was started from a complete lysis of the batch culture of the host bacteria. The concentrations of bacteriophage were determined by using the NanoSight until reaching steady state.

Results from the concentration of bacteriophages at time intervals is shown in Figure 41 and Figure 42, at any dilution rate a steady state is reached at different time periods. However, wash out of bacteriophage particles was not observed, even at dilution rate exceeding the bacteriophage growth rate (2.61 hr^{-1}). This result was similar to Schwienhorst, Lindemann and Eigen (1996). This result can be explained by these studies, Hadas et al (1997), You et al (2002), Rabinovitch et al. (2002), Mizogichi et al (2003) and Fischer et al. (2004). These studies have shown that the faster that the host grows at the time of bacteriophage infection, the faster that the bacteriophage will grow. This corresponds to Abedon (1989) who found that a shorter latent time occurred under high dilution rates and faster progeny production rate resulted.

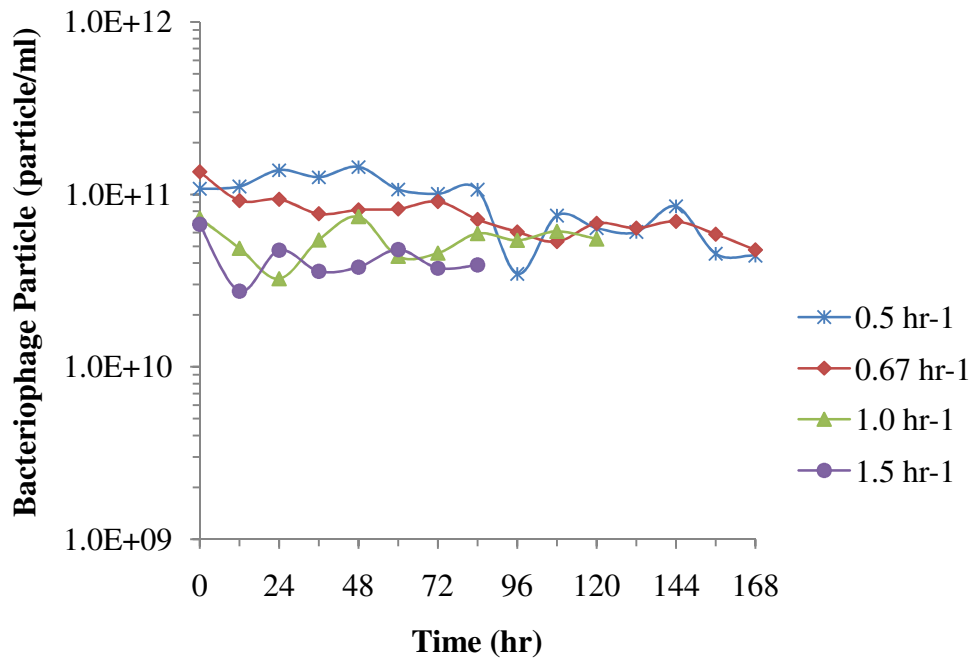

Figure 41 Bacteriophage continuous culture at four different dilution rates [1]

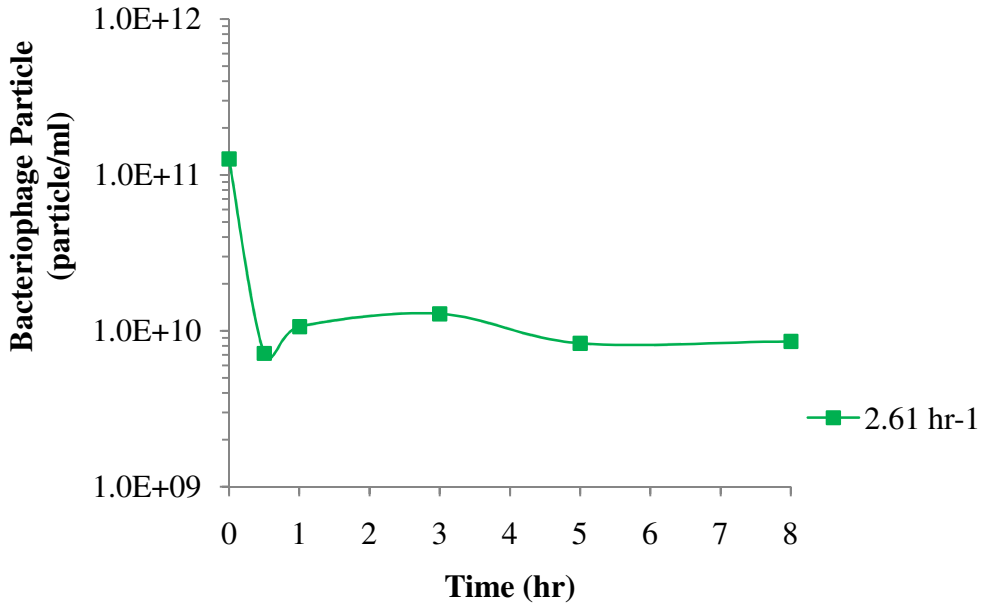

Figure 42 Bacteriophage continuous culture at dilution rate 2.61 hr^{-1} [2]

Figure 43 show the plot of dilution rate against steady state concentration of bacteriophages. The concentration of bacteriophages decreased with increasing dilution rate as expected. This is in agreement with theory of Herbert, Elsworth & Telling (1956) and the study by Mizoguchi et al. (2003) that as the dilution rate increases the concentration of organism falls.

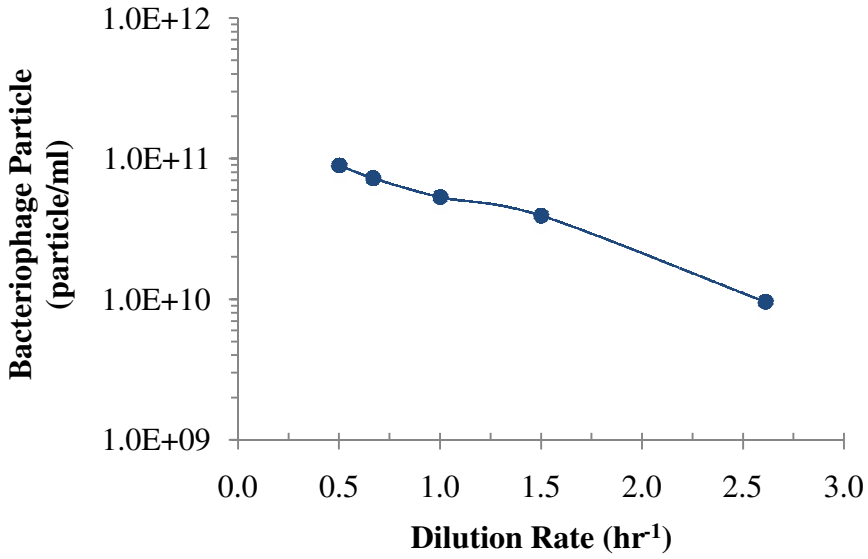

Figure 43 Bacteriophage particles at all 5 different dilution rates of bacteriophage continuous culture

3.4.3.2. Lysis time of bacteriophage produced in continuous culture

Because no wash out was observed in continuous culture even at a dilution rate of 2.61 hr^{-1}, at which dilution rate the bacteriophage lysis time should be exceeded, the one step growth curve of bacteriophages produced in at this dilution rate was carried out by the method described below. This is in order to investigate lysis time (latent period) of the bacteriophages produced as previous works would suggest a shorter latent period.

Method: Bacterial host culture (10 ml) in its exponential growth phase ($OD_{650\ nm}$= 0.2) was centrifuged at 4,000 rpm for 10 minutes. Then supernatant was discarded and the pellet was re-suspended with 1 ml of modified M9 medium (0.5% yeast extract) before infecting by 10^3 pfu of bacteriophage stock. At the time, the bacteriophages were added the concentration of the host cell culture was on average 6.4×10^8 cfu/ml, the bacteriophage titer was 10^3 pfu/ml. After incubating 37°C for 10 minutes, the mixture was centrifuged at 10,000 rpm for 5 minutes. Then the pellet was re-suspended in 1 ml modified M9 medium and was then added to a 250 ml Erlenmeyer flask containing 19 ml of modified M9 medium. The culture flask was incubated at the orbital incubator at 37°C180 rpm. Samples were removed every 5 minutes and immediately serially diluted and determined the number of bacteriophage by plaque assay.

The result of one step growth curve shown in Figure 44 indicates that lysis time (latent period) was from 25-35 minutes, which was the same as the latent period at the start of the experiment. With a conventional bacterial culture one bacterium divides into two so wash out occurs when the average division time is exceeded. However, with bacteriophages a single lysis results in 30 or more progeny so that even a few lysis events, for example the few at 20 minutes will be sufficient to maintain the steady state. Provided 1/30th of the culture lyses before removal from the culture a steady state will result.

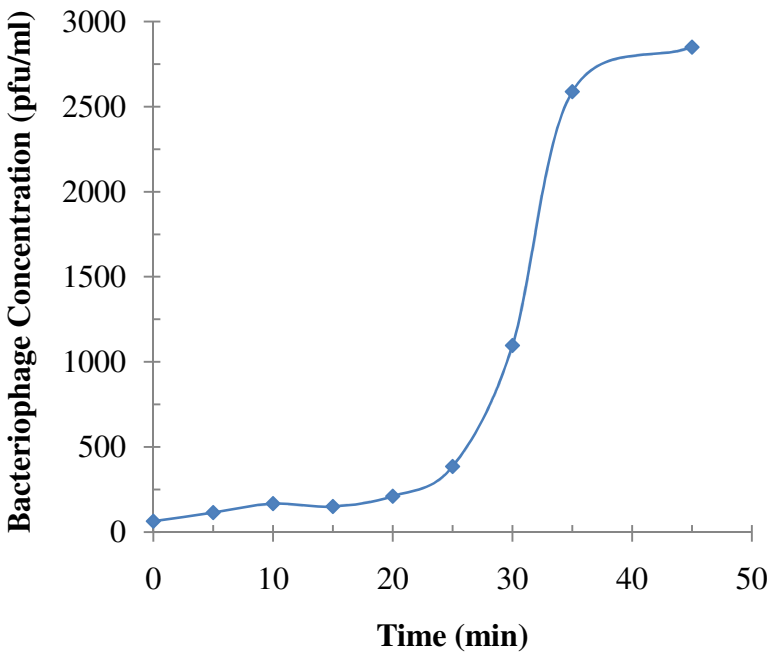

Figure 44 Bacteriophage growth curve of the bacteriophage produced in bacteriophage continuous culture at dilution rate 2.61 hr^{-1}

CHAPTER 4
SUMMARY

Upon examination of the literature in Chapter 1, it became apparent that there exists a distinct lack of studies concentrating on bacteriophage production on a large-scale. Despite this, there has been a resurgence of interest in bacteriophage because of increasing antibiotic resistance in bacteria which has motivated the demand for alternative therapies to control infections and led to the replacement of antibiotics. Research on bacteriophages has increased dramatically, leading to the development of a range of applications for the healthcare, veterinary, agricultural and food sectors include developing rapid detection methods. Moreover, the emergence of many biotech companies researching to commercialize their use. Thus, the main object of this study was to produce the bacteriophage that will be used in commercial production.

4.1 Growth of the bacterial host cell and medium optimisation

Bacterial host cells are a necessary precursor to bacteriophage production; this is because bacteriophages are obligate parasite of bacteria and are only able to multiple in the bacterial host cells using the resources of the bacterial host cells. The studies reported that the faster bacterial host cells grow at the time of bacteriophage infection, the faster bacteriophage will be produced (Fischer et al., 2004; Hadas et al., 1997; Mizoguchi et al., 2003; Rabinovitch et al., 2002; You et al., 2002). This corresponds to a higher growth rate or a short generation time of bacterial host, faster bacteriophage production. In order to achieve the optimum condition for bacteriophage production, the optimum conditions for the bacterial host growth was needed.

For commercial production it is not only necessary to achieve high host growth rates but to do so in a cost effective manner. Initially for screening suitable medium components for growth of the bacterial host factorial experiment design was used.

The advantages of factorial experiment design over one factor-at-a-time experiments are not only because every observation provides information about all the factors in the same study but also because interactions between the factors can also be detected. Overall, factorial experiment design is less time consuming and all possibilities are examined in only one designed experiment compared to multiple one factor-at-a-time experiments.

The bacterial host used, *S. aureus*, is a facultative anaerobe which is able to multiple both in aerobic and anaerobic conditions. Thus, the shake flask culture and the RABIT method were utilised on investigation of its growth. The RABIT method was a useful and convenient to monitor the growth of the bacteria in anaerobic condition without using basic microbial technique, (for example plating count, which takes longer in determining and/or monitoring growth), where as the shake flask culture to monitor the growth of the bacteria in aerobic condition. The result on the growth of the bacteria under aerobic condition was greater than that under anaerobic condition. This is because aerobic growth has a higher efficiency in generating ATP from the energy sources than anaerobic growth.

Another aspect found is that the different medium components are needed for the growth of *S. aureus* culture in anaerobic and aerobic condition. As the result of the RABIT method showed that glucose gave the best result, whereas yeast extract was the best medium component for the growth of the bacteria in shake flask culture. This result was due to different metabolic pathway regulations, in anaerobic conditions the bacteria are able to metabolise glucose via glycolytic pathway but there is no presence and function of enzymes in the TCA cycle in anaerobic growth conditions. In contrast, in aerobic condition, yeast extract was a dominant influence on the growth of the bacteria. The catabolism of yeast extract components via the TCA cycle, which occurs in aerobic condition of the bacteria growth, provides a higher energy efficiency that supports the better growth of the bacteria. This finding supported those previous studies that in aerobic growth, effect of glucose on the growth of bacteria is still highly significant, but that effect is negative. Another investigation to confirm the effect of glucose and yeast extract on the growth (section 3.1.1.2.1), found that only yeast extract supported growth of the bacteria. Thus, this

implies that amino acid or peptides from yeast extract had a positive effect on the growth of the bacteria.

Following this the commercial use of amino acid sources was designed. The result showed that all sources of amino acids were able to support the growth of the bacteria. It also found that for any source of amino acids, at low concentrations the specific growth rate was a function of the substrate concentration but at high concentrations the specific growth rate is independent of the concentration of nutrient as illustrated by Monod (1942). However, yeast extract was chosen as the principal component of medium for the rest of the study, (as previously described in 3.1.2.2).

However, the biomass of the bacterial host in these studies was not directly measured which prevented calculation of the yield factor (Y) of the bacterial host resulting from different sources/concentrations of the medium component utilised in the growth of the bacteria. This would be more helpful in making decisions on the selection of the medium component for the growth of the bacteria. The measurement of the biomass in order to further calculation of the yield factors would be suitable for future work, as it might provide a more complete picture of the suitable medium component/concentration for growth of the bacterial host.

The study of effect of physical factors, temperature and initial pH of the medium, on the growth of the bacterial host was clarified. As the result of temperature on growth showed, the bacteria grew very well at 37°C and 42°C. This results support the fact that *S. aureus* is a mesophile. They will grow at temperatures between 10 and 45ºC with the optimum in the range of 30-37ºC (Baird-Parker, 1972; Kloos & Schleifer, 1986). However, for commercial production, growing the bacteria at 37°C is better than growing at 42°C from an economic viewpoint. According to the result of the experiments on the effect of initial pH of the medium on growth, there were no significant differences (between pH 5-8) on growth despite the buffering effects of the amino acids in the medium. The result agrees with the fact that the bacteria will grow at pH values between 4.2 and 9.3 (optimum 7.0-7.5) (Baird-Parker, 1972; Kloos & Schleifer, 1986). As the initial pH of the medium was 7 without pH adjustment, pH 7 should be recommended for a production process.

In conclusion, the optimum conditions for the bacterial host growth is modified M9 medium containing 0.5% yeast extract at 37°C, without adjusting pH if the initial values are around 7, in aerobic condition. Glucose as the medium component should be avoided as growth would be reduced in its presence.

4.2 Infection and Lysis

Infection and lysis conditions were investigated prior to stage of production of the bacteriophages. The process of infection is regulated by the physicochemical characteristics of bacteriophage genome-encoded functions and the intracellular resources of the bacterial host. The intracellular resources of the host are dependent on the physiological state of the bacteria which is modulated by, for example, growth media and temperature (You, Suthers, & Yin, 2002) or moi (Petty et al., 2006). An examination of the effect of incubation temperature and moi on infection and lysis was carried out. The result found suggests that the optimum temperature for bacteriophage infection and lysis was the same as the optimum for the bacterial host growth (37°C). Meanwhile, infection and lysis at different moi showed that lysis occurred more quickly with a higher moi. Moreover, the state of bacterial host had an effect on infection and lysis, especially when scale up is considered (section 3.2.1 3.4.1); even though the bacterial host is in its exponential phase different result may occur at different scales of growth. Once the bacteria pass into the stationary phase, they are unable to be infected. This leads to incomplete lysis, which was sometimes observed. Thus, using either the bacteria cells in the earlier state of exponential phase or providing a higher moi would assist to achieve complete lysis.

Furthermore, the study of bacteriophage adsorption (section 3.2.2) found that the concentration of bacterial host had an effect on bacteriophage adsorption; in an environment with a high concentration of bacterial host cells, adsorption rates would be high, and vice versa. This is because at high concentrations of bacteria, bacteriophages have a high chance of meeting uninfected host cells. There are several ways to increase adsorption rate therefore:

1) by decreasing the volume of the mixture of bacteria and bacteriophage for adsorption step

2) by using as high as possible concentration of bacteriophage stock solution

3) by decreasing the volume of the bacterial cell culture by centrifuging and re-suspending in a small volume before bacteriophage stock is added, followed by dilution of the mixture to a large volume for infection and lysis after permitting a short time period for adsorption.

In conclusion, the optimum bacterial host growth (temperature 37°C, pH7.0, aerobic condition) also the optimum condition for bacteriophage infection and lysis. Infection and lysis occurred more quickly with a higher moi and also with a higher concentration of bacterial host cell. However, when scaling up, it is not only the state of the bacteria growth phase that must be considered but also the optimum moi used to achieve complete lysis.

4.3 Bacteriophage Immobilisation

In a previous study, immobilised bacteriophages gave more longevity than free bacteriophage while displaying lytic activity (Mattey et al. 2010, personal communication). This study was the preliminary study on bacteriophage immobilisation on nylon membrane by using corona discharge for the further contribution on a wound dressing for using treatment of bacteria infections including MRSA.

The immobilisation of bacteriophage on the nylon membrane strip indicates there were the bacteriophages immobilised on the nylon strip and they were remaining lytic activity. The number of the immobilised bacteriophage presence on the 1cm×1cm nylon strip was 108 particles.

Future work to improve the number of immobilised bacteriophage will concentrate on the level of corona used. The current equipment gives too high a current density such that the polymer chains are broken leading to fragmentation of the surface of

the material. The result is that washing removes fragments of the material with bound bacteriophages as well as the unbound bacteriophages.

4.4 Bacteriophage Production

The data in sections 3.1, 3.2, 3.4.1 and 3.4.2 provided information on the conditions for growth of the bacteria, infection and lysis. For the production of bacteriophages in continuous culture an initial batch culture preparation was made.

Bacteriophage lysate preparation in the fermenter was designed to give a high density of bacteriophages prior to the later steps, in order to achieve high bacteriophage densities in the continuous culture. Higher bacteriophage density was obtained from more concentrated bacterial host cell cultures, but a dramatic decline in the bacteriophage density did occur if there was an infection delay resulting in the bacterial cells passing into late exponential phase or stationary phase (Sergeant & Yeo, 1966). In section 2.9.4.1 infection was performed at an absorbance 0.5 instead of at 0.3 in order to obtain a higher density of bacteriophages (as the result in the section 3.4.2 showed, complete lysis occurred at both of the absorbance but higher bacteriophage density was observed at the absorbance of 0.5). There was no significant time difference between the absorbance of 0.3 and 0.5 in the fermenter.

The bacteriophage continuous culture commenced after achieving complete lysis in the batch step by feeding the bacterial host cells in their stationary phase. Feeding bacterial host cell culture in its stationary phase was used to provide a constant concentration of bacterial host cell at any particular dilution rate. It also modelled the intention to provide the bacterial feedstock by continuous culture in a commercial situation, where the bacterial outflow from its continuous culture would be close to stationary phase with respect to the limiting nutrient.

The result found that all dilution rates achieved steady state although the concentration of bacteriophages declining with increasing dilution rate. Moreover, there was no washing out observed, even at a dilution rate surpassing the expected bacteriophage growth rate. The suggestion was made that the lack of observed wash

out at the (predicted) latent time was the result of the selection of a faster developing subset of the bacteriophages population. As the latent time in a lysing culture is a range (in this case from 15 to 35 minutes) this sounds reasonable. However, the one step growth curve performed to prove this, showed the bacteriophages produced at a dilution rate of 2.61 hr^{-1} (which should have resulted in wash out) had the same time to lysis, both distribution and mean, (25 min) as the growth rate of the bacteriophages at the start experiment. Clearly the idea of selecting a fast growing subset of bacteriophages in the period of the continuous culture experiment is not correct, though it might happen with an extended time scale.

This finding can be explained by the burst size of lysis; with a conventional bacterial culture one bacterium multiplies into two and so wash out occurs when the average division time is exceeded. However, with bacteriophages a single lysis brings about 30 or more progeny so that even a few bacteriophage lysis occurrences, for example the few at 20 minutes will be adequate to maintain the steady state. Provided $1/30^{th}$ of the culture lyses before removal from the culture, a steady state will result.

Interestingly, it was found that feeding in the bacterial host cells in its stationary phase to the bacteriophage production vessel provides an excellent outcome although it was known and anticipated that this might result in a lag period when the bacteria re-entered the log phase from the stationary phase and became susceptible to bacteriophage infection. This would have implications of “premature wash out”, as the effective time to lysis would include the lag period of the host bacteria. Clearly this did not happen, rather the opposite! The growth of *S. aureus* on a peptide medium is however a “special case” in that the lysis products of infected bacteria are similar to the growth medium, so that any lag phase in bacterial growth is minimal. The results show continuous culture was effective without additional nutrient in the bacteriophage production vessel to support the continued growth of the bacteria during infection. This would not be observed in a glucose requiring bacteria such as *E. coli* , as it rarely consume peptides (Sezonov, Joseleau-Petit, & D'Ari, 2007). If a stationary phase culture growing on glucose was used then the nutrient medium would not be replenished by lysis and a long lag phase would ensue where adaptation from glucose to amino acids or peptides took place. The solution would be to include

glucose in the bacterial feed, either directly or by making the bacterial continuous culture limiting nutrient something other than glucose (excess glucose), for example phosphate, which would be released on bacterial lysis.

It is however likely that a general peptides/peptone based media rather than a glucose/ minimal media would be the medium of choice in many bacteriophages production processes. The state of the bacterial host feeding in turned out to be relatively unimportant aspect. This finding means that feeding in with any state of the bacterial host would be effective. Thus, it is unnecessary to grow the bacterial host in the exponential phase continuously to feed in the bacteriophage production vessel as expected.

Overall, these finding have implications for the potential for bacteriophage production in continuous culture (as the result shown in section 3.4.3 and also Table 20). Future work on scale up will be needed. Moreover, there are other things to be considered at the same time for a bacteriophage product in a commercial production. Bacteriophage products tend to have been and are likely to continue to be, prepared in form of a cocktail of bacteriophages, for example PhageBioDerm, Pyophage, Intestiphage, Listex P100 (Fortuna et al., 2008). This study, in particular, will contribute to a particular application; a wound dressing product using immobilised bacteriophages for the prevention and treatment of chronic wound infections. *S. aureus* is not only the pathogen causing the problems in wounds; other pathogens, for example *Enterococcus* spp., *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Enterobacter* spp., *Proteus* spp, including antibiotic resistant strains; (Church, Elsayed, Reid, Winston, & Lindsay, 2006) also contribute. The wound dressing product will only achieve an effective treatment result if the dressing is prepared in form of a cocktail of perhaps 20 different bacteriophages. Bacteriophage cocktails will not only expand the effective host range but also help to prevent the rising of bacteriophage resistances (Cairns & Payne, 2008; Kutateladze & Adamia, 2010; Skurnik et al., 2007). The production of a cocktail of bacteriophages would need more than one fermenter to produce the bacteriophages. In general, the larger fermenter used the more capital cost

investment. Thus, using the smaller fermenters in continuous mode will help reducing the capital cost of production.

Table 20 The bacteriophage production in continuous culture.

Working Volume (l)	Dilution Rate (hr^{-1})	Feed Rate (l/hr)	Residence Time (hr)	Residence Time (min)	Production of the Bacteriophage in Continuous Culture	Time (hr)	24	48	72	96	120
						(Day)	1	2	3	4	5
0.6	0.5	0.3	2.00	120	Total Volume of the Lysate (l)		7.2	14.4	21.6	28.8	36
					Total Bacteriophage (particles)		6.4E+14	1.3E+15	1.9E+15	2.6E+15	3.2E+15
					Bacteriophage Concentration (particles/l)		8.9E+13				
0.6	0.67	0.4	1.50	90	Total Volume of the Lysate (l)		9.6	19.2	28.8	38.4	48
					Total Bacteriophage (particles)		6.9E+14	1.4E+15	2.1E+15	2.8E+15	3.5E+15
					Bacteriophage Concentration (particles/l)		7.2E+13				
0.6	1	0.6	1.00	60	Total Volume of the Lysate (l)		14.4	28.8	43.2	57.6	72.0
					Total Bacteriophage (particles)		7.6E+14	1.5E+15	2.3E+15	3.0E+15	3.8E+15
					Bacteriophage Concentration (particles/l)		5.3E+13				

Table 20The bacteriophage production in continuous culture (cont).

Working Volume (l)	Dilution Rate (hr^{-1})	Feed Rate (l/hr)	Residence Time (T) (hr)	(min)	Production of the Bacteriophage in Continuous Culture	Time hr	24	48	72	96	120
						day	1	2	3	4	5
0.6	1.5	0.9	0.67	40	Total Volume of the Lysate (l)		21.6	43.2	64.8	86.4	108.0
					Total Bacteriophage (particles)		8.4E+14	1.7E+15	2.5E+15	3.4E+15	4.2E+15
					Bacteriophage Concentration (particles/l)		3.9E+13				
0.6	2.61	1.565	0.38	23	Total Volume of the Lysate (l)		37.6	75.1	112.7	150.2	187.8
					Total Bacteriophage (particles)		3.6E+14	7.1E+14	1.1E+15	1.4E+15	1.8E+15
					Bacteriophage Concentration (particles/l)		9.5E+12				

4.5 Commercial Production of Bacteriophages

Bacteriophages have been widely used, either in human or veterinary therapy, which need to be considered in aspects of safe and decontamination of products, agriculture or horticulture or other application. Due to bacteriophage use has become a reality then bacteriophages will have to be manufactured on an industrial scale. The process of manufacturing bacteriophages is unlike any other. In essence the process must involve with the growth of the bacterial host strain, the infection of that bacterial host and the lysis of the bacterial host. The bacteriophages will then have to be purified to some degree level and appropriate to their end product use.

The particular specificity of bateriophages means that any end product is likely to be a cocktail of several different bacteriophages. Each bacteriophage is specific with a different bacterial host, different growth conditions and possibly different downstream processing needs.

Things to consider on growth of the bacterial host as the following:

- Different host bacteria will have different media requirements.
- Some bacterial hosts will use glucose as carbon source, some use amino acids (like *S. aureus*), others bacteria may use different carbon sources. However, nutrient media components chosen should be cheap.
- The volume required will depend on the amount of bacteriophages needed for the particular process.
- Bacteria must be growing to allow infection and lysis.
- Adaptation of the host bacterium to growth on amino acids/peptides might allow re-cycling of the lysate after removal of bacteriophages. Otherwise the culture will have to be fed during process of bacteriophage lysis/production.

Things to consider on production capability; example of decontamination product

• Fermenter size 10 l

• Semi-continuous operation

• Dilution rate 0.5 (5 l/hr)

• In a 12 hr run approximately 60 l bacteria can be grown.

• Estimate time for lysis 2 hr

• Centrifugation 10 min

• Filtration (0.22 µm) 1 hr

• Tangential flow 100kDa; 3 hr with washing (120 l/hr flow rate)

• Final bacteriophage numbers ~10^{13} (60 l at about 5×10^{8} bacteriophages/ml)

• Daily production concentration: 10,000 m/ (10 l); 2×12 hr shifts 20 l

• Maximum daily production with 4 fermenters: 80 l concentration

• Assuming 50% down time: annual production 14-15,000 l concentrate / year

1. For batch fermenter equivalent size; day 1 bacteria growth, day 2 bacteriophage lysis, day 3 clean, day 4 resterilise; would need 500 l fermenter with 1 run per week. Or 2000 l fermenter to equal 4×10 l continuous

2. Capital costs would be high for large batch.

3. Each bacteriophage would require a separate fermenter.

4. Where the bacterial host bacterium is pathogenic. Disposal will require sterilisation of effluent. Then fermentation method is used and this results in scaling being costly.

Quality Control (QC)

- Measure glucose in-out flow (less than 0.01%)
- Microbiological purity by plate assay on each lysis batch.
- Check bacteriophage sensitivity of batch; greater than x % unlysed replace bacterial from working bank. (Actual levels need to be experimentally determined)

Cell Banking

- Bacteria
    - Parent strains, seed stock 120 vials store cryobeads or liquid nitrogen
    - Working bank: replace annually; 24 vials as cryobeads.
    - Subculture monthly from working bank, 10 slopes
- Bcteriophage
    - Parent strain, seed stock 120 vials store liquid nitrogen (cell lysate)
    - Working bank: replace annually; 24 vials
    - Subculture monthly

Lysis condition

- Batch collection for 12 hr into stainless steel holding tank (60 l) 30°C with aeration and agitation (Actual rates need to be experimentally determined)
- Glucose pulse at time 0; 0.1% glucose; agitation aeration and temperature (Actual concentrations and rates need to be experimentally determined)
- Growth time 30 min (Needs to be experimentally determined)
- Bacteriophages from cell bank stock grown to 10^8 -10^9 bacteriophages /ml

- Amount added (Needs to be experimentally determined)
- Time for lysis: 3 hr (Needs to be experimentally determined)

Downstream processing

- Lysis fluid treated by low speed continuous or batch centrifugation; 2000g for 10 min. Solids to waste stream.
- Outflow pumped through 0.22 µm filter (microbiological purity)
- Outflow pumped through tangential flow filter with 100 kDa membrane; 60 l to 1 l; Wash recycle 10 l to 1 l, three times.
- Final bacteriophage concentrate

QC

- Glucose concentration, medium contamination check
- Elisa for protein A (contamination by bacterial debris)
- LAL test for pyrogens (Actual levels need to be experimentally determined)

Corona discharge

- Nylon membrane or others; solid surface membrane for bacteriophage immobilisation use will passed through corona discharge (60 KW) at speed in monolayer. (Actual rates need to be experimentally determined)
- The nylon membrane treated/activated mixed into bacteriophage concentration within 1 min
- Contact time (min) (Need to be experimentally determined)
- Washing method to remove unbound bacteriophage and conditions (Need to be experimentally determined)

- Store (Need to be experimentally determined)
- Shelf life (Need to be experimentally determined)

Effluent disposal

Overall costing will be needed for any process.

4.6 Conclusions

Bacteriophage production was evaluated by optimising medium and conditions for bacterial host growth, infection and lysis. In this study, the bacterial host used was *S. aureus* 8588 to produce the bacteriophage K. However, bacteriophage K was used as a model for bacteriophage production and could be used to conclude the following:

1) Bacterial host growth media will have to be adapted for different bacterial hosts; *S. aureus* 8588 for example requires a glucose free medium whereas glucose maybe the carbon source of choice for other bacteria.

2) The economic cost of the medium is as important as the yield.

3) Optimal conditions for bacterial host growth are also optimal for bacteriophage production.

4) Lysis rates are a balance between bacterial growth and bacteriophage production; the moi determines rate of lysis.

5) Continuous culture of bacteriophage is possible, feeding bacteria leads to a sustainable, stable culture.

6) Wash out does not occur when the mean bacteriophage lysis time is exceeded because the burst size is large compared with bacterial duplication.

7) Bacteriophage products will contain several different bacteriophages in most applications. For regulatory reasons it is likely that each component bacteriophage needs to be manufactured separately. This suggests the capital cost of equipment may be a significant factor and for this reason continuous culture may be the method of choice for large scale production.

CHAPTER 5
FUTURE WORK

Overall, the future work should focus more on these following mentioned below and include experiments conducted throughout this study (for example, medium optimisation and infection and lysis in shake flask/fermenter).

1) The residual substrate in the medium and dry weight of bacterial host should be determined along with viable cell numbers in order to find other growth kinetic parameters e.g. yield factor (Y). The relationship between this parameters and μ_{max} on *S. aureus* 8588 in order to investigate the optimal substrate concentration which will result in a high production yield.

2) Infection and lysis follow by bacteriophage continuous culture on other amino acid sources such as soy peptone should be studied further. Soy peptone is another amino acid source which is non animal products.

3) Bacteriophage production in continuous culture by feeding in the bacteria host in its exponential phase produced by continuous culture at different dilution rates.

4) Bacteriophage production in continuous culture should be studied with the effect of the environmental parameters such as temperature, pH, agitation and aeration in order to find the influence of these environmental parameters on the bacteriophage production.

5) Bacteriophages and bacterial hosts other than *S. aureus* 8588/ Phage K should be examined.

6) The linkage of continuous bacterial culture to bacteriophages lysis should be demonstrated.

7) The economics of potential production processes should be investigated and modelled.

8) Other material substrate e.g. gauzes, celluloses, should be investigated in order to find the other possibility surface substrate for bacteriophage immobilisation.

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APPENDIX

I) Growth determination of the bacteria

Growth determination of *S. aureus* 8588 in shake flask culture, the standard curve is shown in Figure A.1

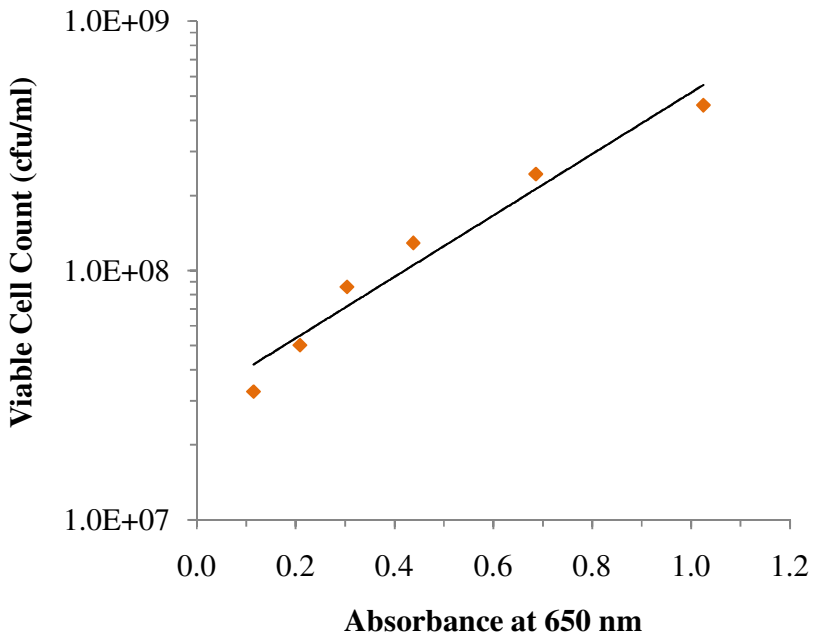

Figure A.1 Standard curve for growth determination of *S. aureus* 8588