

STRATEGIES TO CONTROL BACTERIOPHAGE INFECTION IN A THREONINE BIOPROCESS

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by

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DECLARATION

This Master of Technology: Biotechnology dissertation is my own work and all primary and secondary sources have been appropriately acknowledged. The dissertation has not been submitted to any other institution as part of an academic qualification.

This Dissertation is prepared in partial fulfillment of the requirement of the degree at the Durban University of Technology, Durban, South Africa.

March 2009

DEDICATION

This work is dedicated to my late mom Sitheni Nduli (thanks for all the support, encouragement, motivation, and inspiration. My only regret is that you didn't hang around long enough to see its completion. "Lala ngokuthula Magutshwa" you are always in my heart.), My late Granny Zintombi "Mano" Nduli, My late aunt Nombuso Nduli, and My late best friend, sister & mentor Tshireletso "Junior" Ramothokang.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
LIST OF FIGURES	ii
LIST OF TABLES	iv
ABSTRACT	v

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Bacteriophages.....	3
1.1.1 <u>Physical properties of bacteriophages</u>	3
1.1.2 <u>Infection and reproduction of bacteriophages</u>	5
1.2 Identification of Phage Infection	9
1.3 Phage Infected Plant Clean-up	9
1.4 Bacteriophage Ecology	11
1.4.1 <u>Growth rate of the host</u>	12
1.4.2 <u>Temperature</u>	12
1.4.3 <u>pH</u>	13
1.4.4 <u>Salt concentration</u>	14
1.5 Methods for Isolation and Enumeration of Bacteriophages	14
1.5.1 <u>Host bacteria independent methods</u>	15
1.5.1.1 <i>Electron microscopy</i>	15
1.5.1.2 <i>Genome analysis</i>	16
1.5.2 <u>Host bacteria dependent methods</u>	16
1.6 Identification and Characterisation of Phages	17
1.6.1 <u>Identification of bacteriophages using host range</u>	17
1.6.2 <u>Identification of bacteriophages using electron microscopy</u>	18
1.7 Treatment of Bacteriophages	18
1.7.1 <u>Heat treatment</u>	18
1.7.2 <u>Chemical treatment</u>	19
1.7.3 <u>UV treatment</u>	20
1.7.4 <u>Addition of inhibitors</u>	20
1.8 Prevention of Phage Infection	22
1.8.1 <u>Prevention of phage invasion</u>	23

1.8.2	<u>Employment of phage-resistant mutants</u>	23
1.8.3	<u>Employment of chemicals to halt phage multiplication</u>	24
1.9	Scope of the Present Study	24

CHAPTER 2: ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES

2.1	Introduction	28
2.2	Materials and Methods	32
2.2.1	<u>Sample collection</u>	32
2.2.2	<u>Isolation of bacteriophages</u>	35
2.2.3	<u>Double agar layer method</u>	35
2.2.4	<u>Purification of bacteriophages</u>	36
2.2.5	<u>Enrichment of bacteriophages</u>	37
2.2.6	<u>Preservation of bacteriophages</u>	39
2.2.7	<u>Characterisation of bacteriophages</u>	39
2.2.7.1	<i>Phage infection kinetics</i>	39
2.2.7.2	<i>Electron microscopy</i>	40
2.3	Results	41
2.3.1	<u>Isolation of bacteriophages</u>	42
2.3.2	<u>Purification of bacteriophages</u>	45
2.3.3	<u>Enrichment of bacteriophages</u>	46
2.3.4	<u>Preservation of bacteriophages</u>	48
2.3.5	<u>Characterisation of bacteriophages</u>	52
2.3.5.1	<i>Phage infection kinetics</i>	52
2.3.5.2	<i>Electron microscopy</i>	54
2.4	Discussion	56

CHAPTER 3: DETECTION AND ENUMERATION OF BACTERIOPHAGES

3.1	Introduction	64
3.1.1	<u>Detection and enumeration methods</u>	64
3.1.2	<u>Detection of lysogenic bacteriophages</u>	66
3.2	Materials and Methods	69
3.2.1	<u>Optimal bacterial volume</u>	69

3.2.2	<u>Optimal incubation time</u>	69
3.2.3	<u>Optimal incubation temperature</u>	70
3.2.4	<u>Effect of bivalent ions</u>	70
3.2.5	<u>Comparison of plating methods</u>	71
3.2.6	<u>Detection of lysogenic bacteriophages</u>	72
3.2.6.1	<i>Detection of lysogenic phages using UV light</i>	73
3.2.6.2	<i>Detection of lysogenic phages using green indicator plates</i>	74
3.2.6.3	<i>Detection of lysogenic phages using a mutagen</i>	74
3.3	Results	76
3.3.1	<u>Optimal bacterial volume</u>	76
3.3.2	<u>Optimal incubation time</u>	77
3.3.3	<u>Optimal incubation temperature</u>	78
3.3.4	<u>Effect of bivalent ions on bacteriophages</u>	79
3.3.5	<u>Comparison of plating techniques</u>	80
3.3.6	<u>Detection of lysogenic bacteriophages</u>	81
3.3.6.1	<i>Detection of lysogenic phages using UV light</i>	83
3.3.6.2	<i>Detection of lysogenic phages using green indicator plates</i>	83
3.3.6.3	<i>Detection of lysogenic phages using a mutagen</i>	84
3.4	Discussion	86
CHAPTER 4:	PREVENTION AND TREATMENT OF BACTERIOPHAGE INFECTION	93
4.1	Introduction	93
4.2	Materials and Methods	97
4.2.1	<u>Treatment using heat</u>	97
4.2.2	<u>Treatment using chemicals</u>	97
4.2.3	<u>Treatment using vitamins as inhibitors</u>	98

4.3	Results	100
4.3.1	<u>Treatment of bacteriophages using heat</u>	100
4.3.2	<u>Treatment of bacteriophages using chemicals</u>	102
4.3.3	<u>Treatment of bacteriophages using vitamins as inhibitors</u>	104
4.4	Discussion	107
 CHAPTER 5: GENERAL DISCUSSION		111
 REFERENCES		118
APPENDIX		124

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LIST OF FIGURES

Figure 1.1 Families of bacterial viruses. All the diagrams have been drawn to the same scale and provide a good indication of the shapes and relative sizes of the virions. The name of a well-known, representative member of the family is given in parentheses, but the dimensions and shapes used for the drawing may not be exactly those of the representative virus (Prescott <i>et al.</i> , 2007)	4
Figure 1.2 Infection and reproduction of lytic phages (Prescott <i>et al.</i> , 2007).....	7
Figure 1.3 Infection and reproduction of lysogenic phages (Prescott <i>et al.</i> , 2007).....	8
Figure 1.4 Oxygen uptake trend on pilot fermenters during threonine production. Fermenter L04225 and L04226 had bacteriophage infection as OU trend decrease after ten and 17 minutes respectively. Trends L04227 and L04228 exhibited normal threonine fermentation	25
Figure 2.1 SA Bioproducts production plant layout. Shaded areas 1-5 are the areas where samples were collected. BE Tanks – broth effluent tanks, Thr plant- threonine downstream processing plant; PF – prefermenter, MF- mainfermenter, DTA and DTB- drop tank A and B, PIX- primary ion exchange, Evaps- Evaporation tubes.....	33
Figure 2.2 Lysis plaques of <i>E. coli</i> B following phage infection with DAL. Plate A is an example of undiluted phage sample, with too numerous plaques to count. Plate B was 10 ⁶ dilution of a phage sample	43
Figure 2.3 Plaque size of purified phage sample. After performing DAL three times, single size plaques were achieved (medium sized) on the plate	45
Figure 2.4 Enrichment of bacteriophages. (A) Enrichment of bacteriophages by addition of host bacterium <i>E. coli</i> into phage stock. (B) Enrichment of bacteriophages in a Petri dish by addition of Tris.HCl buffer in DAL plates containing TNTC plates.....	47
Figure 2.5 Preservation of bacteriophages. (A) Phage P1-P3 stored in bacterial medium with the bacterial cells having been removed by centrifugation and filtration at 4°C and -20°C. (B) Phage T1-T3 stored in Tris.HCl buffer at 4°C and -20°C	49
Figure 2.6 Preservation of bacteriophages. Bacteriophages were stored in bacterial growth media and in Tris.HCl buffer at 4°C (A) and at -20°C (B)	50
Figure 2.7 Lysis curve for purified phage stocks (P1-P6) was performed to characterise bacteriophages and was monitored by OD measurement. Arrow represents the point at which bacteriophage samples were added, 3 hours after bacterial incubation. Positive control (CNTRL) representing normal <i>E. coli</i> strain B growth curve was included.....	53
Figure 2.8 Transmission electron micrographs of concentrated and purified samples. All samples showed phages with icosahedral heads and non-contractile flexible tails. P3 appears to possess a base plate. Scale used was 100 nm (P1 and P5) and 200nm (P2, P3, P4, and P6).....	54

Figure 3.1 Diagram outlining the optimised DAL method. 100 µl of bacteriophage and bacteria were added into a test tube with Ca-Mg solution. This mixture was pipetted onto an agar plate, soft agar was added, and the plates were incubated at 37°C for 20 hours72

Figure 3.2 Effect of bacterial cell concentration on pfu numbers with bacteriophage samples P1, P2 and P3. Various amounts of exponentially growing bacterial cells were added with 0.1 ml bacteriophage sample and DAL was done. Data represents averages of tests done in triplicate77

Figure 3.3 Effect of incubation time on pfu counts with bacteriophage samples P1, P2, and P3. 100 µl of phages and 100 µl of bacterial cells were used and incubated at 37°C. Plates were read after 16, 20 and 24 hours78

Figure 3.4 Effect of incubation temperature on pfu counts determined using bacteriophage sample P1, P2, and P3. DAL plates were incubated at 34, 37, and 39°C. Results shown were averages of tests done in triplicate after 20 hours of incubation79

Figure 3.5 Influence of addition of bivalent ions on pfu counts. Bacteriophage samples P1, P2, and P3 were used for this experiment. Individually prepared Ca and Mg ions were mixed and added to a phage-host DAL. A control (phage-host DAL plate) for each phage sample was performed as well80

Figure 3.6 Comparison of plating techniques. Pfu counts determined by Adams DAL method was compared to pfu counts after improvements in the plating technique. Phages P1, P2, and P3 were used for this experiment. Results showed were averages of tests done in triplicate81

Figure 3.7 Detection of lysogens with Mitomycin C. Concentrations of Mitomycin C used were 0.2, 0.5, 1.0, and 2.0 µg/ml. Presumptive resistant *E. coli* strain B isolates C5A and C5B were used.....85

Figure 4.1 Treatment of bacteriophages using heat. Phage samples P1-P6 were mixed and exposed to different temperatures: 70, 80, 100, and 120°C using a heating block. Complete bacteriophage inactivation was achieved at 120°C after 30 minutes. Results were the average of tests done in triplicate101

Figure 4.2 Bacteriophage inactivation using inhibitors. Different kinds of vitamins were used individually and then mixed. Phages were incubated with the vitamins at 37°C for 30 min and DAL was done. Results are an average of tests done in triplicate.....105

Figure 4.3 Effect of phage-inhibiting vitamins on the bacterial growth. Growth of the host bacterium showed no difference in the presence of inhibiting vitamins when compared to the control, host bacterium without the vitamins106

LIST OF TABLES

Table1.1 Three physiological classes of coliphages isolated from aquatic habitats (Primrose <i>et al.</i> , 1982)	13
Table 2.2 Levels of bacteriophages in the environment detected by DAL	44
Table 2.3 Sample collection and handling	41
Table 3.1 Isolation of potentially phage-resistant bacterial cells	82
Table 3.2 The detection of lysogenic phages using UV light	83
Table 3.3 The detection of lysogenic phages using the green plate method	84
Table 4.1 Treatment of bacteriophages and <i>E. coli</i> strain B using chemicals	103
Table 5.1 Parameters for the bacteriophage-host bacteria system	113

ABSTRACT

Production of numerous biotechnologically-important products such as threonine is based on cultivation of bacterial cultures. Infection of these bacterial cultures by bacteriophages has a detrimental effect in the production of these bioproducts. Despite this, most people controlling these bioprocesses do not recognize the early signs of bacteriophage infection. SA Bioproducts (Pty) Ltd was no exception and has suffered tremendous loss of production time after bacteriophages infected threonine producing *E. coli* strain B. This study was aimed at developing assays to control and prevent bacteriophage infection at this company. These included determining the source of phages by monitoring the process plant environment, optimising the detection and enumeration methods so as to monitor the levels of bacteriophages in the environment, identification of bacteriophages in order to determine the number of bacteriophages capable of infection threonine producing *E. coli* strain B, treatment and of phages, and possible prevention of phage infection. Adam's DAL method was very efficient at detecting phages in the samples collected at various areas (sumps, odour scrubber, process water, and soil) around the plant for 16 weeks. High levels of phages were found in the sumps and this was identified as the source of infection. Samples collected were grouped together according to their source. The samples were enriched and purified in order to characterise them. The prevalent phage in all samples was identified as a T1-like phage. Bacterial strains that grew on the plate in the presence of phages were assumed to be resistant to phages or contained lysogenic phages which would explain the new lytic cycles that were observed whenever these resistant strains were used for production. UV light, green

indicator plates, and a mutagen (Mitomycin C) were used to detect lysogens. Mitomycin C at 1 µg/ml was found to be most effective in detecting lysogenic phages. This was shown by new plaque forming units that were visible on the DAL plates. Temperature (heat), chemicals, and inhibitors (vitamins) were investigated as strategies for prevention and treatment of bacteriophage infection. Bacteriophage samples were exposed to 70, 80, 100, and 120°C. At these temperatures pfu counts in the samples were reduced significantly. At 120°C there was a complete inactivation of bacteriophages within 30 minutes. Chemicals investigated such as sodium hydroxide and Albrom 100T were capable of complete deactivation of bacteriophages at a very low concentration (0.1%). Therefore, these chemicals can be used to clean the plant area and sumps. Vitamins C, K and E solutions were investigated to determine their inhibitory effect on bacteriophages. Vitamin C, K and E reduced pfu counts by 3, 2, and 4 logs, respectively. Therefore vitamin C and E solutions were mixed and to determine if mixing them would enhance their inactivation capabilities. This resulted in a reduction greater than 9 logs of phage in the sample (from 7.7×10^9 to 3 pfu/ml). The host bacterium was also exposed to this mixture to determine effect of the vitamin mixture on its growth. It was found that there was no effect exerted by this mixture on the host bacteria. This proved to be an ideal mixture for combating phages during fermentation. However, vitamin E is not cost effective for co-feeding in 200 m³ fermenters, and therefore vitamin C solution was a cost-effective alternative. It was concluded that bacteriophage contaminated bioprocessing plant should be properly cleaned using a combination of heat and chemicals. Bacteriophage infection should be prevented by employing inhibitors.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Many biotechnologically-important products are produced by cultivation of bacterial cells. Therefore, bacterial cells are of outmost importance in research and biotechnology (Los *et al.*, 2004). Serious problems occur in the laboratories and factories when cultures are contaminated by natural parasites of bacteria such as bacteriophages (Callanan and Klaenhammer, 2002).

Bacteriophage infection of bacterial cultures leads to detrimental problems which includes complete loss of bacterial culture and subsequently bioproduct formation and the spread of bacteriophages throughout the laboratory or factory (Ogata, 1980). This is critical for the microbiology laboratory as the problem with lysis of cultures may reappear frequently, even after extensive cleaning process. The effect of phage contamination is more detrimental when bacterial cultivation is performed on a large scale. Decontamination is very difficult if not impossible in large factories. If the phage is propagated in the bioreactor, it can spread throughout the plant and may survive for a lengthy period, especially in places that are difficult to decontaminate. This will cause reappearance of phage infections in the factory even after it has been previously treated (Primrose, 1990).

Apart from lysing the bacterial cells, there is another problem caused by these viruses. Upon infection of its host, some phages can insert their genetic material

into the host chromosome, forming prophages, instead of lysing the cell (Los *et al.*, 2004). Jones *et al.* (2000) reported that lysogenic strains (i.e. those bearing integrated prophages) may grow slower than the non-lysogenic strains, and the efficiency of synthesis of bioproducts in the lysogenic strains can be decreased.

The location of a bioprocessing factory should be considered carefully (during plant design) as it can greatly influence the chances that infections occurring. Bioprocesses using genetically modified bacteria especially *E. coli* are increasing. These factories should not be close to the sewage disposal plants. Coliphages are present in great numbers in these sewage disposal plants and aerosols produced by activated sludge plants can result in wide dispersal of phage particles. Even plants located in agricultural areas are not safe because farmers spread sewage and farmyard waste in their farms. Plant operators should make sure that there are no pools of production organisms in waste channels and under sampling ports because these can breed unwanted phages (Primrose, 1990; Los *et al.*, 2004).

This study involved the development of strategies to control bacterial infections in the S.A. Bioproducts bioprocessing plant during threonine production. Strategies to decontaminate the bioprocessing plant were investigated as well as strategies for preventing phage infection during fermentation process.

1.1 Bacteriophages

1.1.1 Physical properties of bacteriophages

Bacteriophages are viruses that infect and lyse bacterial cells. Bacteriophages are diverse in shape and size (Figure 1.1). Most bacteriophages have head-tail morphology. Structural variation includes, contractile vs. non contractile tails, presence or absence of base plates, collars, etc. (Prescott *et al.*, 2007).

Primrose (1990) and VanDemark and Batzing (1987) observed that all viral particles consist of protein and nucleic acid. However, some phages might have a lipid coat. The nucleic acid is either DNA or RNA (but not both) and can be single or double stranded and is located in the head. The phage length generally ranges from 50-200 nm and the head averages from 50-90 nm in width. Therefore, bacteriophages can pass through a bacteriological filter with a pore size of 0.2 μm (Prescott *et al.*, 2007).

The phage particle is fundamentally a survival mechanism for a phage genome to survive the brutality of the environment to which it is exposed to when it lyses the host cell before infecting another. It is clearly efficient as some phages can survive in the bacterial lysate for long periods at normal temperature, provided they do not dry out. A large number of phages are resistant to drying. Although freezing and thawing can cause a reduction in viral titres, survival is still very high (Atmar *et al.*, 1993).

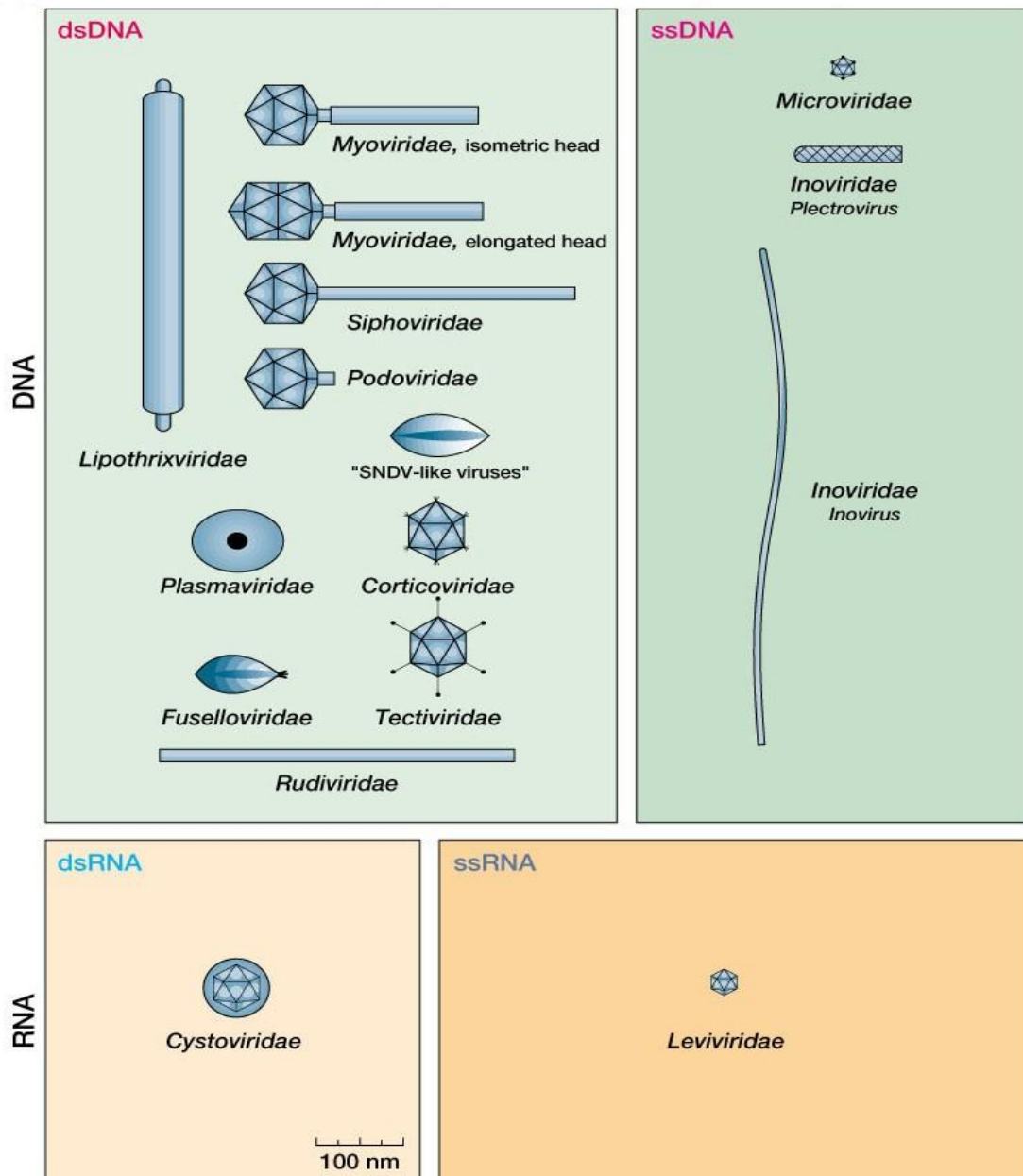


Figure 1.1 Families of bacterial viruses. All the diagrams have been drawn to the same scale and provide a good indication of the shapes and relative sizes of the virions. The name of a well-known, representative member of the family is given in parentheses, but the dimensions and shapes used for the drawing may not be exactly those of the representative virus (Prescott *et al.*, 2007).

1.1.2 Infection and reproduction of bacteriophages

Infection occurs when a phage collides with a host bacterial cell that has a specific chemically complementary site, to which a phage can be adsorbed to. The phage will then be adsorbed or attached to the bacterial surface. Viral nucleic acid then enters the bacterial cell. The phage nucleic acid undergoes replication, where viral genes are transcribed and translated and structural proteins are synthesized. The nucleic acids and proteins assemble to form a complete phage particle. Virally-specific lysozyme is synthesized with the phage assembly. This enzyme attacks the peptidoglycan layer of bacterial cell walls. The cells eventually rupture due to digestion by the lysozyme and internal osmotic pressure of the cell. This bacterial cell rupture will release new phage particles into the environment along with bacterial cell contents (Holder and Bull, 2001; Budzik, 2003).

There are two types of bacteriophages that are distinguished according to the way they infect the bacterial cells (figure 1.2): lytic and temperate. Lytic phages destroy host cells as described above (section 1.1.2). Temperate phages do not instantaneously destroy their host bacterial cell. This effect on bacterial cells is called lysogeny (Budzik, 2003). The viral DNA is integrated into the bacterial chromosome and is now called a prophage. The bacterial host cell continues to replicate and grow, despite having a prophage integrated among its own genes. The phage genes replicate as part of the bacterial chromosome. Under the right

conditions, the prophages may occasionally become spontaneously lytic, lysing the host bacterial cell as illustrated in Figure 1.3 (Lu *et al.*, 2003; VanDemark and Batzing, 1987). There is a rare type of lysogeny where the viral DNA enters the host cell but is not inserted into the host cell chromosome. Instead, the bacteriophage DNA exists in a bacterial cell as a plasmid. *E. coli* phage P1 typically carry out this type of lysogenic life cycle (Forde and Fitzgerald, 1999).

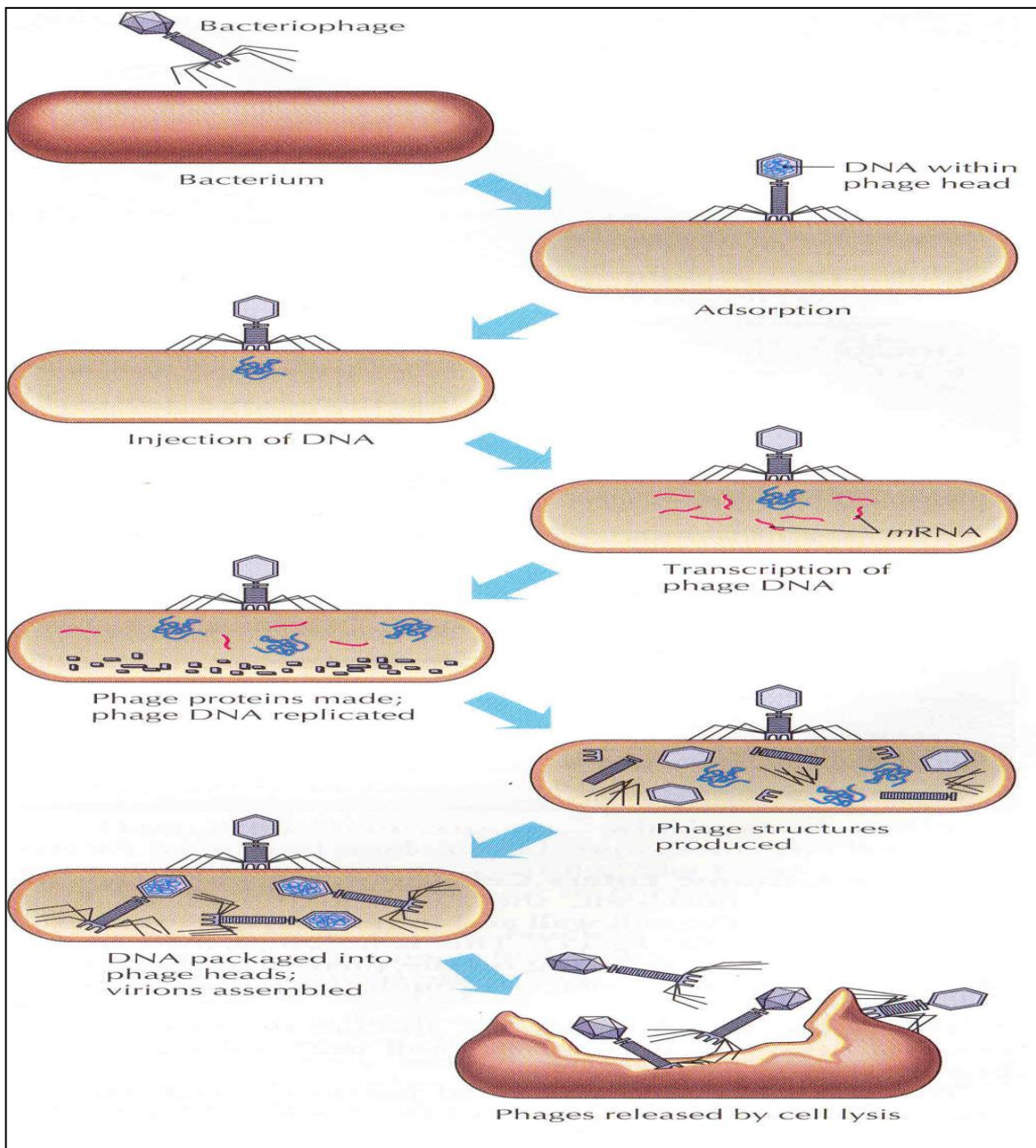


Figure 1.2 Infection and reproduction of lytic phages (Prescott *et al.*, 2007)

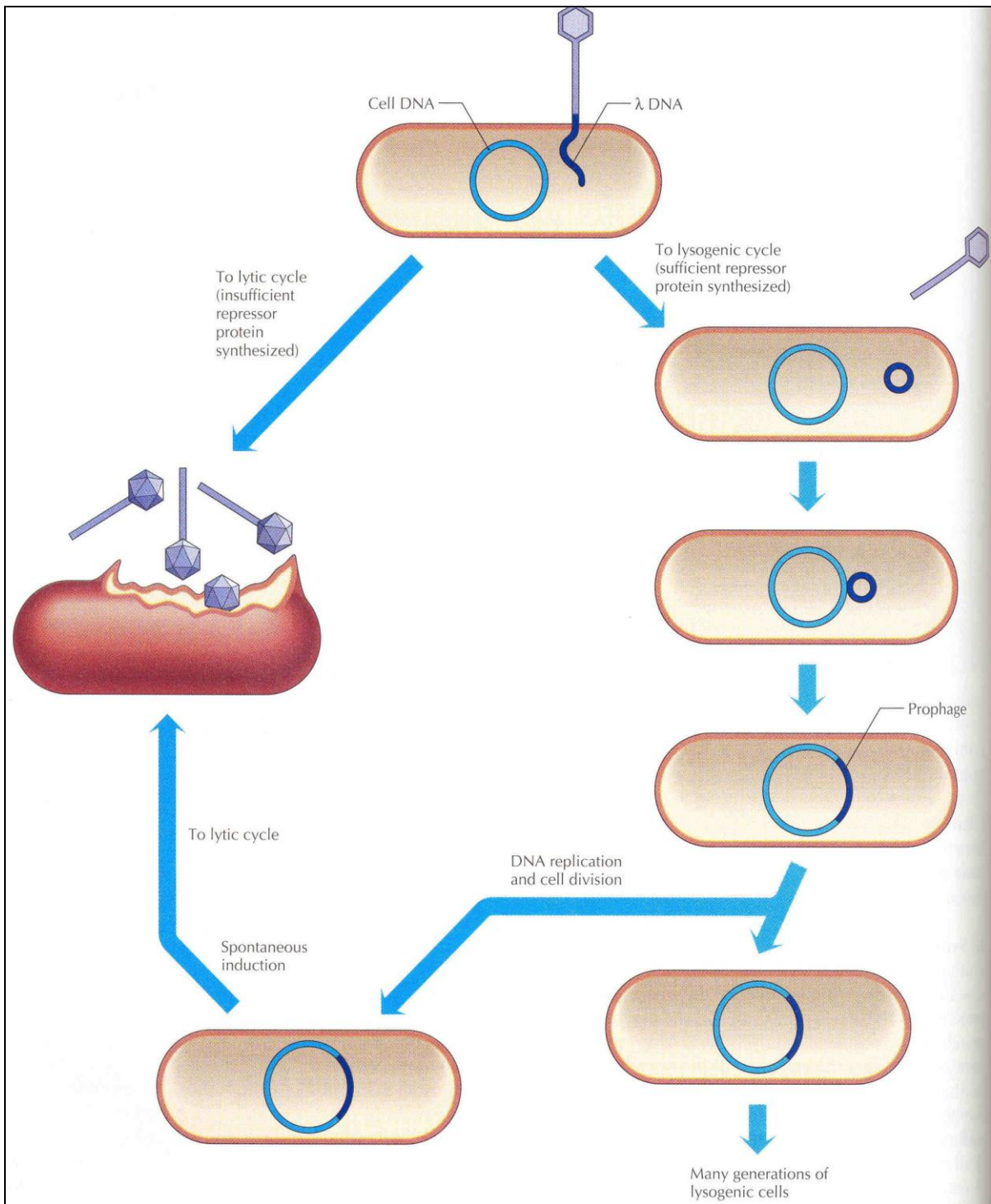


Figure 1.3 Infection and reproduction of lysogenic phages (Prescott *et al.*, 2007).

1.2 Identification of Phage Infection

When a phage multiplies in a bacterial culture, the number of infected cells can grow from a few percent to 100% in as little as 30 minutes. It is very difficult to identify phage infection when only few cells are infected (Jones *et al.*, 2000). Symptoms at the beginning of the phage infection include a delay in sugar consumption, an abrupt change in pH and weak gas production. However, during the later cycle of infection, metabolic rate of the bacterial cells decrease sharply. This is evident by the sharp decrease in oxygen uptake (OU) as well as a sharp decrease in production of gases such as CO₂. As cells lyse, biomass concentration decreases causing the broth to appear transparent, and clumps and threads in the culture will be visible (Primrose, 1990). In aerated cultures, excessive foaming may occur due to the release of host cell proteins into the growth medium. Whenever there is any microbial contamination, such as bacterial contamination in the bioprocess, deviations in parameters are observed. However, bacteriophage contaminations cause very large deviations in very short periods of time (Los *et al.*, 2004; Ogata, 1980).

1.3 Phage Infected Plant Clean-up

Decontamination of a large scale plant is very difficult. As soon as the bioreactor is suspected of phage infection, it must be shut down immediately because continued aeration of the culture medium will dispense large numbers of phages into the environment of the plant making clean up more difficult. Also, there must be no excess foaming (foam over) as this will facilitate dispersal of phages.

Spread of phages will be facilitated if the exit gas lines from a bioreactor are manifold. If a bioprocess plant has air intakes and air exits in a close proximity, then the entire aeration system, e.g. filters, compressor, etc. will also be contaminated (Primrose, 1990; Wunsche, 1989).

An assay for phage particles must be developed. Then, the contents of the bioreactor must be sterilized. This can be done by increasing temperature as high as the plant design allows and the bioreactor must be held at that temperature for as long as possible. Before discarding the contents of the bioreactor, a phage assay should be used to ensure the absence of viable phages. Pipelines and bacterial filters connected to the reactor, air inlets upstream and downstream of the compressor must be extensively steamed because bacteriophages can pass through most of the bacterial filters, if not all. When possible, liquid samples or surface swabs should be taken and assayed for phage. Ideally, exterior surfaces of all pipes and the plant have to be disinfected. This is dependent on the size and design of the plant. If design and size of the plant permits, the entire plant should be washed down with water which is as hot as is practicable. Again, samples of the washings should be analysed with a phage assay to determine the extent of contamination and the efficiency of the washing procedure in phage reduction. If open drains exist in which pools of liquid might collect and serve as a reservoir for infections, they should be decontaminated as well (Los *et al.*, 2004; Primrose, 1990; Ogata, 1980).

1.4 Bacteriophage Ecology

Viruses are obligate intracellular parasites, therefore it is impossible to understand their ecology without considering their hosts. Virus ecology may be defined as the interaction of the virus with its host cells in a particular environment and the effect this interaction has on the productivity and composition of that particular environment (Lu *et al.*, 2003; Primrose *et al.*, 1982). Bacteriophages need not only susceptible host cells to multiply, but cells which are actively growing. Therefore, the number of a particular phage in an environment can be correlated to the number of its host cells (Primrose, 1990).

In order to study virus ecology, knowledge about their distribution in the environment and the effect the environment has on their ability to infect cells and produce must be acquired. Viruses have the potential to reduce the life span of their host or kill it. If the life of the bacterial host is weakened by bacteriophage infection, it might lose its competitiveness in the environment and even its productivity (Berget and Chidambaram, 1989).

Factors which affect phage multiplication can concurrently affect the rate at which new phage particles are produced. Furthermore, if these factors are known then they can be used in phage inactivation. They include specific growth rate of the host, temperature, pH, and salt concentration. If these factors are studied, then better understanding of the phage environment can be attained (Primrose, 1982; Oki *et al.*, 1967).

1.4.1 Growth rate of the host

Bacteriophages are dependent on their host for all of their precursor molecules. Therefore, the growth rate of the host could influence the length of the eclipse and rise periods and the burst size (i.e. the average number of new phages produced by single bacteria during the lytic growth cycle). Most of the studies of phage multiplication kinetics have been done with cells growing near their maximum specific growth rate, e.g. *E. coli* growing with a specific growth rate of 2.0 to 1.0 h⁻¹ (Tanji *et al.*, 2002; Primrose *et al.*, 1982; Anderson, 1948). In nature, it is unlikely that these growth rates are attained, as even in the human gut, enteric bacteria have a specific growth rate of 0.09 to 0.07 h⁻¹. Temperate phages may even be more complex. On infecting the cell, a choice has to be made between the lysogenic or lytic cycles. This choice is influenced by the cellular level of cyclic AMP which is controlled by a number of physical factors including growth rate. Also, in a chemostat culture certain nutrient limitations can result in lysogenic induction (Abedon, 1990).

1.4.2 Temperature

Temperature can affect phage infection and multiplication as well as affecting the growth rate of the host. In studying the effect of temperature, it is necessary to take into account the temperature of the environment and the upper and lower temperatures for growth of the host. For example, *E. coli* can grow in the range 10 – 46°C, but the temperature of aquatic habitats in Britain seldom rises above 20°C (Primrose *et al.*, 1982). Seeley and Primrose (1980 – cited by Primrose *et*

al., 1982) showed that coliphages fall into three physiological classes (Table 1), depending upon the effect of temperature on their efficiency of plating. These three classes were called low temperature (LT), mid-temperature (MT), and high temperature (HT).

Table1.1: Three physiological classes of coliphages isolated from aquatic habitats (Primrose *et al.*, 1982)

Classes	Minimum plating temperature (°C)	Maximum plating temperature (°C)
Low Temperature (LT)	<15	30
Mid Temperature (MT)	<15	45
High Temperature (HT)	25	45

1.4.3 pH

Different natural habitats have widely different pH values. One effect of pH to viral particle is to alter the charge on the protein capsid and this could influence the rate of adsorption. Therefore, pH can affect the adsorption of phages onto their host bacterial cells. Phages tolerate more extended ranges of environmental conditions than their bacterial host cells. The sensitivity of acidophilic, methanol-utilizing bacteria and its phage to the pH value of the medium, investigated by Wunsche (1989), illustrated that the phage was more sensitive to pH than the host bacteria (Primrose *et al.*, 1982).

1.4.4 Salt concentration

Most bacteriophages require divalent cations for adsorption. It was established by Berg (1987) that there are bacteriophages present in marine and estuarine waters. Also, some bacteriophages are halophilic by nature and therefore salt can enhance their adsorption rate, and thus production rate (Logan *et al.*, 1980).

In order to fully study the ecology of phages, several methods are required, i.e., methods for isolation, enumeration, purification, concentration and identification of phages.

1.5 **Methods for Isolation and Enumeration of Bacteriophages**

Methods to isolate and enumerate bacteriophages require a successful transmission of a viral particle to virus-free host cells and their subsequent multiplication therein (Muniesa and Joffre, 1989; Primrose *et al.*, 1982). The problem with studying bacteriophages is their extremely small size, which makes them difficult to visualize. Visualization requires sophisticated techniques such as electron microscopy. But electron microscopy is rarely used because there is no behavior, kinetics, or reactions that can be seen. Therefore, it is only used to determine phage structures (Luria *et al.*, 1951). The size of bacteriophages necessitates the use of indirect techniques of detection and enumeration using reactants or host bacteria. These indirect techniques make it possible to obtain necessary information about the evolution and of the population of the phages and physiological state of the phages. These methods must be precise and

reproducible in order to be considered scientifically valid. When selecting a method, financial aspects must also be considered (Coffey *et al.*, 2001; Atmar *et al.*, 1993).

It is not possible to get a method that can fulfill all the above specifications. Therefore, a compromise needs to be made when selecting the best method to use. Enumeration of phage methods are divided into two groups. The first group consists the techniques that do not need the presence of the host bacterium i.e. electron microscopy, and techniques using molecular biology. The second group is the microbiological detection techniques which all require the presence of the host bacterium. Therefore, these methods do not directly detect the presence of phage but rather the lysis of the bacterial host (Rossi, 1994).

1.5.1 Host bacteria independent methods

1.5.1.1 ***Electron microscopy***

This method can be used to detect viral concentration above 10^4 phages/ml. It cannot be used therefore for systematic counting of diluted samples. It cannot determine the physiological state of the phage observed. This method is not selective but detects all the phages in the sample. As a result a particular phage of interest might not be detected because it would be lost in a bacteriophage population of phages. Therefore, it a purification step is necessary when using this method (Serwer *et al.*, 2004; Ewert and Paynter, 1980; Hancock and Reeves, 1975; Walker and Anderson, 1970).

1.5.1.2 **Genome analysis**

This method is based on the selective amplification of the genetic material. It is very sensitive for the detection of phages. But, the quantification of phages in the sample is still too complex for this method to be useful for quantification of a large number of samples. PCR is therefore a great tool for detecting presence/absence of the phage. But the cost of source material and enzymes used in this method is high (Coffey *et al.*, 2001; Atmar *et al.*, 1993).

1.5.2 Host bacteria dependent methods

Viruses are obligate parasites whose assay involves the presence of the living cells. Therefore, to determine the number of bacteriophages present in the sample the host has to be present (Purdy *et al.*, 1984). This method measures the lysis of bacterial cells. If conditions are favorable for bacteriophage infection, the liquid or solid culture becomes translucent. The most commonly used method to date is the one described by Adams in 1959 using a double agar layer (DAL). This method uses a petri dish with a first layer of solid medium (1% agar). The bacteriophage and host bacteria are then poured onto the surface of this agar with some soft molten agar (0.6%) at 45-50°C. In this method, the phage and bacteria are trapped in the medium soft enough to allow their diffusion but solid enough to inhibit any movement of fluids. This will then allow the formation of clear zones in the agar called lysis plaques (Cornax *et al.*, 1990; Oki *et al.*, 1967).

1.6 Identification and Characterisation of Phages

Bacteriophages, unlike the animal and plant viruses do not fall into well defined groups. The head and tail structure is the common morphological group. But almost every conceivable variation of this structure can be encountered. Assigning a filamentous and icosahedral bacteriophage to its correct family might be easier, but this is not true if it has a tail. Physico-chemical characterisation of a phage might assist in its identification, but it is such an extensive process. The most vital properties of phages that assist in identification are morphology and host range (Rossi, 1994).

1.6.1 Identification of bacteriophages using host range

Determination of the host range is usually a simple procedure. The common method involves applying drops of phage suspension to a lawn of bacteria and look for zones of lysis. This method does not take into consideration the fact that some phages may kill the cells without multiplying in them Morrison and Malmay, (1971 – as cited by Primrose *et al.*, 1982). Also many phages have capsid depolymerases on their outer membranes. This can cause zones of turbid lysis in the spot test and can be mistaken for lysogeny. A suitable method involves making phage dilutions and putting small drops of each dilution on a single lawn of each bacterium (Sewer *et al.*, 2004).

1.6.2 Identification of bacteriophages using electron microscopy

Electron microscopy can give a significant amount of knowledge about the phage being studied. Nevertheless, incorrect methods for sample preparation can give false results. For preliminary analysis, it is possible to examine phages isolated directly from a plaque. For high resolution though, a highly purified and concentrated bacteriophage preparation is required (Serwer *et al.*, 2004; Ewert and Paynter, 1980).

1.7 **Treatment of Bacteriophages**

Viral destruction or inactivation is an important part of phage control in a bioprocessing plant. Physical parameters that may directly affect the phages are temperature, chemicals, UV and inclusion of inhibitors in production broth (Los *et al.*, 2004). Viruses can lose their virulence due to the following reasons (Rossi, 1994):

- the alteration or destruction of phage capsid or tail;
- when specific receptors are destroyed, making it impossible for a phage to recognize the host;
- due to chemical or physical alteration of the phages genetic material; and
- the phage binding with complex organic molecules.

1.7.1 Heat treatment

Most phages are sensitive to heat, and temperatures above 70°C will inactivate most phages completely with sufficient exposure time. At 100°C, they are almost

instantly inactivated. Below 65°C, some phages can be inactivated but most are not (Callanan and Klaenhammer, 2002). The medium in which the phages are inactivated plays a major role in inactivation. Inactivation in pure water is rapid, but addition of salts like calcium or magnesium and proteins reduces the rate of inactivation significantly (Primrose, 1990). In general, the sensitivity of bacteriophages to environmental factors correlates with the corresponding properties of their host bacteria. For instance, phages infecting the extremely thermophilic, anaerobic, sulphur reducing archaebacterium *Thermoproteus tenax* are able to lyse their host at a temperature of 88°C which is usually used to cultivate this bacterium (Wunsche, 1989; Ogata, 1980).

1.7.2 Chemical treatment

As phages consist of protein molecules which envelop their genetic material, they are sensitive to protein-denaturing chemicals. Because of this property, solutions of bleaching powder have been recommended as powerful phage inactivators applicable in a technical cleaning process. Some detergents such as sodium dodecyl sulfonate are known for their phagocidal effect (Wunsche, 1989). Most of the surface disinfectants that are used against bacteria can be effective in phage inactivation. Thus, compounds which react with proteins and nucleic acids should be effective in inactivation of phages. Under certain circumstances, phage particles exist as aggregates. Adding surface disinfectant to phage aggregates may fail to inactivate phages (Primrose, 1990). Also dead microbial cells can shield the phage from chemical inactivation. Similar protective effects exerted on

the phage was seen in the study by Stagg *et al.* (1977 – as cited by Primrose *et al.*, 1982) when a phage was associated with inorganic clay. This indicated that phages temporarily inactivated by association with solids, may be protected from other form of inactivation (Primrose *et al.*, 1982).

1.7.3 UV treatment

Use of UV lamps can be very helpful in keeping production rooms and laboratories microbiologically clean (Los *et al.*, 2004; Wunsche, 1989). Exposure of bacteriophages to UV can result in their inactivation. UV rays are capable of disrupting phage DNA which can result in phage inactivation. But some studies have shown that some phages have the ability to reverse the DNA damage due to UV radiation by repairing of DNA lesions (Kokjohn, 2005).

1.7.4 Addition of inhibitors

Murata *et al.* (1985) studied the usage of vitamins to inactivate phages. They had previously studied the effect of water and fat soluble vitamins (Vitamin C, Vitamin K₅) on a wide variety of phages (Murata *et al.*, 1983). They showed that addition of vitamin E and vitamin K₁ inactivates a wide range of *E. coli* phages. Although these vitamins are considered practically water insoluble, Murata *et al.* (1983) showed that a 10⁻⁵ M concentration may provide >90% inactivation. Ascorbic acid can be selectively toxic and cause virus inactivation (Wang and Van Ness, 1989). Chio (1983 – as cited by Wang and van Ness, 1989) demonstrated that mixing

ascorbic acid and copper generates reactive oxygen species which are capable of damaging proteins and DNA, and can therefore be used to inactivate phages. Richter and Loewen (1982) treated bacteriophages T7 with ascorbic acid. This resulted in the rapid accumulation of single-stranded breaks in DNA with double-stranded breaks appearing only after 20 minutes of incubation. The single strand breaks were responsible for rapid inactivation of the phage as assayed by immediate plating of phage-bacteria. The bacteria/phage mixture was incubated in liquid medium to allow the host cell reactivation process to repair the nicks and reactivate the phage. The liquid incubation recovery period provided time for the DNA repair process which can repair several types of damage to both host and phage DNA. This phage DNA repair would have not taken place if cell growth was immediately initiated on agar plates, giving rise to apparent inactivation following ascorbate treatment. Non-reactivation of the phage was a slower process which could be correlated with the appearance of a double-strand break in phage DNA.

Samuni *et al.* (1983) claimed that ascorbic acid, Cu (II) / Fe (III) and oxygen inactivated T-uneven phages. This was done at temperatures >44°C. They showed that T-phages adsorbed to Cu (II) with high selectivity.

Shalitin and Katchalski (1962) showed that T2 coliphage can be irreversibly inactivated by incubation with polylysine at 37°C followed by tryptic digestion. The T2 particles treated appeared intact but had lost their ability to form plaques.

They demonstrated that the phage could still adsorb and inject its DNA into the host cell but the host cell did not die.

Apple pectin, a complex organic medium was used by Reiter (1956) to protect *E. coli* strain B from lysis by bacteriophage T2. The pectin was neither bactericidal nor virucidal. The rate of phage adsorption was not altered, but part of the initially adsorbed phage could be eluted with distilled water, as a second irreversible step of adsorption was inhibited by pectin. It was shown in a single-step growth and single cell burst experiment that phage multiplication was reduced. The release of any formed phage from the host was not affected. It was concluded that the protective effect of the pectin resulted from the failure of some of the phage particles to penetrate into the host cell and from its action in reducing phage synthesis in those cells where penetration did take place. It was suggested that this non-specific polysaccharide may exert its protective action because of its polymeric electrolyte nature.

1.8 Prevention of Phage Infection

In order to prevent phage infection, measures have to be taken and they include preventing phage invasion, isolating and employing phage resistant mutants, and employing chemicals that halts the multiplication and spread of phages.

1.8.1 Prevention of phage invasion

Phage invasion may be prevented by good laboratory hygiene. It is important to wash and sterilize all equipment and apparatus. It is also important that the raw materials to be used are sterile. Examination of phage concentration in the plant environment (air, soil, and sewage) must be done regularly. Sterile and clean working conditions must be employed. Also, test the glycerol stocks for phages by DAL regularly and destroy spoiled cultures and old cultures by autoclaving them (Los *et al.*, 2004; Ogata, 1980).

1.8.2 Employment of phage-resistant mutants

One possible method to deal with phage contamination is to isolate, and use in the fermentation process a bacterial mutant resistant to phages. Mutants must be selected carefully such that they do not lose their production capabilities. Mutants must be able to perform in the fermentation process as the original strain. Mutants must be treated with antisera to ensure that various phages do not attach to their cell surface and should be free from any phages. Lysogenic strains are often encountered among resistant strains. The mutants must not be used because they can release the phages into the broth. Resistant mutants alone cannot cope with new phage infections. It may contribute in the stabilization of the fermentation process, but it is still important to ensure that all phages in the vicinity of the fermentation sites are eliminated (Los *et al.*, 2004; Jones *et al.*, 2000).

1.8.3 Employment of chemicals to halt phage multiplication

There are numerous chemical agents that can be used in the bioreactors to inhibit phage multiplication without interfering with bacterial growth. For example, in order to prevent infection by phages that require divalent cations for adsorption or DNA injection, chelating agents can be added to the fermentation broth. Antibiotics can also be added in the fermentation broth. The antibiotics must have a limited effect on the host bacterium than on the bacteriophage (Ogata, 1980).

1.9 **Scope of the Present Study**

SA Bioproducts is a bioprocessing plant that produces amino acids by large scale fermentation. One of the amino acids produced is threonine, using an *E. coli* strain B. bacteriophage was first suspected during threonine fermentation in the pilot fermenters. During fermentation, a flask is inoculated and then transferred into a prefermenter. The prefermenter is used to inoculate main fermenters. During fermentation, in the fermentation lab (pilot plant), a prefermenter was used to inoculate three pilot main fermenters. Two of the three fermenters fermented well with normal oxygen uptake (OU) trend. One fermenter had exponential growth for about 8 hours and a sudden OU decrease was observed. The OU was about 40 mmol/h at 8 hours and this dropped to zero after 5 hours. This phenomenon was seen intermittently in the runs that followed. The fermentation performance was variable and the sudden drop of OU trend was often seen (figure 1.4). This trend was also seen on large scale bioreactors during threonine production (Parry, 2004).

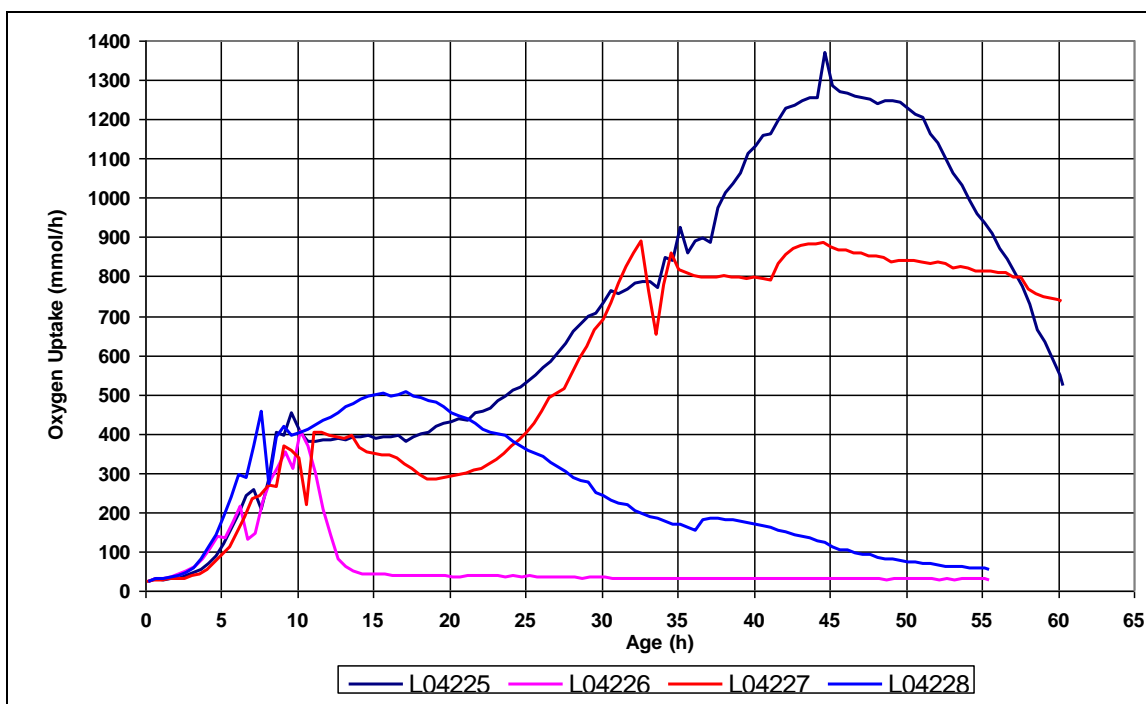


Figure 1.4 Oxygen uptake trend on pilot fermenters during threonine production. Fermenter L04225 and L04226 had bacteriophage infection as OU trend decrease after ten and 17 minutes respectively. Trends L04227 and L04228 exhibited normal threonine fermentation.

After the loss of more fermentation runs due to this drop in OU trend. This phenomenon was investigated as it was randomly attacking fermentations. After ruling many reasonable causes, the possibility of phage infection was investigated. Samples were taken of the failed fermentations. When observed under the microscope, a few live cells and cell debris were visible. Also, samples were sent to the University of Free State for analysis. They had observed plaque formation on agar plates (Parry, 2004).

The above evidence confirmed that the laboratory fermenters at SA Bioproducts were being infected with bacteriophages that completely destroyed the batch by

lysing the cells. The same phenomenon was seen in the production plant as well, during threonine production. This was detrimental to threonine fermentation as it is entirely dependant on the host cell's viability to be long enough for biosynthesis to take place.

A phage attack is catastrophic in industrial bioprocess and results in loss of production thus threatening the liquidity of a bioprocessing plant. Despite this, engineers and technologists in charge of operating a bioprocessing plant are seldom taught basic phage biology. Consequently, they cannot recognise the early signs of a phage infection. They do not know how to carry out post infection clean-up or how to minimise or prevent future attacks.

The above mentioned reasons motivated this study which aimed to develop strategies to control bacteriophage infections in the plant or lab during threonine production. This meant defining and adapting various methods for production, enumeration and identifying phages. A series of tests were done to determine the method suitable for deactivation of phages in various parts of the plant and prevention of future attacks.

The objectives of this study were:

- to develop a method for bacteriophage isolation;
- to characterise the isolated bacteriophages;
- to determine and optimise a method for detection and enumerating bacteriophages;

- to identify the sources of bacteriophages;
- to determine a method for identifying phages; and
- to develop methods for deactivating phages.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF BACTERIOPHAGES

2.1 Introduction

Bacteriophages like all viruses are obligate intracellular parasites that need a host cell to multiply. They require a susceptible host cell that is actively growing to multiply (Lu *et al.*, 2003). Bacteriophages have adapted to infect most, if not all, members of the bacterial kingdom (Rossi, 1994). There is at least one bacteriophage specific for every bacterium, and this has proven the ubiquity of these bacteriophages. Bacteriophages can be isolated from almost all habitats. The favorite environment for a bacteriophage is the host bacterium but they can survive for extended periods outside their hosts (Primrose *et al.*, 1982). When a bacteriophage invades a bacterium, its genetic material may physically associate with the bacterial genome. In this latent form, the bacteriophage (called a prophage) is dormant. It multiplies passively with each bacterial division. These phages are referred to as temperate phages (Prescott *et al.*, 2007). Induction inside the host cell only follows some particular external stimulation such as change in temperature or UV-irradiation of the host cell. This ability to integrate into the host bacterial cell genome is not common to all bacteriophages (Budzik, 2003). All other bacteriophages that do not have this ability are called virulent or lytic phages. Their infection cycle occurs immediately after entry into the host cell. The cycle ends with the cell lysing and releases the phages (Rossi, 1994; Primrose, 1990).

The size of bacteriophages necessitates the use of indirect techniques of detection and isolation. These indirect techniques make it possible to obtain necessary information about the evolution and the population of phages as well as their physiological state. These techniques must be precise and reproducible in order to be considered scientifically valid. In addition, when selecting a technique, the financial aspects must also be considered (Coffey *et al.*, 2001; Atmar *et al.*, 1993). It is not possible to get an isolation method that can fulfill all the required specifications (Rossi, 1994). Therefore, a compromise needs to be made when selecting a best method to use.

Methods to isolate bacteriophages require successful transmission of a viral particle into a virus-free cell and their subsequent multiplication therein (Muniesa and Jofre, 1998). A variety of methods exist for isolation of bacteriophages. They include different techniques for analysis on petri dishes, as well as those that involve concentration such as filtration-elution, physico-chemical precipitation, and filtration. (Atmar *et al.*, 1993; Purdy, 1984; Seeley and Primrose, 1982). The most widely used method is an indirect method that allows the enrichment of bacteriophages in liquid medium (Serwer *et al.*, 2004; Lu *et al.*, 2003; Coffey *et al.*, 2001). The indirect method refers to microbiological detection techniques which require the presence of a host bacterium. It does not directly detect the presence of bacteriophage but rather the lysis of bacterial host. If bacteriophages specific for the host bacterium are present in the sample, then they will multiply (Rossi, 1994; Primrose *et al.*, 1982).

The problem with studying bacteriophages is their extremely small size, which makes it difficult to visualize them. Visualization requires sophisticated techniques of electron microscopy. However, electron microscopy cannot be used for isolation of bacteriophages because there are no behaviors, kinetics, or reactions that can be seen. Therefore, it is mainly used for characterisation as it shows phage structures (Bratbak *et al.*, 1990).

Electron microscopy can give a significant amount of knowledge about the bacteriophage being studied, by looking at the phage structure. It can be useful in bacteriophage detection, as well although it is not an ideal method for detection (Rossi 1994). Nevertheless, incorrect method for sample preparation can give false results (Serwer *et al.*, 2004). Bacteriophages exist in natural environment, sometimes in high concentrations and the number of different bacteriophages can be too numerous. Therefore, for preliminary analysis it may be possible to examine phages isolated directly from a plaque (Ewert and Paynter, 1980).

The purpose of this chapter was to determine the areas in the production plant that might contain high numbers of bacteriophages, which could be the possible source of all the infections that were experienced in this plant. This chapter involved the use of indirect techniques to isolate the bacteriophages from the environmental samples. In addition, the isolated bacteriophages were characterised so as to better understand them. The method for enriching

bacteriophages, the conservation of stocks, and the preparation of purified samples of bacteriophages are presented in this chapter.

2.2 Materials and Methods

2.2.1 Sample collection

The plant environment [SA Bioproducts at Umbogintwini Operation Site (UOS)] was sampled for bacteriophages. The following points were sampled: ground outside the compressor house (soil), Odour scrubber (SCR), Process water, from the process water hose located next to main fermenter 2 (PROC), Plant wash-down sump (PWDS), and Tank farm sump (TFS) (figure 2.1).

The solid samples (soil) were collected in sterile beakers. The beakers were sterilised by soaking in nitric acid overnight and autoclaved at 121°C for 30 minutes. 10 g of the soil surface located in front of the compressor house was placed into 100 ml diluent (5 g/l NaCl, 1 g/l nutrient broth) in a sterilised Erlenmeyer flask. The flask was then placed in a shaker (Innova 4300) at 150 rpm for 30 minutes, in order to release any phage particles attached on the soil particles into the diluent. The contents of the flask were then collected in a sterile centrifuge tube and centrifuged at 2500 x g, for 20 minutes to remove soil particles. The supernatant was collected and filtered through 0.2 µm sterile syringe filters (Millex GP) to remove any bacteria that may be present (Rossi, 2004).

Liquid samples (from odour scrubber, plant wash-down sump, process water, and tank farm sump) were collected with sterile 500 ml Schott bottles that were soaked over night in nitric acid and then autoclaved at 121°C for 30 minutes. 100

ml of liquid was collected from the sample points. The samples were placed into sterile centrifuge tubes. Samples were centrifuged at 5000 x *g* for 10 minutes to remove any particulates, if any was visible. The supernatant was collected and filter sterilised through 0.2 µm sterile syringe filters to remove any bacterial cells that may be present.

Samples were collected from the above mentioned sites weekly for 16 weeks and a total of 70 samples were collected. For all samples collected, bacteriophage isolation (section 2.2.2) was done immediately and then tested for bacteriophage presence using DAL (section 2.2.3). Samples that showed presence of bacteriophages were stored in the fridge at 4°C. Samples that did not have bacteriophages were discarded. A total of 70 samples were collected, 45 samples exhibited presence of bacteriophage and 25 samples did not have bacteriophages.

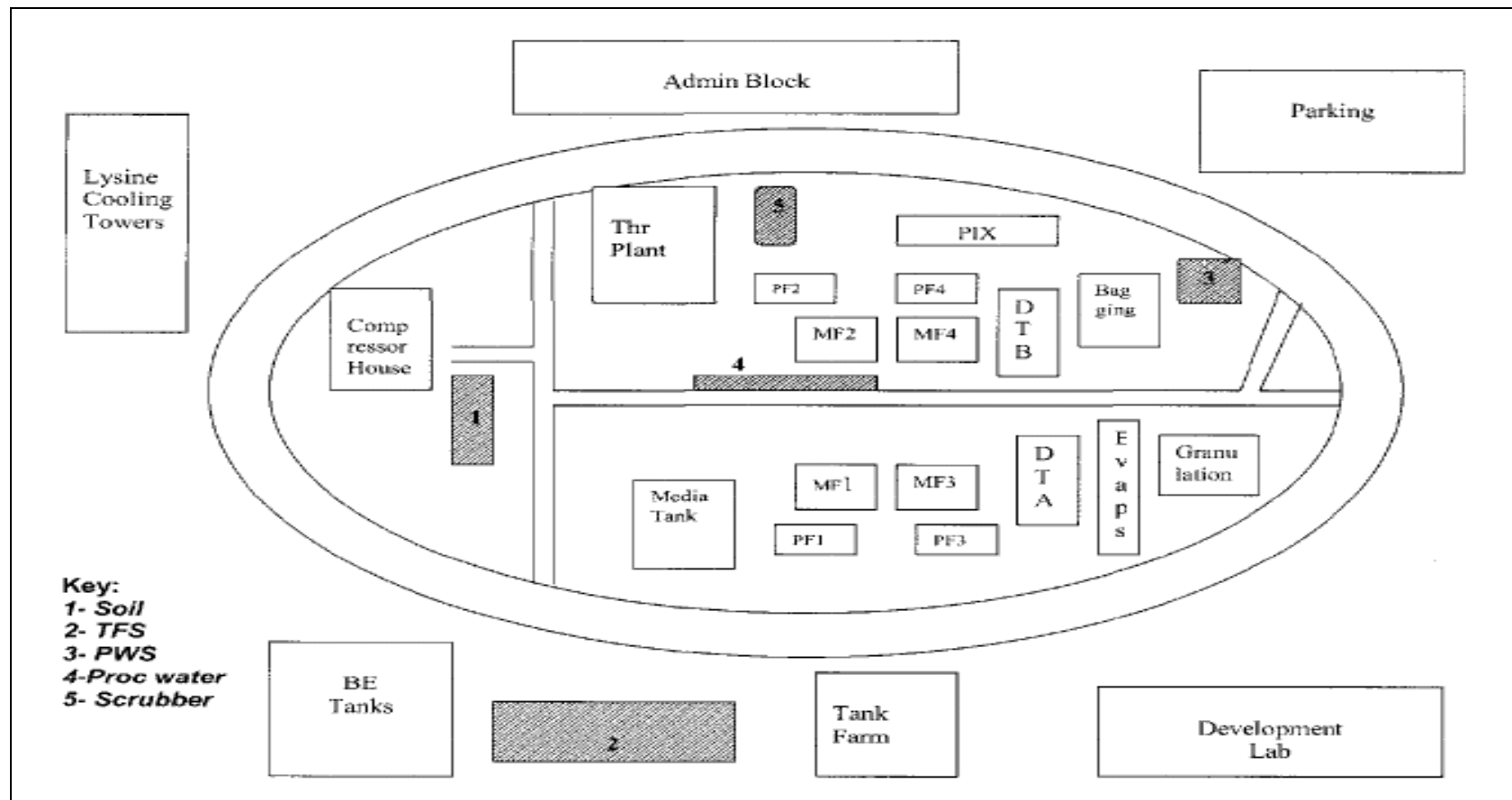


Figure 2.1 SA Bioproducts production plant layout. Shaded areas 1-5 are the areas where samples were collected. BE Tanks – broth effluent tanks, Thr plant- threonine downstream processing plant; PF – prefermenter, MF- mainfermenter, DTA and DTB- drop tank A and B, PIX- primary ion exchange, Evaps- Evaporation tubes.

2.2.2 Isolation of bacteriophages

The phages were isolated by adding a culture of the host bacterium to the sample. This was to ensure that phages specific for the bacterium present in the sample, were allowed to multiply.

Host bacterium (*E. coli* strain B, for threonine production) was used for isolating bacteriophages from the sample collected. Two ml of an overnight culture of *E. coli* B was placed into 250 ml Erlenmeyer flasks containing 20 ml of growth medium Luria broth (Biolab) (tryptone 12 g/l; NaCl 12 g/l; yeast extract 6.0g/l pH of 7.2). Flasks were incubated in a shaking incubator at 37°C, 240 rpm until an OD₆₀₀ 0.2 was reached (approximately 2 hours). Five ml of phage samples, from each sample point were added into the flasks with exponentially growing culture. The flasks were incubated for 12-18 hours or until the culture turned clear. After the incubation period, the contents of the flasks were then centrifuged at 5000 x *g* for 10 minutes. The supernatant was collected and filter sterilised through 0.2 µm filters to remove any bacterial cells that may be still present. These samples were then used for all analysis using the Adams double agar layer (DAL) method described in section 2.2.3. All samples were refrigerated at 4°C.

2.2.3 Double agar layer method (DAL)

In the Adams (1959) DAL method, a small volume (0.1 µl) of phage suspension and a small quantity (0.2 µl) of host cells grown to high cell density, (sufficient to give 10⁷-10⁸ CFU/ml), are mixed in 2.5 ml of molten 0.6% agar medium at 46°C.

The resulting suspension is then poured over an appropriate LB agar medium (casein peptone 10g/l; yeast extract 5.0g/l; NaCl 10 g/l; agar 15 g/l; pH 7.2) to form a thin layer of top layer which hardens and immobilizes the bacteria. During incubation, uninfected bacteria multiply to form a confluent film of growth over the surface of the plate. Each infected bacterium, however, bursts after a short time and liberates progeny phages that in turn infect adjacent bacteria, which are in turn lysed. This chain reaction spreads in circular motion until brought to a halt when all bacterial cells have been infected and lysed. The result is a visible, circular area of clearing in the confluent bacterial growth known as a plaque. Counting of the number of plaques, and multiplying by the appropriate dilution factor gives an indication of the number of plaque forming units (pfu) in the original phage sample

2.2.4 Purification of bacteriophages

Plaques on the DAL samples from isolation step, were classified as small (<1.5mm), medium (2mm), and large (>3mm). Plaque size that was prevalent on the isolation plates were selected and purified.

Two or three purification stages were necessary to obtain similar-sized plaques (medium) in an agar plate. This was done to ensure that each sample contained not more than one phage type. Bacteriophages were detected in a total of 45 samples. The samples from each sample point were grouped and mixed together to make one sample, and a total of 5 samples (P1-P5) resulted. Preliminary DAL was done with representative plaque size from each of the 5 samples and the

prevalent lysis plaque from each sample was selected and cut out from the petri dishes of the first analysis, prior to purification. These plaques produced mixed sized plaques and most of them were medium sized. The medium size plaques were therefore seen to be prevalent on all the samples as well, and were purified. Each plaque was added into 20 ml of growth medium with 2 ml of exponentially-growing culture of the host bacteria. The mixture was incubated at 37°C, 150 rpm for 12-18 hours. After bacterial lysis, a sample of the culture was serially diluted and added into soft agar and plated on a petri dish. New plaques were formed and were cut out and the purification was repeated twice to ensure a single type of phage was finally isolated. It was observed that after three purification steps plate P5 had medium and large sized plaques. Therefore it was decided that both sizes were to be enriched. This plaque was labelled P6.

2.2.5 Enrichment of bacteriophages

A single plaque was selected and cut out from each purification plate (section 2.2.4), and two plaques were used from plate P5. The plaque was then enriched by initially suspending it in 2 ml of Tris.HCl (1 M, pH 7.2) buffer in a test tube. The test tubes were left to stand at room temperature for 1 hour. This was then mixed by shaking the test tube with a vortex mixer.

One ml of the phage sample was added into 10 ml of an exponentially-growing culture of the host bacterium (threonine producing *E. coli*) in McCartney bottles. The mixture was incubated in a shaker incubator at 150 rpm at 37°C for 12 hours

(Innova 4300). Positive (exponentially growing *E. coli* strain B without bacteriophage) and negative controls (10 ml LB broth with 1 ml of phage sample from each sample) were included. After 12 hours, the contents of the McCartney bottle were centrifuged at 5000 x *g* for 10 minutes. The supernatant was collected and 0.3% v/v of chloroform was added. This was then shaken vigorously for 2 minutes, and centrifuged again (10000 x *g*, 20 min). The supernatant was filtered through 0.2 µm (Millex GP) syringe filter. DAL was done to determine the phage concentration in each sample. (Rossi, 1994; Luria *et al.*, 1951).

The remaining 1 ml of the phage sample was used to perform a total of 8 DAL plates per sample. Some of the plates had too numerous to count (TNTC) plaques and others were clear, showing that the phage destroyed all the bacterial cells. Five ml of Tris.HCl buffer was pipetted to these plates. The plates were left at room temperature for 5 hours, during which the bacteriophages would have diffused into the buffer. Supernatant was then collected by pipette. The samples were labelled T1-T6. Samples were streaked on Luria-Bertani (LB) agar to ensure that they were free of bacteria. Hundred µl of each bacteriophage sample harvested from the clear plates was added on the plates with *E. coli* to ensure the presence of bacteriophages.

2.2.6 Preservation of bacteriophages

Samples P1, P2, and P3 were randomly selected and used to determine preservation parameters for bacteriophages. Samples that were stored in Tris.HCl buffer were labelled T1, T2, and T3.

First method involved using enriched bacteriophages stored in the bacterial growth medium after the enrichment process, after removal of bacterial cells by centrifugation and filtration with 0.2 μm (Millex GP) sterile syringe filter (P1, P2, and P3). The second method used bacteriophage samples that were prepared by adding Tris.HCl buffer to harvest bacteriophages from DAL plates (T1, T2, and T3). One ml aliquots of bacteriophage samples were dispensed into fourteen cryovials. Seven of these vials were stored at 4°C and the other seven samples were stored at -20°C. Glycerol (50%) was added as cryo-protective agent in frozen samples stored at -20°C. Samples were tested for viability by performing DAL (section 2.2.3) every two weeks for a period of twelve weeks, using a new cryovials each time.

2.2.7 Characterisation of bacteriophages

2.2.7.1 Phage infection kinetics

An overnight *E. coli* B culture was inoculated into LB broth and incubated overnight until late stationary phase. One ml of this culture was transferred into a 250 ml Erlenmeyer flask containing 100 ml of LB broth. The culture was incubated until the OD₆₀₀ 0.6 was reached and 1 ml of enriched bacteriophage

sample was subsequently added to the culture. The flasks were incubated in a shaker at 37°C. The OD₆₀₀ was measured hourly for 12 hours. Negative (flask containing phage only) and positive (flask containing *E. coli* culture only) controls were included.

2.2.7.2 *Electron microscopy*

Phage characterisation was done at the EM unit, University of KwaZulu-Natal using TEM microscopy. The purified and concentrated phage samples (P1-P6) were negatively stained by uranyl acetate. A drop of concentrated phage sample was deposited in a copper grid with carbon-coated Formvar films, stained with 2% uranyl acetate pH 4.2. This was then visualised using a transmission electron microscope (Phillips CM120 Biotwin) operated at 100 kV.

2.3 Results

Table 2.3 Sample collection and handling

Steps:	SOIL	TF SUMP	PWD SUMP	ODOR SCRUBBER	PROCESS WATER
Samples collected	16	15	16	9	14
Isolation: Samples with phages & kept	8	15	16	7	9
Mixed phages	P1	P2	P3	P4	P5
DAL	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques
Purification done with a prevalent plaque type resulted in	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques
2nd Purification done with medium plaque	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques	Mixed plaques
3rd Purification done with medium plaque	Medium plaques only	Medium plaques only	Medium plaques only	Medium plaques only	Two types (medium & large)of plaques persistent resulted in P5&P6 respectively
Enrichment	P1	P2	P3	P4	P5 & P6
Preservation: Bacterial media Tris.HCl	P1 T1	P2 T2	P3 T3		
Characterisation	P1	P2	P3	P4	P5 & P6

2.3.1 Isolation of bacteriophages

The double agar layer (DAL) method as described by Adams (1959) was used to determine the presence of bacteriophages in the samples collected. The areas that were sampled were soil from the ground outside the compressor house, tank farm sump, plant wash down sump, odor scrubber, and process water. Samples collected were handled as described in table 2.1. Bacteriophages were isolated using *E. coli* strain B used in threonine production as a host bacterium. There were 3 sizes of lysis plaques i.e. small (<1.5mm), medium (2mm), and large (>3mm) visible on the DAL plates from the environmental samples (figure 2.2). The prevalent plaque size on most samples tested that showed evidence of bacteriophage presence was medium. It was determined that when samples were not diluted, the plaques were too numerous to count (figure 2.2 A) or the plates appeared clear. Dilution of samples showed well defined and countable plaque forming units (figure 2.2). The plaques formed were mostly clear, but some plates had turbid zones with clear centers (figure 2.2 B). The plaques shown in figure 2.2 were visible after 18 hours of incubation at 37°C.

The plant environment was sampled weekly for 16 weeks. DAL was done to detect bacteriophages in all the samples that were collected. The samples that showed presence of bacteriophages were refrigerated. The samples that did not yield any plaques on DAL plates were discarded. The plaques on the plates were counted and pfu/ml was determined. This was done to monitor the environment and determine the areas that had the highest number of phages. The DAL results

showed that the tank farm sump had the highest level of bacteriophages (table 2.2). Samples from the odor scrubber and process water had the lowest bacteriophage counts. The total of 70 samples was collected during this period, of which 45 exhibited the presence of bacteriophages and 25 had no bacteriophages (table 2.2). They were then grouped into five categories according to the place they were samples. These samples were then purified. The prevalent bacteriophage (medium sized plaques) from each purification plate was isolated. A total of six plaques were selected from purification plates and were used further in this study. These samples were labeled P1-P6.

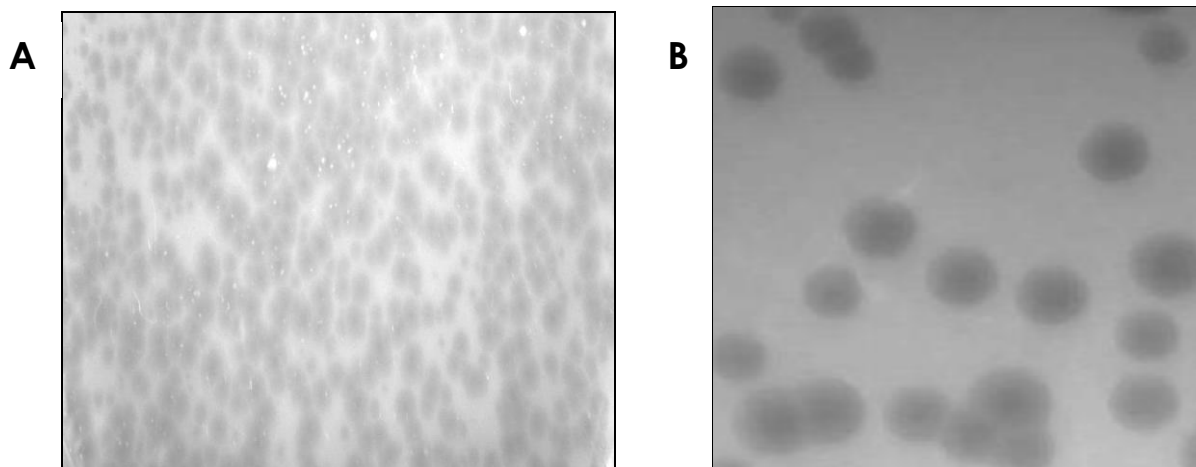


Figure 2.2 Lysis plaques of *E.coli* B following phage infection with DAL method. Plate A is an example of undiluted phage sample, with too numerous plaques to count. Plate B was 10^6 dilution of a phage sample.

Table 2.2 Levels of bacteriophages in the environment detected by DAL

Week	Soil Sample⁺	Tank Farm Sump⁺	Plant Washdown Sump⁺	Odor Scrubber⁺	Process Water⁺
1	6.00E+03	2.00E+05	3.00E+05	ND	ND
2	8.30E+01	1.14E+05	4.10E+01	ND	ND
3	5.40E+04	1.14E+02	4.00E+02	3.00E+02	5.00E+01
4	8.00E+04	2.10E+02	9.00E+03	1.60E+01	2.00E+01
5	4.00E+01	ND	4.00E+03	1.00E+01	1.30E+01
6	3.50E+03	1.00E+03	2.00E+03	ND	2.50E+01
7	0.00E+00	2.60E+04	2.00E+03	ND	0.00E+00
8	5.00E+04	2.00E+05	2.35E+02	ND	7.50E+01
9	0.00E+00	8.90E+03	7.00E+04	3.00E+03	1.30E+02
10	0.00E+00	4.30E+04	7.00E+02	1.00E+02	2.00E+01
11	0.00E+00	4.00E+04	5.50E+02	1.50E+02	0.00E+00
12	1.80E+03	2.25E+04	2.50E+02	7.50E+02	7.25E+03
13	0.00E+00	7.25E+03	3.33E+03	ND	0.00E+00
14	0.00E+00	5.75E+04	3.00E+04	0.00E+00	0.00E+00
15	0.00E+00	1.16E+03	2.00E+05	ND	0.00E+00
16	0.00E+00	4.65E+04	5.50E+04	0.00E+00	1.00E+01

ND: not determined

⁺ Phage count in pfu/ml

2.3.2 Purification of bacteriophages

The samples (P1-P6) were purified to ensure that methods developed for controlling bacteriophages (e.g. characterisation, enrichment, preservation, etc) were optimised using pure bacteriophages. DAL had to be repeated three times in order to achieve pure phage stocks i.e. similar size as plaque size was used to initially differentiate the plaques that formed on the DAL plates (figure 2.3). Since the samples produced plaques that were morphologically similar, size was used to differentiate them since it was only characteristic that was different. Preliminary experiments showed that even when a single plaque was cut out of a DAL plate and used to prepare a new DAL plated, different sized plaques resulted, mostly medium sized plaques. This led to the selection of only medium sized plaques as they were prevalent to all plates and therefore purified further until only medium sized plaques were visible on a DAL plate.

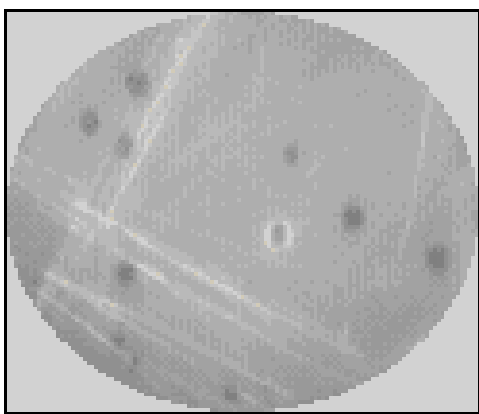


Figure 2.3 Plaque size of purified phage sample. After performing DAL three times, single size plaques were achieved (medium sized) on the plate.

2.3.3 Enrichment of bacteriophages

Bacteriophages were enriched using two methods; one method involved adding host bacterium to bacteriophage samples. The other method involved harvesting bacteriophages by adding Tris.HCl to a DAL plate with too numerous plaques. Samples P1-P6 were used to determine the method that yielded higher concentrations of bacteriophages in the sample. Samples enriched by petri dish method were correspondingly labeled T1-T6. DAL was done to determine the number of bacteriophages before and after enrichment. Results represented a mean of two samples as plaque forming unit per ml of bacteriophage sample (pfu/ml)

Samples enriched in liquid medium had higher bacteriophage concentration than the samples enriched using petri dish method (figure 2.4). Samples enriched in liquid medium showed that P1-P6 achieved about 1×10^{10} pfu/ml after enrichment from 1×10^4 and 1×10^3 pfu/ml before enrichment (figure 2.4A). Bacteriophage samples enriched in petri dishes (T1-T6) had an average increase of 5 logs in pfu counts (figure 2.4B).

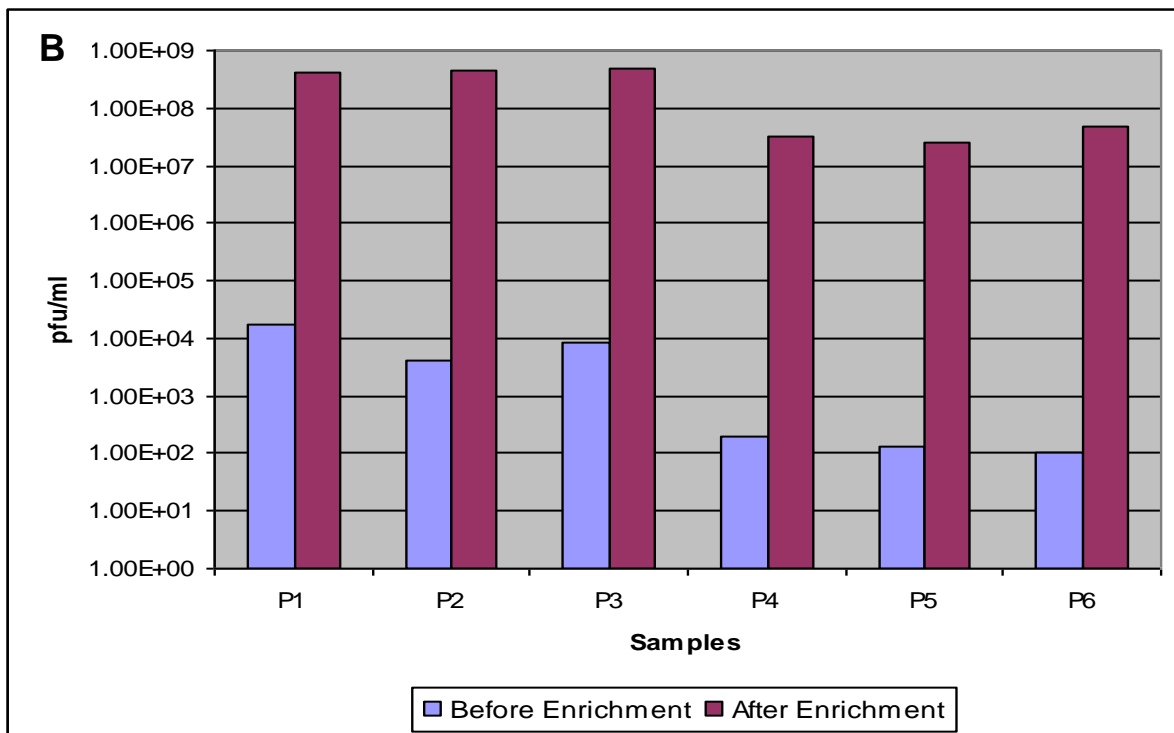
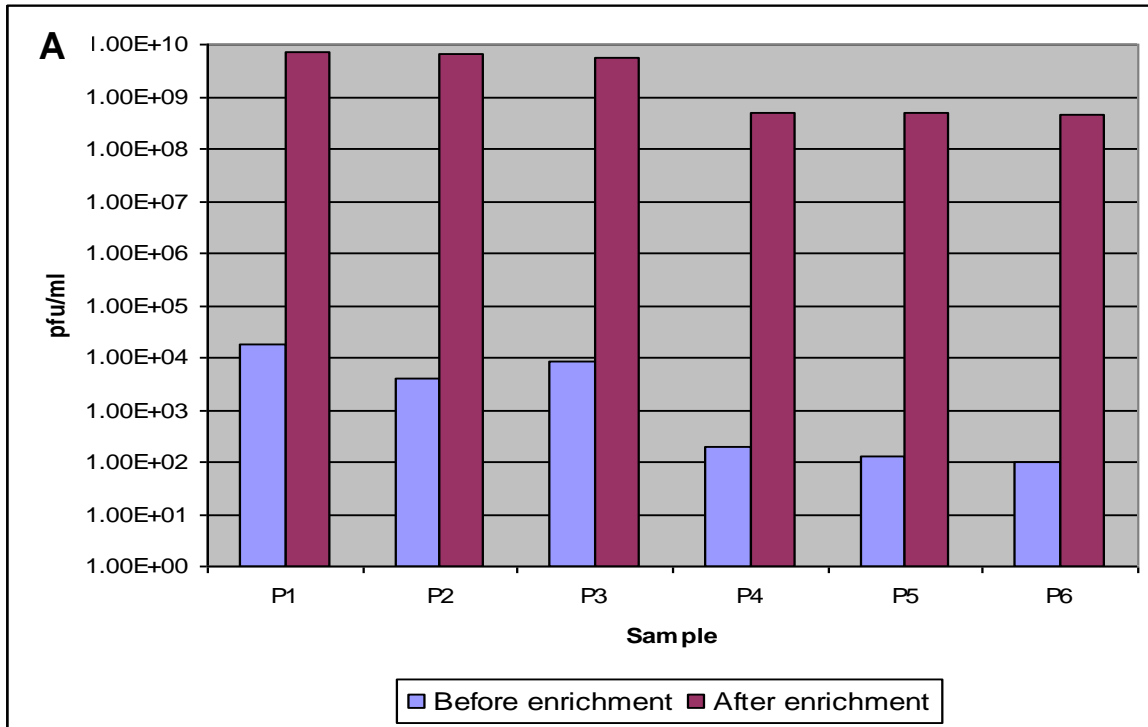


Figure 2.4 Enrichment of bacteriophages. (A) Enrichment of bacteriophages by addition of host bacterium *E.coli* into phage stock. (B) Enrichment of bacteriophages in a Petri dish by addition of Tris.HCl buffer in DAL plates containing TNTC plates.

2.3.4 Preservation of bacteriophages

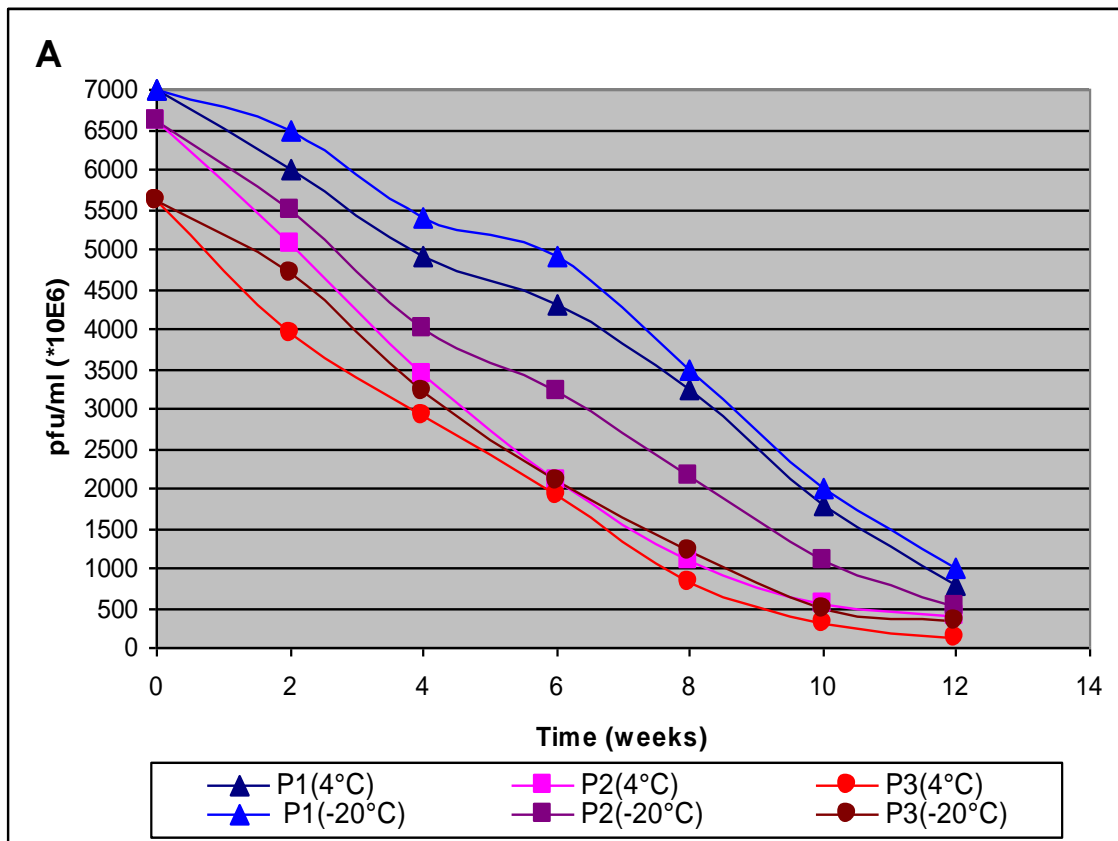
Samples P1-P3 and T1-T3 were randomly selected and were stored at 4°C and -20°C. The samples were stored for 12 weeks and sampled every two weeks to determine phage viability using the DAL method.

Samples P1, P2, and P3 (figure 2.5 A) were stored in bacterial growth media with bacterial cells removed by filtration. P1 had initially 7×10^9 pfu/ml and after 12 weeks of preservation had 8×10^8 pfu/ml, which was 89% reduction in bacteriophage counts at 4°C and 1×10^9 pfu/ml had ~86% decrease bacteriophage counts at -20°C. P2 had 6.6×10^9 pfu/ml before storage and after 12 weeks of storage at 4°C had 3.9×10^8 pfu/ml, that was about 94% decrease in bacteriophage counts and at -20°C, had ~92% decrease in bacteriophage counts (5.2×10^8 pfu/ml). P3 had 5.6×10^9 pfu/ml after enrichment and after 12 weeks at of storage at 4°C had 1.2×10^8 pfu/ml, about 98% decrease in bacteriophage counts and at -20°C, had ~94% decline in plaques counts (3.3×10^8 pfu/ml).

Samples T1, T2, and T3 (figure 2.5 B) were preserved in Tris.HCl buffer. T1 decreased from 4.2×10^9 pfu/ml to 3.62×10^8 pfu/ml at 4°C and 8.9×10^8 pfu/ml at -20°C. There was ~94% and 79% reduction in bacteriophage counts at 4°C and -20°C, respectively after 12 weeks. T2 initially had bacteriophage counts of 4.5×10^9 pfu/ml and after 12 weeks it was reduced to 4.12×10^8 pfu/ml at 4°C and 1.1×10^9 pfu/ml at -20°C. At 4°C bacteriophage counts were reduced by 15.2%

more than at -20°C. T3 initially had 4.69×10^9 pfu/ml and was reduced by 89.2% to 5.05×10^8 pfu/ml at 4°C and by 71% to 1.32×10^9 pfu/ml at -20°C.

Tris samples were harvested by adding Tris.HCl buffer onto a DAL plate had initially lower concentration of bacteriophages than the samples stored in bacterial growth media. However, results showed these samples were more stable in comparison with the samples stored in the bacterial growth medium. Also, samples that were stored in both Tris.HCl and bacteria growth media were more stable at -20°C than at stored at 4°C.



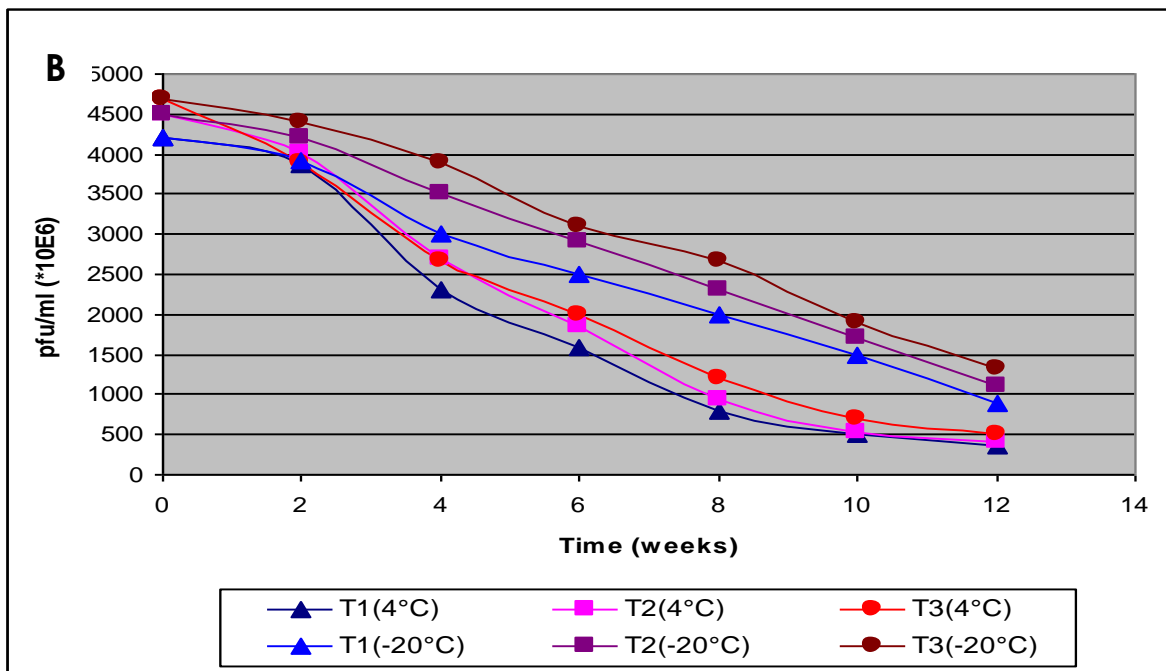
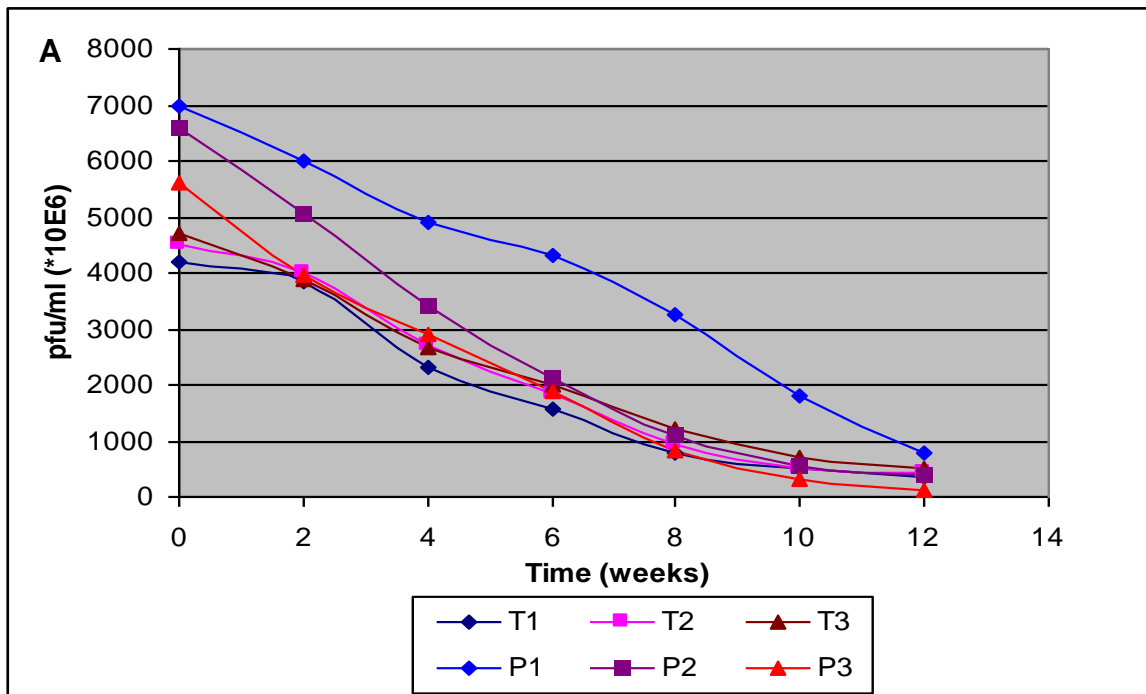


Figure 2.5 Preservation of bacteriophages. (A) Phage P1-P3 stored in bacterial medium with the bacterial cells having been removed by centrifugation and filtration at 4°C and -20°C. (B) Phage T1-T3 stored in Tris.HCl buffer at 4°C and -20°C.



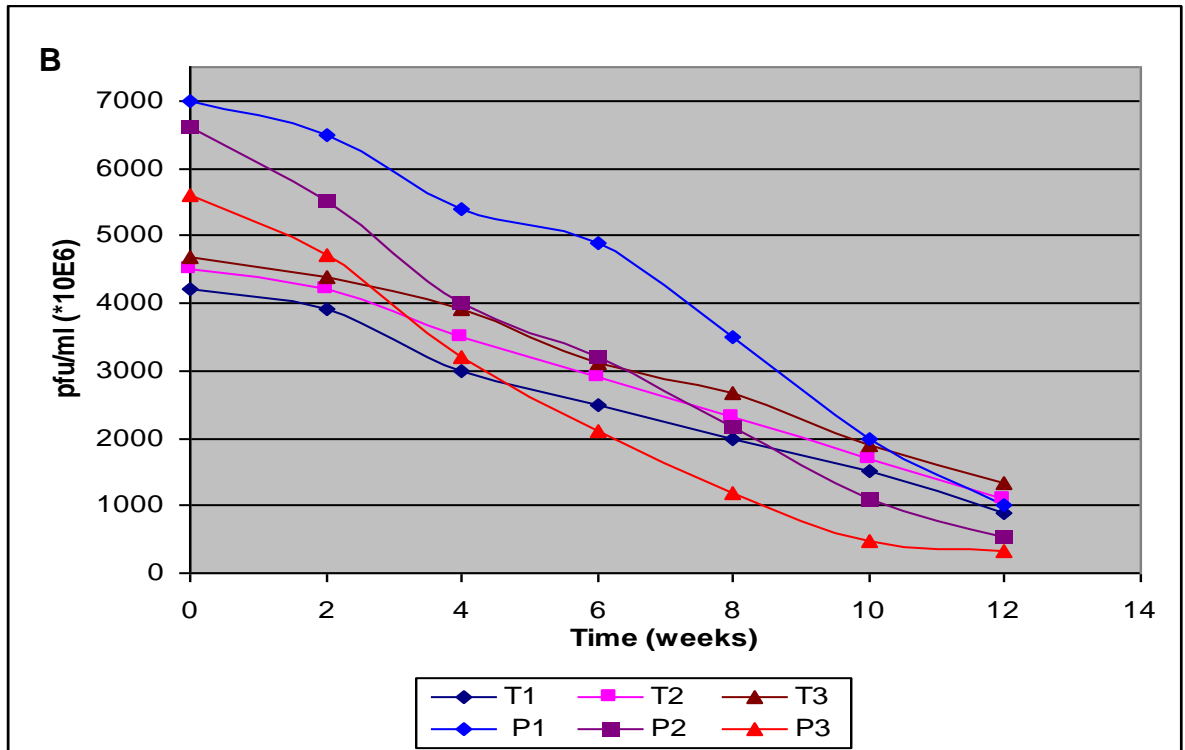


Figure 2.6 Preservation of bacteriophages. Bacteriophages were stored in bacterial growth media and in Tris.HCl buffer at 4°C (A) and at -20°C (B).

2.3.5 Characterisation of bacteriophages

2.3.5.1 ***Phage infection kinetics***

Lysis curve of purified stocks of bacteriophages P1-P6 was performed to characterise the samples. This was done by infecting exponentially-growing *E.coli* strain B with pure bacteriophage samples P1-P6. A positive control was included, consisting of an uninfected *E. coli* strain B culture used in threonine production. OD₆₀₀ was measured hourly over a period of 12 hours and was reported as a mean of duplicate experiments. Bacteriophages were introduced after 3 hours of bacterial incubation at OD₆₀₀ 0.56. CNTRL represented positive control and shows the normal *E. coli* strain B growth curve. All samples followed the same infection trend, but the rate of infection was different. Sample P1, had the lowest average OD₆₀₀ of 0.52, indicating the highest infection rate. Sample P6 had the highest average OD₆₀₀ of 1.02, indicating the lowest infection rate. Infected bacteria trends showed a sharp decrease in bacterial concentration three hours after infection for all the samples. Samples P1, P2 and P3 reached latent state after 9 hours (figure 2.7).

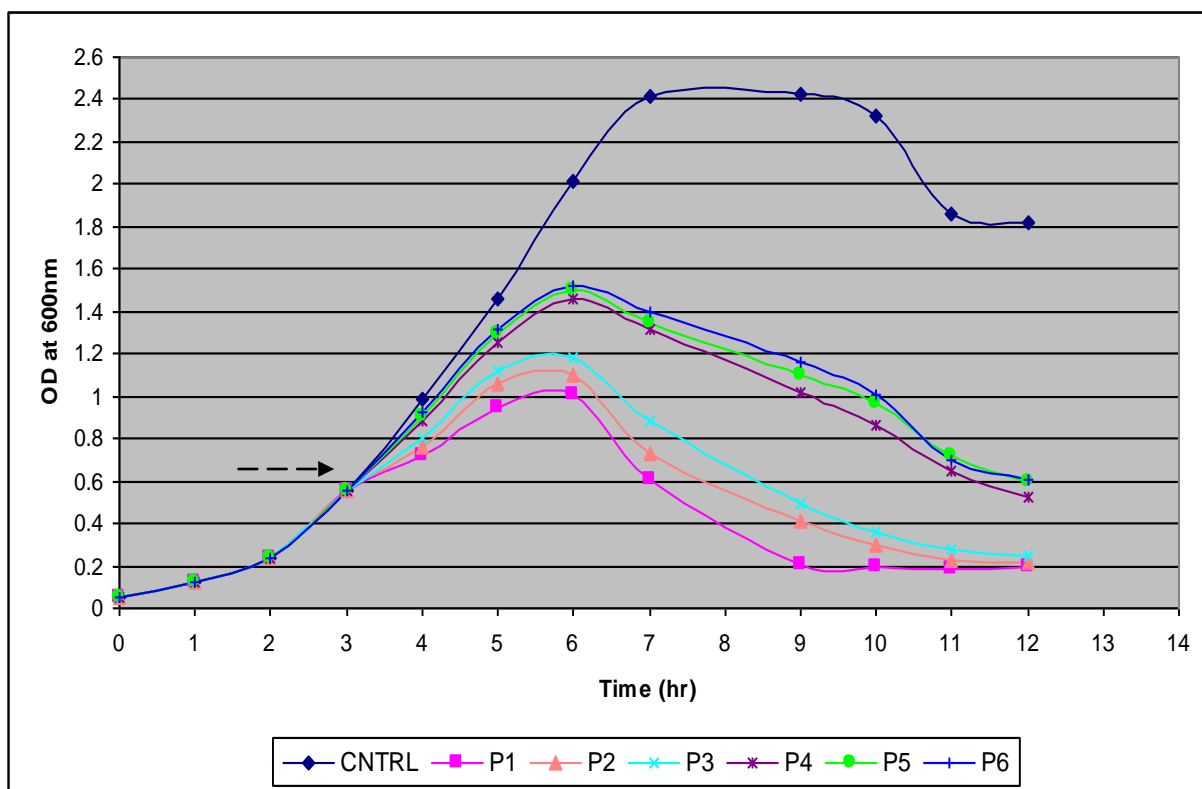
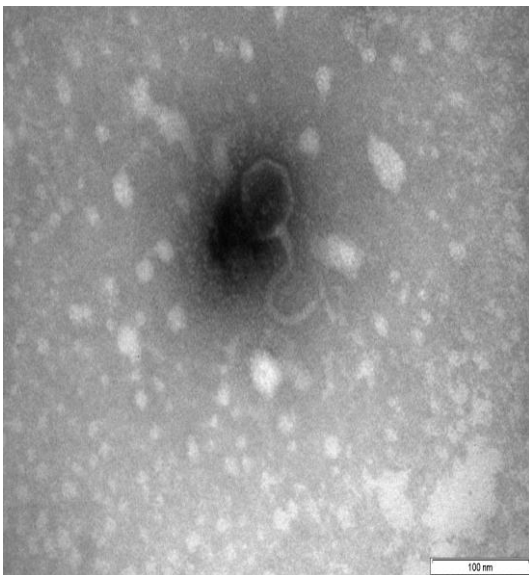


Figure 2.7 Lysis curve for purified phage stocks (P1-P6) was performed to characterise bacteriophages and was monitored by OD measurement. Arrow represents the point at which bacteriophage samples were added, 3 hours after bacterial incubation. Positive control (CNTRL) representing normal *E. coli* strain B growth curve was included.

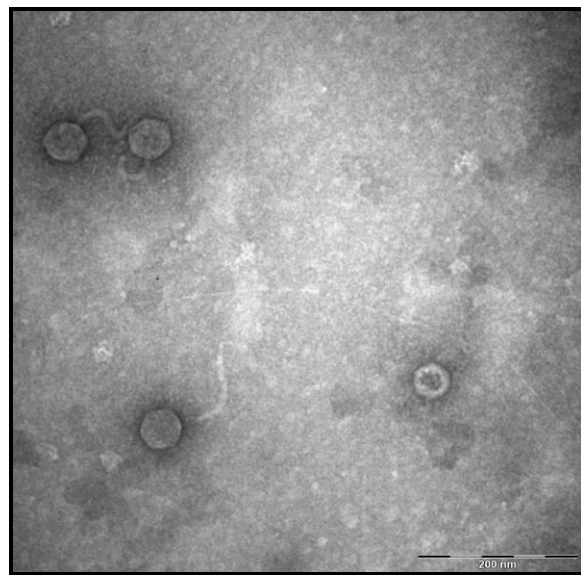
2.3.5.2 *Electron microscopy*

Pure enriched samples (P1-P6) were sent to University of KwaZulu Natal (UKZN) in Pietermaritzburg for characterisation with transmission electron microscopy (TEM). Electron micrographs (figure 2.8) showed that all the samples had similar bacteriophages that had icosahedral heads and noncontractile flexible tails. Phage heads were ~60 nm across and tails between 140 and 160 nm in length. P3 showed what looked like a base plate on its tail. This was only seen in this one micrograph. Other micrographs of the same sample did not show this base plate (Appendix B).

P1



P2



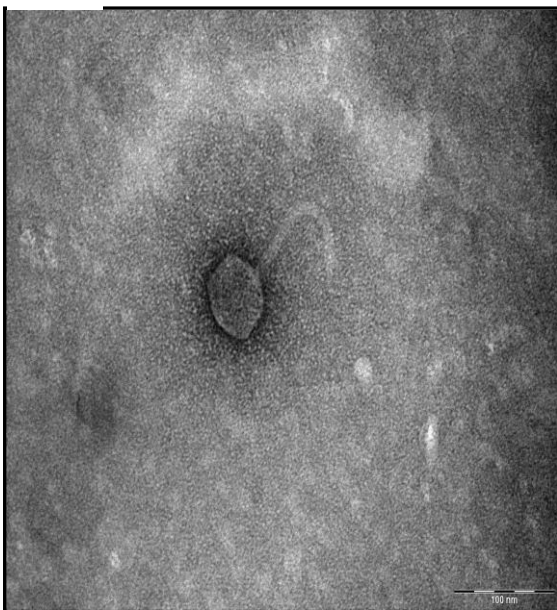
P3



P4



P5



P6



Figure 2.8 Transmission electron micrographs of concentrated and purified samples. All samples showed phages with icosahedral heads and non-contractile flexible tails. P3 appears to possess a base plate. Scale used was 100 nm (P1 and P5) and 200nm (P2, P3, P4, and P6).

2.4 Discussion

The double agar layer (DAL) method was used to isolate phages in the environmental samples, using exponential cultures of *E. coli* strain B strain used in threonine production as the host bacterium. This method is widely used in bacteriophage isolation (Lu *et al.*, 2003). Bacteriophages are prevalent in the environment (Wommack *et al.*, 1992), therefore the only bacteriophages of interest for this study were those that can infect the threonine producing *E. coli* strain B strain. Sites sampled (figure 2.1) included the ground outside the compressor house, since the compressors supplies air into the fermenters. It is possible that if the soil outside had high bacteriophage counts, they could be easily be transferred into the fermenters. The odour scrubber, used to remove odour from off-gas produced in the fermenters, was also sampled. The odour scrubber could be responsible for spreading bacteriophages throughout the plant because it blows the air from the fermenters into the environment and is located above the fermenters. The other site that was sampled was the plant wash down sump which is a sump where all the waste generated from the plant flows, and is located on the plant, also near the fermenters. The tank farm sump where all the waste generated all over the plant , fermenters and other tanks is collected before it is pumped into effluent tanks, was sampled. Process water, which is the water used in the plant for all operations that requires water including dissolving nutrients added into the fermenters, was also sampled. All these sites were sampled as external factors that can introduce bacteriophages into the

fermenters and to determine the level of bacteriophages in the plant environment and to possibly determine the source of bacteriophage contamination.

The formation of plaques on the DAL plates was evidence of the presence of active bacteriophages in the samples. It was shown that when undiluted bacteriophage samples were used, numerous plaques were visible in the plates (figure 2.2A). Prior to enumeration of plaques dilutions up to 10^{-6} were necessary (figure 2.2B). Plaques of three sizes were formed by all these samples after 18 hours of incubation, and were categorised as small (<1.5mm), medium (2mm) and large plaques (>3mm). The plaques formed had turbid zones with clear centers (figure 2.2B). Since the plaques formed morphologically similar plaques, size was the only differentiating characteristic.

The plant environment was sampled weekly over a period of 16 weeks. DAL was done on every sample collected to determine the presence of bacteriophages in the sample. The samples that showed plaques on DAL plates, indicating the presence of phages, were stored at 4°C. A total of 70 samples, were analysed during this period, of which 45 exhibited the presence of bacteriophages and 25 had no bacteriophages. It was found that the sumps had the highest bacteriophage levels (table 2.2). This was as expected because the sumps were the storage site for waste from the plant and fermenters. There was not much that could be done with the sumps to prevent bacteriophage infestation except ensuring that the waste that was collected in them did not contain threonine

producing *E. coli* strain B. Although bacteria as well as phages are prevalent in the environment, the most suitable habitat for the phages is the host bacterium (Primrose, 1990). The odour scrubber and process water showed lower phage levels. These two areas were suspected to be responsible for the spreading of phages throughout the plant as well as introduction of phages into the fermenters. The odour scrubber could spread the phages because it blows out air continuously, and if the air contained bacteriophages, they could spread throughout the plant. Also, the odour scrubber is situated next to the fermenters. The process water could disperse phages throughout the plant through vapors that are produced from the cooling towers. Also, process water is used when preparing initial charge for the fermenters and can therefore introduce bacteriophages into the fermentation media. All samples were then grouped into five categories according to the site from which they originated.

Purification was carried out following the isolation of phages from the samples. DAL was done using the samples that have been categorised into 5 samples. The resulting plaques also showed three different sizes, and the prevalent plaque size from each plate was used in this purification step. Each plaque used, resulted in mixed plaque sizes therefore making it necessary to repeat purification steps three times. Medium sized plaques were chosen from the mixture as they were more prevalent than the other sizes. Purification was done to ensure that each bacteriophage sample had one type of phage in it. After

repeating the purification steps three times, similar sized plaques were seen in the DAL plates (figure 2.3). The effectiveness of the method was shown by the TEM micrographs (Appendix), which showed that the bacteriophage present in each samples were morphologically similar. Also, phage kinetics (figure 2.6) showed that there was a single type of phage in each purified sample as all the bacteriophage samples followed the same lysis trend and they were all lytic phages. This method was also simple and could be used with ease in any simply-equipped microbiology laboratory. It also allowed rapid preparation of purified stocks of bacteriophages.

Purified stocks of bacteriophages were then enriched. Enrichment was done in a petri dish by directly adding Tris.HCl into a DAL plate or into liquid medium by infecting exponentially growing *E. coli* and then harvesting bacteriophages. The liquid medium method proved to produce more bacteriophages than the petri dish method (figure 2.4). The petri dish method was performed for the preservation step. It was necessary to add Tris.HCl directly on a plate in order to avoid diluting the bacteriophages further, which would have been the case if the bacteriophages enriched by liquid medium were used. The petri dish method produced lower bacteriophage concentrations than the liquid method. The phage suspensions from this method were free of bacterial cellular debris. This method proved to be faster than the liquid method. Both methods proved to be efficient in enriching bacteriophages as both methods had over 50% increase in bacteriophage counts after enrichment.

Bacteriophage samples were preserved at 4°C and -20°C, in bacterial medium (with bacterial cells having been removed by centrifugation and filtration) and in Tris.HCl buffer. Storage of all samples at these two temperatures showed that bacteriophages were unstable. This was also observed by Rossi (1994). However, storing bacteriophages in Tris.HCl buffer at -20°C proved to be a better method than the other methods. This method was 16% more stable than Tris.HCl at 4°C (figure 2.5 B). The samples that were stored at -20°C were not severely affected by freezing as they had glycerol added as cryoprotective agent. Repeated freezing and thawing of samples can have a severe effect on bacteriophage viability (Prescott *et al*, 2007). This was prevented by dispensing samples into cryovials and then freezing them. A new vial was used each time a viability assay was performed.

Characterisation of bacteriophages was done by two methods i.e. infection kinetics and electron microscopy. The findings of this study were compared to the characterisation study done by Theron (2006). Bacterial lysis curves for phages P1-P6 were performed to determine the rate of infection of each sample in attempt to characterise the bacteriophages in the samples according to the rate they infect host bacteria. The lysis curve (figure 2.6) showed that all the samples followed the same trend. After six hours there was an exponential decrease in bacterial concentration. This occurred as bacteriophages were reproducing thus affecting more bacterial cells (Budzik, 2003). Also, it was evident that bacterial reproduction was slowed down dramatically as the phages infected bacterial

cells; this was evident in figure 2.6 as the infected bacteria trend had lower OD₆₀₀ than the control. This was also observed in a study by Primrose (1990). After 9 hours bacterial lysis was stable. This may be due to the fact that at this stage most; if not all bacterial cells have been lysed. It is also possible that the bacterial cells that were left have acquired resistance against bacteriophages as shown by VanDemark and Batzing (1987). Cells which have been infected by T-even phages but have not yet lysed are restrictive to secondarily adsorbing homologous phages (Abedon, 1990). Thus, lysis may have been decreased due to the exposure to progeny bacteriophages. Because of the similarity in infection kinetics exhibited by all six phage samples, there was a suspicion that there was a single type of very aggressive and prevalent virulent phage in all these samples.

The electron micrographs showed that all samples had morphologically-similar bacteriophages that had icosahedral heads and long noncontractile flexible tails (figure 2.7). It was not clear whether P3 had tail fibres because the base was not seen or it was just other tails from other virions that were attached to its tail. The other micrographs from P3 did not exhibit this base. Therefore, it was concluded that the base like characteristic might have been tails from other bacteriophages that were stuck on its tail. These micrographs showed that a single type of phage was prevalent and it morphologically resembled T-even phages in the samples. This was also evident in the study by Theron (2006).

A detailed study of phage characterisation was done by Theron (2006). The study included electron micrographs of bacteriophages sampled from the infected fermenters from SA Bioproducts. It was found that all samples had morphologically similar virions with long, flexible non-contractile tails and icosahedral heads. It was determined that the heads were 60 nm across and tails between 140-160 nm in length, with a diameter of approximately 14 nm. The study was able to determine that these virions were morphologically similar to T1-like genus of *Siphoviridae* family. It further characterised bacteriophages genetically using the method of Sambrook *et al.* (1989) for DNA extraction and digested the DNA with restriction enzymes. It was found that the genome size of the phages was 48.1 kb. The study also found that *EcoRI* cut the phage DNA at least twelve times. *BamHI* did not restrict the phage DNA, and *HindIII* cut only once. According to Roberts *et al.* (2004 –as cited by Theron, 2006), the T1 genome contained no *BamHI* sites, three *HindIII* sites, and two *EcoRI* sites, suggesting that the phage present in the samples was genetically distinct from phage T1. This difference in *EcoRI* sites could be explained by the mosaic nature of T1 genome documented by Roberts *et al.* (2004) to contain recombinant hotspots. Also, according to Regenmortel *et al.* (2000- as cited by Theron, 2006) there are more than 50 viruses that have been classified in the T1-like particle on a purely morphologically basis.

All of these characterisation methods revealed that there was one type of phage that was prevalent in this plant. It was determined that this type of phage was the

same phage that was infecting the threonine fermentations. It was further concluded that the phage belonged to the T1-like particle.

CHAPTER 3

DETECTION AND ENUMERATION OF BACTERIOPHAGES

3.1 Introduction

3.1.1 Detection and enumeration methods

The main problem when studying bacteriophages are their extremely small size. They generally range between tens and hundreds of nm. Therefore, an indirect technique involving a host bacterium has to be used for their enumeration (Bratbak, 1990). There are numerous methods used today for bacteriophage detection. They include plaque formation using host bacteria, electron microscopy, PCR, and immunology. Electron microscopy can be used to detect viral concentrations above 10^4 pfu/ml (Wommack *et al.*, 1992). The PCR method is based on selective amplification of genetic material. It is very sensitive for the detection of viruses. This method is very complex for analyses of samples containing different types of viruses and not cost effective (Atmar *et al.*, 1993).

Immunological detection of bacteriophages includes dilution plates and reaction with specific antibodies. This method is also very sensitive but Tartera and Jofre (1987 – as cited by Rossi, 1994) showed that this method is not as sensitive as the Adams DAL method. The disadvantages of this method are that the antibodies are very specific, and therefore cannot detect other bacteriophages in the environmental samples. It is also very expensive.

According to the literature, DAL is still the most commonly used method (Budzik, 2003; Lu *et al*, 2003; Coffey *et al.*, 2001). Efforts have been made to improve it by eliminating the bottom agar layer. Grabow and Coubrough (1986) treated hundred ml water samples mixed with soft agar and distributed this into petri dishes. This method was very tedious and was not better in reproductivity than the Adams DAL method. Kott (1966) developed an enumeration method in liquid cultures. The samples were serially diluted in liquid cultures. A culture that becomes clear after incubation signals that at least one bacteriophage is present at the beginning. This study used the most probable number to determine the initial concentration of phages. This method is not suitable for analysing numerous samples. Also, Cornax *et al.* (1990) showed that a DAL technique gives better results than serial dilutions in a tube.

Several authors have used filtration-elution methods to detect and count viral particles in large volumes of water (Logan *et al*, 1980; Seeley and Primrose, 1979). These procedures are very complex. They require that the filters be washed first, then adsorption and desorption of viruses, and sometimes concentration of them, and then finally counting on petri dishes. This method is tedious if there is a large number of samples to be analysed. Purdy *et al.* (1984) showed that these methods lose viral particles in each step. Some authors add another step (pre-filtration) to eliminate suspended solids and this also reduces viral counts (Rossi, 1994).

Another method of detection is placing the host bacteria directly in the water sample to be analysed. Bivalent ions (Ca^{2+} , Mg^{2+}) are added to increase efficiency of the method. After a period of latency, permitting adsorption of phages onto their host, samples are centrifuged. The cell pellet is then diluted and analysed by the DAL method. This method has advantages over the other methods because the adsorption of phages to the bacteria occurs in the liquid environment. The possibilities of contact are better than those existing in gelled media (Purdy *et al.*, 1984).

Although the Adams DAL method is widely used, it needed to be optimised and adapted for each bacteriophage–host bacteria (BHB) system. The DAL method can also be criticised because in this method, the phage and bacteria are placed in the molten agar at 45-50°C before pouring into the petri dish. This temperature might present some risks for the phages. Also, it is impossible to empty out all the contents of the test tube when pouring the thick mixture into the petri dish. This leaves some phages behind in the tube. Another disadvantage is that this method was not optimised for a specific BHB system (Rossi, 1994).

3.1.2 Detection of lysogenic bacteriophages

The lysogenic cycle is an alternative phage cycle whereby the phage DNA, following injection, integrates into the host bacterial chromosome in a site-specific manner. Homologous attachment sites on the bacterial and phage genomes mediate site-specific recombination with the aid of phage-encoded integrase

activity (Prescott *et al.*, 2007; Budzik, 2003). Stresses like UV light, Mitomycin C, and heat can evoke excision of the phage DNA from the bacterial chromosomes, resulting in phage replication and host cell lysis. As a consequence of this life cycle switch, lysogenic strains have been suspected to be the source of new lytic cycles (Coffey *et al.*, 2001).

Bacterial cells infected with unstable lysogenic viruses (pseudolysogens) can be distinguished from stable lysogens using indicator plates (green plates). These green plates contain aniline blue and alizarin yellow dyes. On these plates, the pseudolysogens appear dark green whereas non-lysogens are yellow. This method can be used also to “cure” the pseudolysogens by streaking a dark green colony on green plates and choosing a yellow segregant (Berget and Chidambaram, 1989; Bochner, 1984). Green plates are poorly buffered and rich plates. Being rich media, auxotrophic strains grow, but the dyes can interfere with strains mutant replication and recombinant functions. The excess glucose causes the strains to make more acid than usual when strains lyse. The acid released causes the plates to turn dark green. Non-lysogenic colonies will turn the plate light green/yellow after sufficiently long periods (more than 24 hours). Thus, green plates have to be examined promptly. Single-phage infected strains lyse and make small dark green colonies. Non-lysogenic cells will make larger, light green/yellow colonies. Phage-free bacterial strains may be inoculated across over phage sample on green plates. The portion of the streak before the phage will be healthy and light green/yellow. After the phage, non-lysogenic phage will

lyse, so the portion of the streak after the phage will be spotty and dark green (Jeff, 2005).

Another method for detecting lysogenic phages is by using the mutagen, Mitomycin C. This method can be used to screen large number of bacterial strains for the presence of lysogenic phages within a minimum of expended time and effort. This method involves replicating young colonies (7-8 hours) to a master plate containing nutrient agar and also a second plate of medium supplemented with Mitomycin C. After overnight incubation of the plates, the lysogenic cells produce partially lysed or mottled colonies on Mitomycin C plates. Alternatively, phage induction can be carried out by incubating early exponentially growing cultures in the presence of Mitomycin C followed by incubation. After centrifugation, the supernatant is collected and filter sterilised. This can be tested for phage presence by the DAL method (Tenreiro *et al.*, 1993; Siddiqui *et al.*, 1974).

The objective of this chapter was to optimise and adapt the method for detection and enumeration of bacteriophages for this bacteriophage-host bacteria system (BHB). It was also suspected that there might be lysogenic bacteriophages in our sample because of resistance that was observed in the *E. coli* strain B after exposure to bacteriophage samples. Therefore, a method for detection of lysogenic bacteriophages had to be developed and optimised.

3.2 Materials and Methods

3.2.1 Optimal bacterial volume

Bacterial cell concentration was optimised to determine the amount of bacterial cells required to obtain an optimal number of bacteriophages. A fixed volume of bacterial cells (50, 100, 200, and 400 μ l) was mixed with 100 μ l of phage samples. Diluted phage samples of P1, P2, and P3 (See section 2.2.1) were randomly selected for this study. DAL was done using the Adams (1959) method (section 2.2.3). Plates were incubated overnight and pfu counts were determined for each plate. A control for each volume was included, and was pipetted into hard agar plate. Soft agar was poured onto the plates and swirled to mix agar with bacterial cells. The results reported were an average of a test done in triplicate, as pfu/ml.

3.2.2 Optimal incubation time

Incubation time was used to determine the most appropriate time to read plates after incubation in order to obtain highest number of plaques. Phage samples P1, P2, and P3 were randomly selected and diluted. 100 μ l of bacterial cells was added into a test tube containing 100 μ l of bacteriophage samples. DAL was done using the Adams (1959) method (section 2.2.3). Plates were incubated at 37°C and were taken out of the incubator at different intervals, i.e., 16, 20, and 24 hours and pfu counts were determined for each plate. The results reported were an average of a test done in triplicate, as pfu/ml.

3.2.3 Optimal incubation temperature

This experiment was done to determine the influence of incubation temperature on the number of plaques formed. Phages samples P1, P2, and P3 were randomly selected and diluted. DAL was done using the Adams (1959) method. Plates were incubated for 20 hours at different temperatures, i.e., 34, 37, and 39°C and pfu counts were determined for each plate. The results reported were an average of a test done in triplicate, as pfu/ml.

3.2.4 Effect of bivalent ions

This experiment was done to determine the effect of adding the bivalent ions Mg^{2+} and Ca^{2+} on the number of plaques formed. The salts were added in an attempt to increase detection efficiency of DAL. 0.5 mM of $CaCl_2$ and 0.5 mM $MgCl_2$ were added into the host-phage mixture. DAL was done using the Adams (1959) method. Plates were incubated for 20 hours at 37°C and pfu counts were determined for each plate. Diluted phage samples P1, P2, and P3 samples were used for this experiment. A control, comprising of phage-host mixture without bivalent ions, was included. The results reported were an average of a test done in triplicate, as pfu/ml.

3.2.5 Comparison of plating methods

Phage samples P1, P2, and P3 were randomly selected and used in this experiment. This study compared the classic Adams (1959) DAL (section 2.2.3) and the proposed improvements that were done to optimise it (figure 3.1). In the optimised method, 100 µl of bacteriophage and 100 µl of host bacteria were mixed together with 50 µl of 5 mM Ca-Mg solution. This mixture was mixed with a vortex mixer and pipetted onto LB agar plates. Three ml of soft molten agar (40°C) was added to the plates and swirled to mix with the phage-host mixture. Plates were left at room temperature long enough for the soft agar to solidify. Once solidified, the plates were incubated at 37°C for 20 hours. The results reported were an average of a test done in triplicate, as pfu/ml.

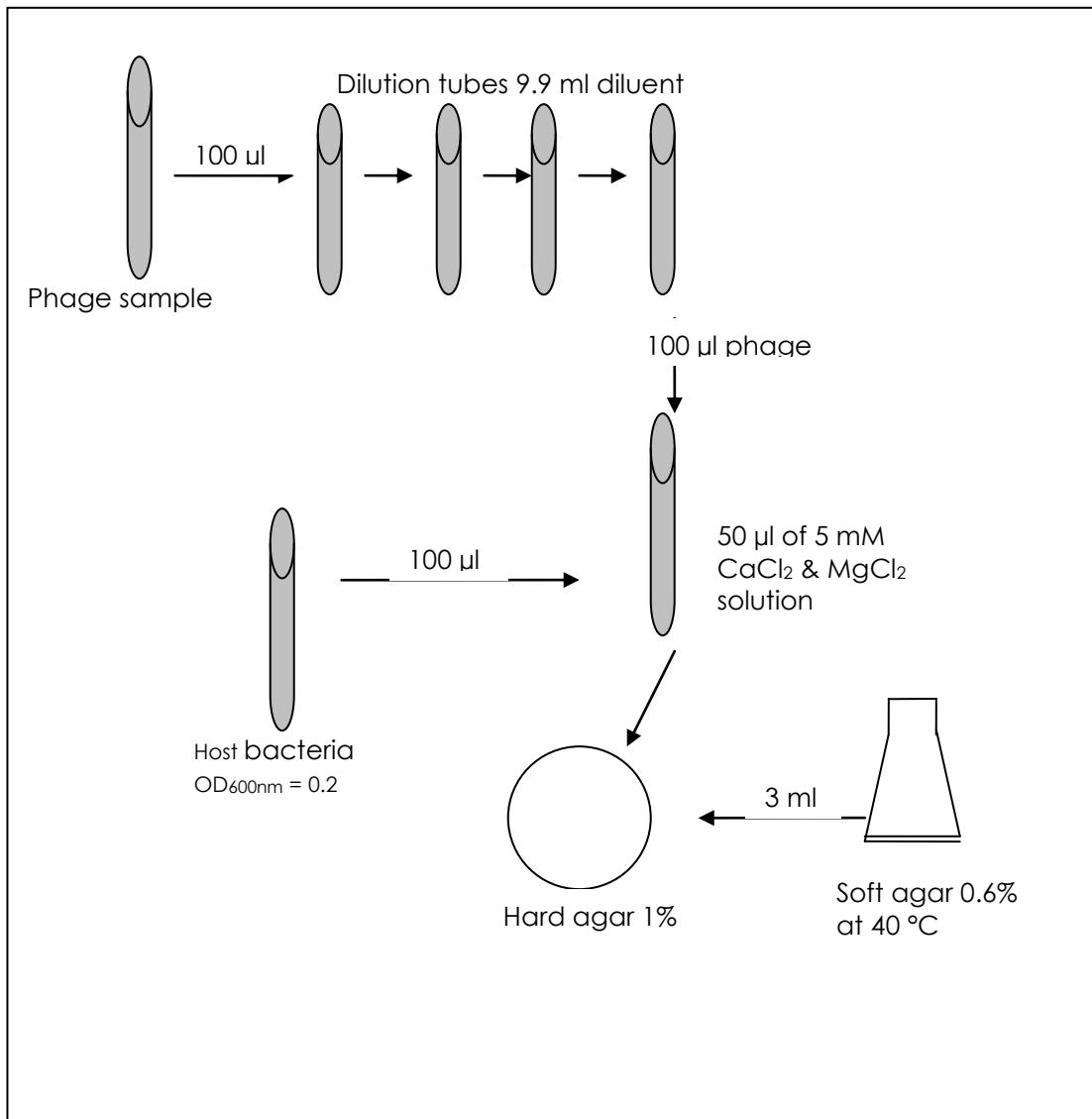


Figure 3.1 Diagram outlining the optimised DAL method. Hundred µl of bacteriophage and bacteria were added into a test tube with Ca-Mg solution. This mixture was pipetted onto an agar plate, soft agar was added, and the plates were incubated at 37°C for 20 hours.

3.2.6 Detection of lysogenic bacteriophages

E. coli strain B was exposed to a mixture of phages P1-P6. Ten colonies that grew on the DAL plates were potentially phage resistant and were individually grown by streaking them on ten LB plates. Two colonies were picked from each

plate, labeled and the suspended in 100 µl of diluent. These colonies were then cross-streaked against individual bacteriophage (P1-P6) samples to verify their resistance. The colonies that showed resistance to all individual bacteriophage samples were then used in the development of a method for detecting lysogenic bacteriophages. Two isolates (labeled C5A and C5B) were found to be resistant to all bacteriophage samples.

3.2.6.1 ***Detection of lysogenic phages using UV light***

Exponentially-growing isolates C5A and C5B were exposed to UV light at 220 nm (Thermorex UV steriliser cabinet) overnight. 200 µl of bacterial cells were pipetted onto agar plates, and 3 ml of soft agar was added. The plates were gently swirled to mix bacterial cells and soft agar, in order to ensure even distribution of cells through the plate. The plates were incubated at 37°C for 16 hours. After 16 hours, plates were removed from the incubator and placed in the UV steriliser cabinet and checked after 20, 24, and 48 hours. A positive control (LB plate with phage-susceptible *E.coli* strain B) was included. The appearance of plaques in the potentially phage-resistant colonies and the absence of plaques in the control was evidence that there were lysogenic phages present in the bacterial cells.

3.2.6.2 *Detection of lysogenic phages using green indicator plates*

Green plates were prepared by adding and mixing two dyes (1:1) i.e., aniline blue 1.3 g/l and alizarin yellow 62 g/l. LB agar was prepared as per manufacturer's instruction (Biolab) and then cooled. One ml/l of each dye mixture was added into LB agar and poured into petri dishes. Presumptive phage-resistant bacterial cells (C5A and C5B) were streaked on these plates. *E. coli* strain B that was susceptible to bacteriophages was used as a control. If the bacterial cells were non-lysogenic, it was expected that light green or yellow colonies would be produced. If the cells had unstable lysogens, small dark green colonies were to be produced (Hughes and Maloy, 2007; Berget and Chidambaram, 1989; Bochner, 1984).

3.2.6.3 *Detection of lysogenic phages using a mutagen*

The mutagen Mitomycin C was used in this study. Presumptive phage-resistant bacterial cells (C5A and C5B) were grown to early exponential phase in LB broth in an Erlenmeyer flask. Different concentrations of Mitomycin C (Sigma Aldrich) (0.2, 0.5, 1, 2 µg/ml) were added to the culture. This mixture was then incubated at 37°C, 150 rpm for 12 hours. A control was included which comprised of a flask with bacterial cells only (without Mitomycin C). 200 µl of bacterial cells from each flask was pipetted onto three agar plates, and 3 ml of soft agar was added in each plate. The plates were gently swirled to mix bacterial cells and soft agar, in order to ensure even distribution of cells throughout the plate. Also, the

remainder of the mixture was centrifuged and the supernatant was filter sterilised. This was done in order to isolate any bacteriophages present in the mixture. The supernatant was used to perform DAL. This was done to confirm the result of the previous test (Tenreiro *et al.*, 1993; Siddiqui *et al.*, 1974).

3.3 Results

3.3.1 Optimal bacterial volume

Fixed volumes of bacterial cells (50, 100, 200, 300, and 400 μl) from an exponentially growing culture (5.6×10^7 cfu/ml) were added into the test tube containing 100 μl of diluted bacteriophage sample. This mixture was used to perform the DAL. A control with only bacterial cells for each fixed volume was performed.

There were no plaques visible on all control plates. The control plate with 50 μl of bacterial cells did not form a uniform carpet of growth covering the plate. Control plates with 100 and 200 μl of bacterial cells formed a uniform carpet of bacterial growth on the plate. Control plates with 300 and 400 μl of bacterial cells formed a very thick layer of growth on the plates.

It was shown that pfu counts varied with the variation in volume of host cells. There was a noticeable decrease in the pfu counts when the cellular volume was above 100 μl . When cell volume increased from 50 μl to 100 μl , pfu counts increased from an average of 3×10^5 to 6.13×10^9 , respectively. Bacterial cell volume greater than 100 μl produced lower pfu counts than at 100 μl . They were on average 1.63×10^8 ($\pm 3.06 \times 10^7$), 1.7×10^8 ($\pm 3.00 \times 10^7$) and 5.07×10^7 ($\pm 2.08 \times 10^6$) at 200, 300, and 400 μl respectively. The plaques that were visible on plates with the higher cell volume (300 and 400 μl) too small in diameter and

were very difficult to count, even though the bacteriophage samples were diluted, therefore they were not counted (figure 3.2).

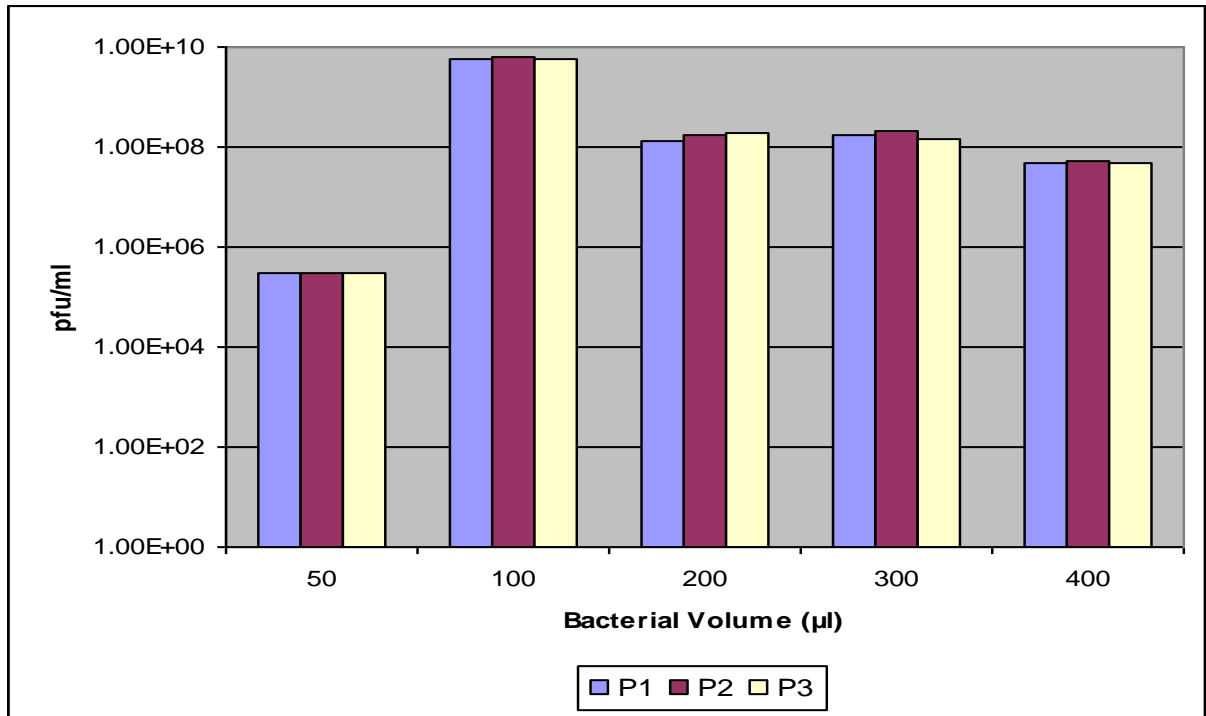


Figure 3.2 Effect of bacterial cell concentration on pfu numbers with bacteriophage samples P1, P2 and P3. Various amounts of exponentially growing bacterial cells were added with 0.1 ml bacteriophage sample and DAL was done. Data represents averages of tests done in triplicate.

3.3.2 Optimal incubation time

This was done to determine whether incubation time had any effect on the plaques formed on DAL plates. DAL plates were incubated at 37°C and were taken out to determine pfu counts after 16, 20, and 24 hours. The pfu counts increased after 16 hours (average 3.52×10^8 pfu/ml $\pm 1.58 \times 10^8$) of incubation. The difference between pfu counts counted after 20 and 24 hours was insignificant (average $3.34 \times 10^9 \pm 6.5 \times 10^8$ and 3.1×10^9 pfu/ml $\pm 6.56 \times 10^8$, respectively). The only difference noted with an increased incubation

temperature, was on the size of the plaques that were bigger after 24 hours of incubation (figure 3.3).

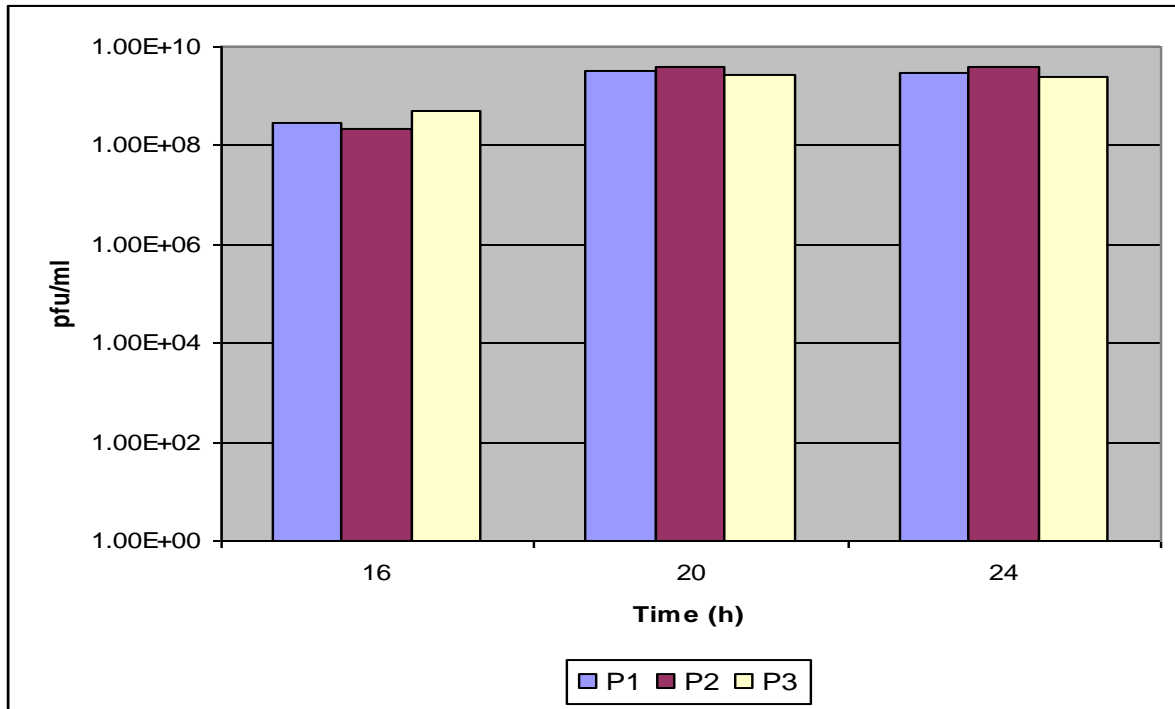


Figure 3.3 Effect of incubation time on pfu counts with bacteriophage samples P1, P2, and P3. 100 μ l of phages and 100 μ l of bacterial cells were used and incubated at 37°C. Plates were read after 16, 20 and 24 hours.

3.3.3 Optimal incubation temperature

This experiment was done to determine the reaction of our BHB system to the changes in the incubation temperature. The DAL plates were incubated at 34, 37, and 39°C for 20 hours (section 3.3.2). The highest pfu counts with all bacteriophage samples were obtained at 37°C (average 5.47×10^9 pfu/ml $\pm 7.63 \times 10^8$). The plaques that were seen on these plates were well defined and clearer to count. There were less pfu counted at 34 (average 7.6×10^8 pfu/ml $\pm 7.9 \times$

10^7) and 39°C (average 3.37×10^8 pfu/ml $\pm 1.03 \times 10^8$). It was noted, also, that growth of the host bacterium was slower at 39°C (figure 3.4).

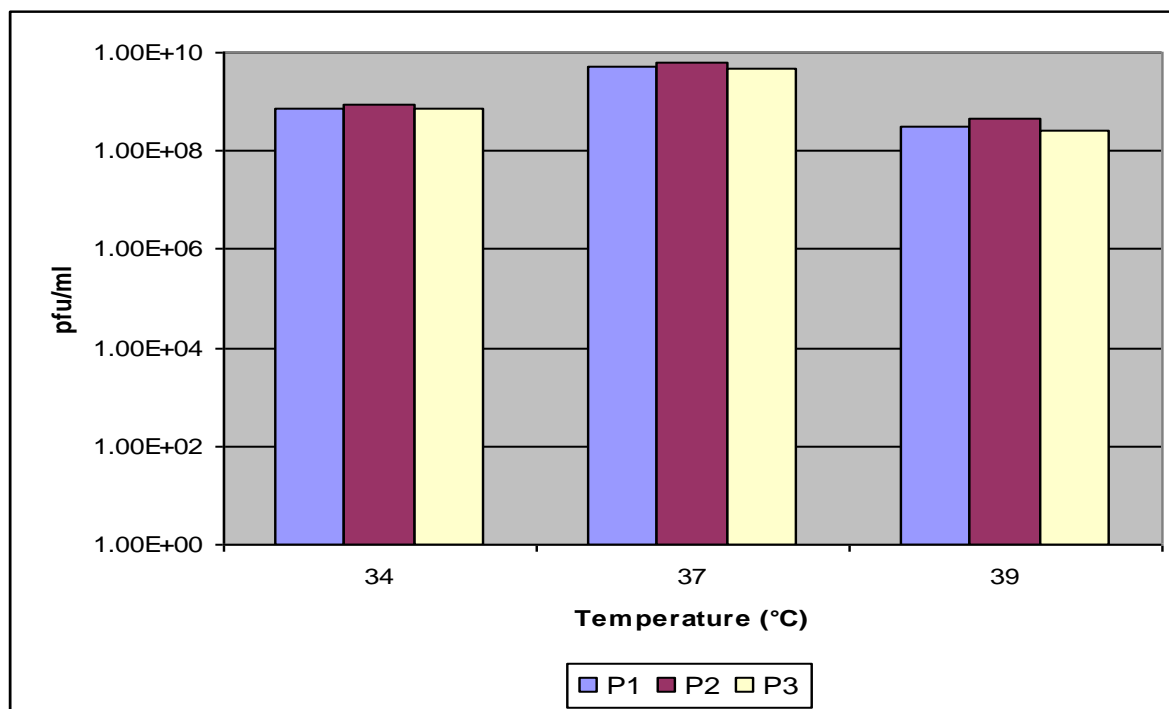


Figure 3.4 Effect of incubation temperature on pfu counts determined using bacteriophage sample P1, P2, and P3. DAL plates were incubated at 34, 37, and 39°C. Results shown were averages of tests done in triplicate after 20 hours of incubation.

3.3.4 Effect of bivalent ions on bacteriophages

Influence of Ca^{2+} and Mg^{2+} ions was determined on this study using the method of Adams (1959). Plates were incubated at 37°C (section 3.3.3) overnight and pfu counts were determined for each plate. There was a slight increase in the pfu counts when Mg^{2+} ions (average 4.77×10^8 pfu/ml $\pm 9.29 \times 10^7$) were used. Ca^{2+} ion addition increased pfu counts almost two fold (average 8.7×10^8 pfu/ml \pm

1.01×10^8). However, Ca-Mg solution showed a significant increase in pfu counts (average 8.33×10^9 pfu/ml $\pm 1.33 \times 10^9$) (figure 3.5).

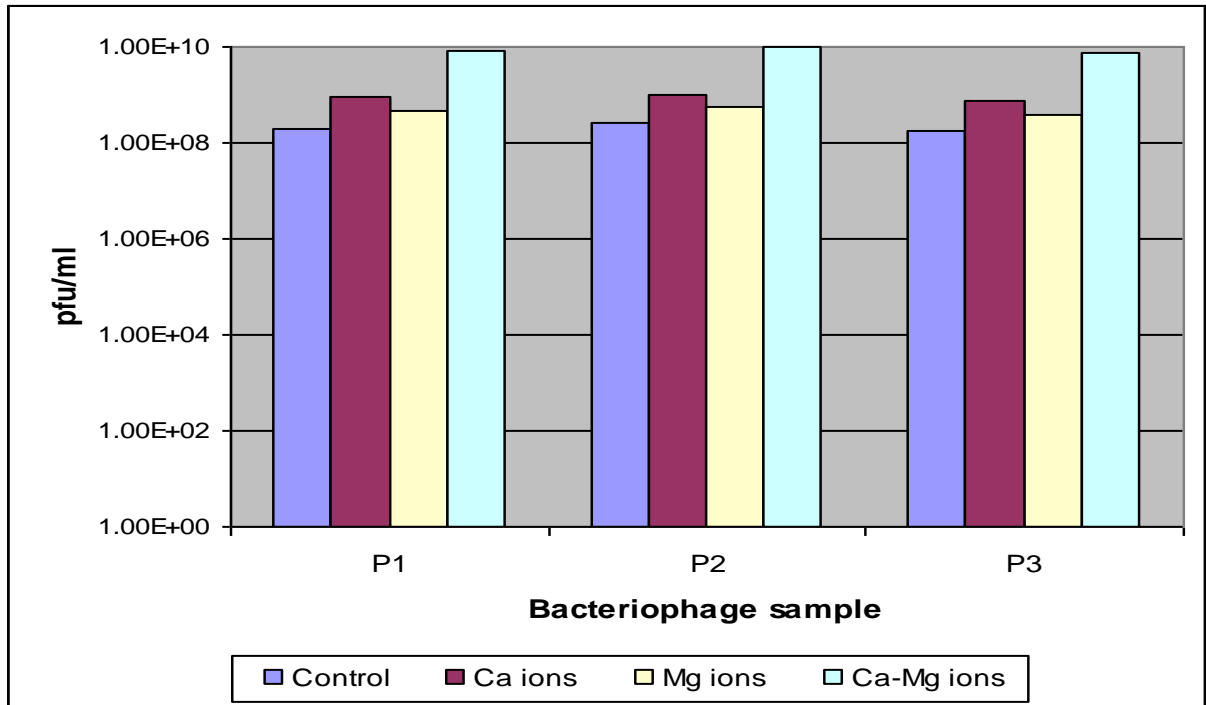


Figure 3.5 Influence of addition of bivalent ions on pfu counts. Bacteriophage samples P1, P2, and P3 were used for this experiment. Individually prepared Ca and Mg ions were mixed and added to a phage-host DAL. A control (phage-host DAL plate) for each phage sample was performed as well.

3.3.5 Comparison of plating techniques

This study was done to compare Adams (1959) DAL method and the DAL method that was optimised for our BHB system (section 3.2.5). Sample P1, P2, and P3 were used. The optimised DAL method had significantly higher pfu counts (average 3.40×10^9 pfu/ml $\pm 7.21 \times 10^8$) than Adams classic DAL method (average 2.97×10^8 pfu/ml $\pm 8.33 \times 10^7$) (figure 3.6).

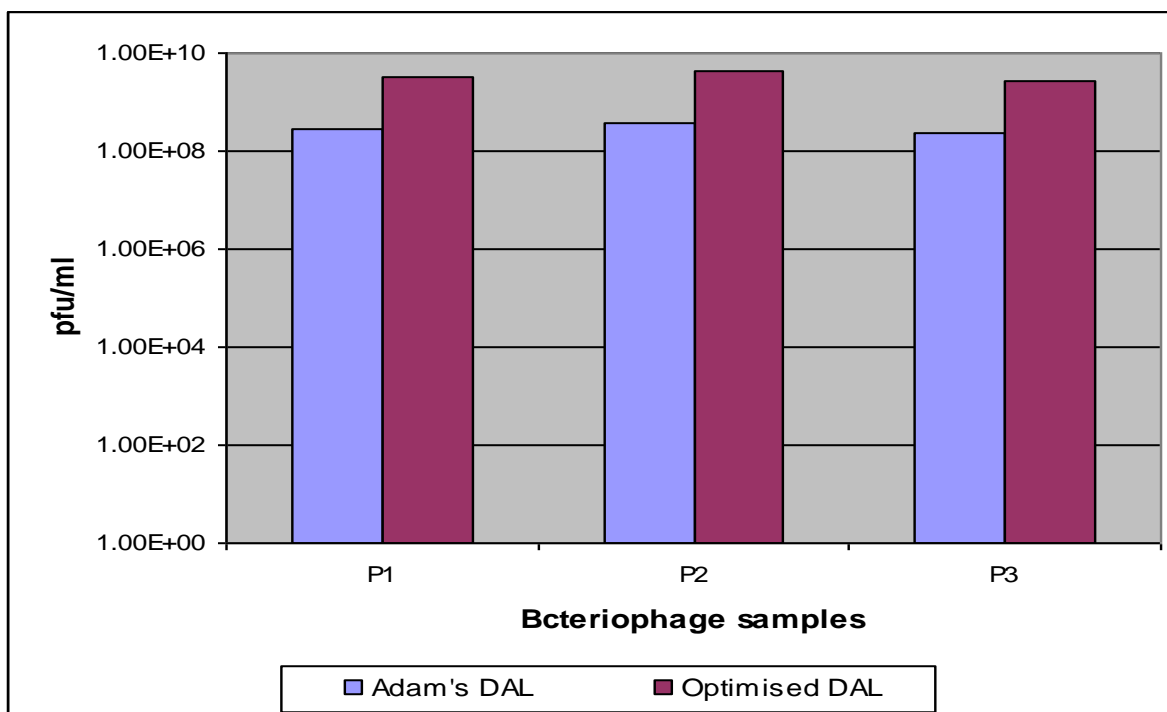


Figure 3.6 Comparison of plating techniques. Pfu counts determined by Adams DAL method was compared to pfu counts after improvements in the plating technique. Phages P1, P2, and P3 were used for this experiment. Results showed were averages of tests done in triplicate.

3.3.6 Detection of lysogenic bacteriophages

Presumptive phage-resistant colonies were isolated by picking colonies that grew on the DAL plates in the presence of mixed bacteriophages and individually streaking them on five LB plates. Two colonies were picked from each LB plate and cross-streaked against individual phage samples to determine their resistance.

Only isolates C5A and C5B had total resistance against all the bacteriophage samples. Seven isolates were resistant to 50% of the bacteriophage samples (C1A, C2A, C3B, C4B, C6A, C9B, and C10A). Isolates C9A and C10A were

susceptible to all the bacteriophage samples. Isolates C3A and C7A were resistant to 33% of bacteriophage samples. Isolate C4A confirmed resistance to most of the phage samples (83%). Isolates C8A, C8B, C7B, and C1B were susceptible to most of the phage samples and only resistant to 17% of the phage samples (table 3.1).

Table 3.1 Isolation of potentially phage-resistant bacterial cells

Potential Phage-Resistant Colonies	Bacteriophage Samples					
	P1	P2	P3	P4	P5	P6
C1 A	+	-	+	-	+	-
C1B	+	-	-	-	-	-
C2 A	+	+	+	-	-	-
C2B	+	+	-	-	+	+
C3 A	-	+	-	-	-	+
C3B	-	-	-	+	+	+
C4A	+	-	+	+	+	+
C4B	-	-	+	-	+	+
C5A	+	+	+	+	+	+
C5B	+	+	+	+	+	+
C6A	+	-	+	-	+	-
C6B	+	-	-	-	-	-
C7A	-	-	-	+	-	+
C7B	+	+	-	-	+	+
C8A	+	+	+	-	+	-
C8B	+	+	+	+	-	-
C9A	-	-	-	-	-	-
C9B	+	+	-	+	-	-
C10A	+	-	+	-	+	-
C10B	+	-	-	-	-	-

+ = Resistant, no plaques

- = Susceptible, plaques present

3.3.6.1 *Detection of lysogenic phages using UV light*

The plates were exposed to UV light at room temperature for 16, 24, and 48 hours. There were no plaques visible on the plates after 16 hours. After 24 hours of UV exposure, there were 20 pfu/ml visible and 50 pfu/ml after 48 hours of exposure for sample C5A. Sample C5B and the controls did not show any plaques on the plates (table 3.2).

Table 3.2 Detection of lysogenic phages using UV light.

	Pfu/ml 16 h	Pfu/ml 24 h	Pfu/ml 48 h	Description
C5A	0	20	50	Small and medium plaques
C5B	0	0	0	Lawn of bacterial growth, no plaques
Control	0	0	0	Bacterial growth, no plaques

3.3.6.2 *Detection of lysogenic phages using a green indicator plate*

Potential phage-resistant bacteria C5A and C5B were used for this experiment. Bacterial cells were streaked on the green agar plate. Each strain was streaked on 5 green plates. Plates streaked with isolate C5A showed a mixture of light green and dark green colonies. Most of the colonies on these plates were light

green, very few dark green colonies were observed. Isolate C5B plates only showed yellow colonies, similar to those shown by control plates (table 3.3).

Table 3.3 Detection of lysogenic phages using the green plate method.

<i>E. coli</i> B		Observation*
C5A	Control	Yellowish colonies
	Test	Mixture of light and dark green colonies but mostly light green.
C5B	Control	Yellowish colonies
	Test	Light yellow colonies

* Light green/yellow colonies: contain stable or no lysogens
 Dark Green colonies: contain stable lysogens

3.3.6.3 ***Detection of lysogenic phages using a mutagen***

Isolates of *E. coli* strain B that were resistant to all six phages and labeled C5A and C5B were used in this experiment. Different concentrations of Mitomycin C were added into the flasks that had growing isolates. Both isolates showed the presence of lysogenic bacteriophages. There were no plaques visible on the control plates. At a lower concentration (0.2 µg/ml), pfu counts were the lowest (average 4.75×10^5 pfu/ml $\pm 6.01 \times 10^5$). There was an exponential increase in pfu counts with an increase in Mitomycin C concentration. At 0.2 µg/ml an average of 4.75×10^5 pfu/ml ($\pm 6.01 \times 10^5$), 0.5 µg/ml had an average of 1.1×10^6 pfu/ml ($\pm 1.41 \times 10^6$, and at 1 µg/ml there an average of 1.65×10^7 pfu/ml ($\pm 2.05 \times 10^7$) was achieved. However, at higher concentrations (2 µg/ml) pfu counts decreased exponentially to an average of 5.75×10^6 pfu/ml ($\pm 6.01 \times$

10⁶). The highest counts for both C5A and C5B were at 1 µg/ml with 3.1 x 10⁷ and 2 x 10⁶ pfu/ml, respectively (figure 3.7).

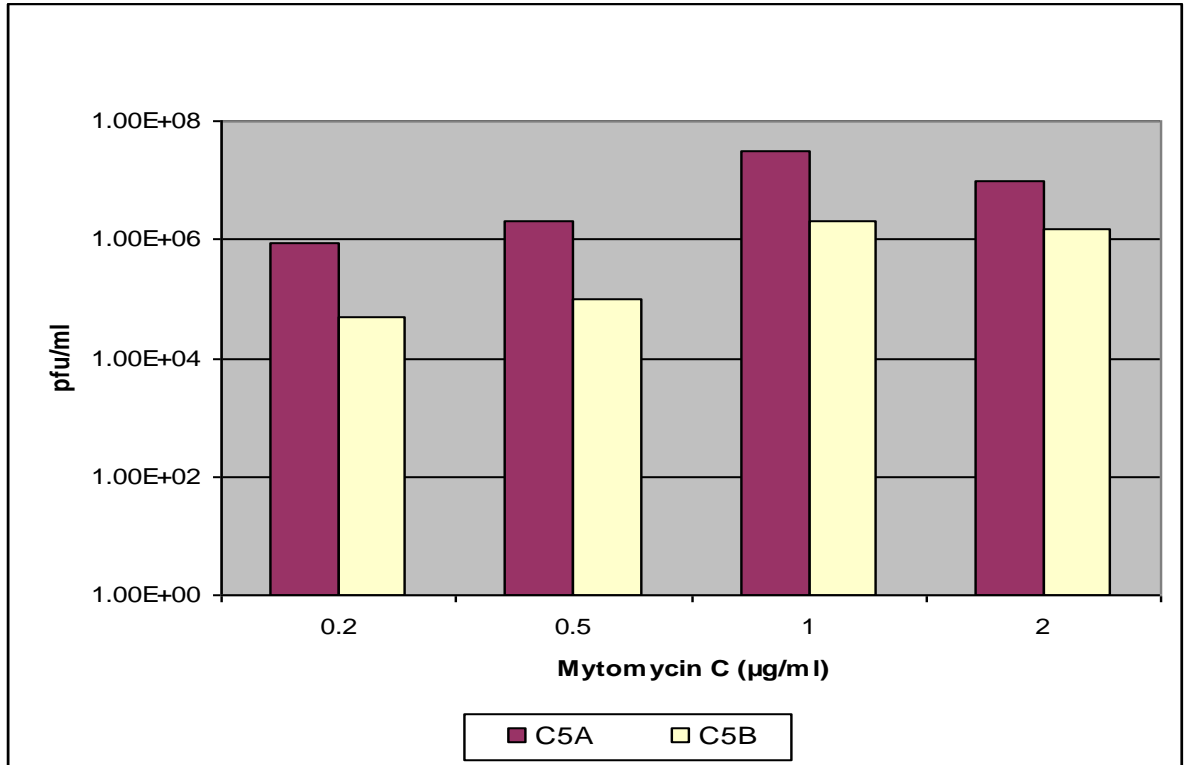


Figure 3.7 Detection of lysogens with Mitomycin C. Concentrations of Mitomycin C used were 0.2, 0.5, 1.0, and 2.0 µg/ml. Presumptive resistant *E. coli* strain B isolates C5A and C5B were used.

3.4 Discussion

Optimisation was done in order to adapt Adams DAL method to this BHB system. Rossi (1994) showed that it is impossible to define the physico-chemical conditions that would be optimal for the enumeration of all bacteriophages. Each BHB system has its own peculiarities. Enumeration must, therefore, be preceded by an optimisation stage. The aim of optimisation was to make it possible to count the maximum number of phages, regularly and reproducibly.

Optimal bacterial concentration was determined by performing DAL using different quantities of host bacteria. It was essential to determine an appropriate bacterial cell volume because if the amount of cells added is low, the pfu counts would be low as well. When a higher volume of cells is used, the amount of pfu's counted would be low because the bacterial growth would out-grow and cover any plaques that were formed (Cornax *et al.*, 1990). Fixed volumes (50, 100, 200, 300, and 400 µl) of an exponentially growing *E. coli* strain B was used with OD₆₀₀ of 0.2 (5.6×10^7 cfu/ml), was added to 100 µl of diluted phage samples (P1, P2, and P3). When 50 µl of bacterial cells were used, uniform growth of cells over the DAL plate was not formed. This proved that 50 µl of 5.6×10^7 cfu/ml was too low to use for DAL. This may be due to the fact that with Adams DAL method bacterial cells and bacteriophage had to be added in the soft molten agar in a test tube. Thus, it was impossible to empty the entire contents of the test tubes. Also, the molten agar temperature was at 45°C, which was capable of denaturing bacterial cells. Hundred µl of cells was used and a uniform layer of growth was

formed in soft agar and it covered the whole plate. Also, at this volume maximum pfu counts of 6.13×10^9 pfu/ml were obtained. When higher volumes ($>100 \mu\text{l}$) of bacterial cells were used, the pfu counts decreased (figure 3.2). This may be due to the bacterial growth being so high that it was masking the lysis plaques, i.e., phage multiplication was drowned by the proliferation of bacteria (Cornax *et al.*, 1990). It was also observed that usage of large volumes of bacteria was difficult to work with as the soft agar started to solidify before it was emptied into the LB agar plates.

Optimal incubation time was determined by incubating DAL plates and counting plaques after 16, 20, 24, and 48 hours of incubation. It was shown that there were plaques formed after 16 hours (3.52×10^8 pfu/ml). But these plaques were small and not clear. After 20 hours of incubation, the plaques were clearer and bigger, which made it easier to count. There were new plaques that were visualised after 20 hours, and this increased pfu counts (average 3.1×10^9 pfu/ml). There was little difference in the plaques counted after 20 and 24 hours, 3.34×10^9 and 3.1×10^9 pfu/ml, respectively (figure 3.3). The only difference was the increased plaque size of some of the plaques after 24 hours.

Influence of incubation temperature was determined by incubating DAL plates at different temperatures (34, 37, and 39°C). This temperature range was chosen since the optimum growth temperature for this *E. coli* strain B is 37°C . At this temperature range, the pfu counts increased drastically between 34 (7.6×10^8

pfu/ml) and 37°C (5.47×10^9 pfu/ml - figure 3.4). At 39 °C, the pfu counts were also lower ($3.3.7 \times 10^8$ pfu/ml). This may be due to the host bacterial growth being slower at these temperatures. Lu *et al.* (2003) showed that in some BHB systems the optimal temperature for host bacterial growth was different from that of bacteriophage production. This was not the case in this BHB system because the highest pfu counts were at 37 °C, which was the optimal bacterial growth temperature. Although, the pfu counts were lower at 39°C than at 37°C, the plaques were clearer and well defined. Rossi (1994) showed that in some BHB systems, a temperature above optimum bacterial growth temperature slowed down the bacterial development allowing lysis plaques to appear more easily. The optimal temperature (37°C) was determined graphically by the highest pfu counts obtained. Also, it was observed that after 20 hours at 39°C, some of the agar plates dried out.

Influence of addition of bivalent ions was done by adding 5 mM of Ca^{2+} and Mg^{2+} ions into the phage-bacteria mixture (Cornax *et al.*, 1990). The use of a similar concentration was suggested by Tartera and Jofre (1987 – as cited by Cornax *et al.*, 1990). There was a small difference in pfu counts when only Mg^{2+} ions were added. There was 2.7×10^8 pfu/ml increase in pfu counts. When Ca^{2+} ions were added, there was an increase in pfu counts of 6×10^8 pfu/ml. Pfu counts increased even further when Ca-Mg ions were added. Plaques counted increased from 2.07×10^8 to 8.33×10^9 pfu/ml (figure 3.5). There was an appreciable increase in pfu counts when only calcium ions were added. The pfu

counts increased even further when Ca-Mg ions were added (figure 3.5). This was because the bivalent ions increase the efficiency of adsorption of phage onto the host bacteria by changing the electrical charge of the cell wall making it more favorable for bacteriophages receptors (Purdy *et al.*, 1984).

Although Adams DAL method is widely used (Coffey *et al*, 2001), it needed to be optimised and adapted for BHB used in this study. A disadvantage of this DAL method is that the phage and bacteria are placed in the molten agar at 45-50°C before pouring into the petri dish. This temperature might present a risk for the phages and bacterial cells. Also, it is impossible to empty out all the contents of the test tube when pouring the thick mixture into the petri dish. Therefore, some phages and bacterial cells were left in the tube. Another disadvantage is that this method was not optimised for a specific BHB system (Rossi, 1994). An attempt made to improve the plating of the DAL method. The changes introduced to the method were that the host bacteria with the phage, were mixed first, and then were poured onto the agar plate and then the soft agar was poured on the mixture. Also, this allowed for the temperature of the molten agar to be reduced from 45 to 40°C. There was a significant increase in pfu counts when optimised method (3.4×10^9 pfu/ml) was compared to the classic DAL method (2.97×10^8 pfu/ml) (figure 3.6). This was because with optimised method there were fewer bacteriophages and bacterial cells lost because the test tube could be emptied. Also, in the optimised method, the phage and bacteria are mixed together in

liquid medium that contained bivalent ions. This increased adsorption efficiency of the bacteriophages.

It was of great importance for this study that a method for detection of lysogens be developed because bacterial cells containing lysogenic phages (lysogens) are immune to infection by other bacteriophages (Jeff, 2005). Therefore, the bacterial strain could be mistaken as being phage-resistant. Also, lysogens have been suspected to be responsible for introducing new lytic cycles when used in the production process (Coffey *et al.*, 2001). The resistant strains that were isolated would have to be tested so as to determine whether they were truly resistant or they were resistant due to the lysogenic phage incorporated in their DNA. Three methods for detection of lysogenic phages (UV light, green plates, and Mitomycin C) were tested in this study.

E. coli strain B was exposed to the mixture of phage samples P1-P6 and the colonies that appeared to possess resistance to this phage mixture were selected. Their resistance was re-tested using individual phage samples and only isolates that were resistant against all phage samples were selected. These isolates were labelled C5A and C5B (table 3.1).

Exposure of these strains to UV light was able to induce plaques on isolate C5A but not on isolate C5B and control (table 3.2). The presence of plaques indicated that UV light exerted stress which evoked excision of the phage DNA from the

bacterial chromosomes resulting in phage replication and host lysis. This is a well-known phenomenon called induction (Coffey *et al.*, 2001). These results corresponded with the results from the green plate method (table 3.3). The green plate method is used to detect unstable lysogenic virus from stable or non-lysogenic strains. Dark green, small colonies after 24 hours represent the presence of unstable lysogenic bacterial cells. Yellow or light green colonies are evidence of the presence of stable or non-lysogenic bacterial cells (Bochner, 1984; Berget and Chidambaram, 1988). Isolate C5A contained a mixture of both light and dark green colonies. However, the amount of light green colonies was higher than dark colonies. This was an indication that isolate C5A contained unstable lysogenic phages. Isolate C5B did not show any evidence of unstable lysogenic phages, as it produced light yellow colonies. This method was not suitable for this study as it could not clearly show that isolate C5B was free of lysogenic phages. Therefore, if used, it would require an additional step for confirming that the isolate was not a lysogen. After using UV and green plates, C5B was not confirmed as a lysogen. It could still be resistant to bacteriophages via other mechanisms like mutation, thereby preventing entry of bacteriophages. A disadvantage of the green plate method for this study was that it detected unstable lysogenic phage presence. It did not distinguish between stable lysogens and the phage free isolates. The UV method was very time consuming and tedious. The green plate method is therefore suitable for screening numerous strains.

The Mitomycin C method was efficient in detecting lysogens (figure 3.7). After exposure to different Mitomycin C concentrations, phages were detected on both phage-resistant isolates (C5A and C5B) even at very low concentrations. Mitomycin C as a mutagen induced lytic cycle of lysogenic bacteriophage and they in turn lysed bacterial cells (Tenreiro *et al.*, 1993). The number of bacteriophages detected increased with the increase in Mitomycin C concentration. At higher concentrations (2 µg/ml), pfu counts decreased to 1×10^7 pfu/ml for C5A and 1.5×10^6 pfu/ml for C5B. This may be due to the fact that Mitomycin C is toxic to bacterial cells at high concentrations. The optimal concentration proved to be 1 µg/ml, as it gave the highest pfu counts of 3.1×10^7 and 2×10^6 pfu/ml for C5A and C5B, respectively. The controls that were included did not show any evidence of the presence of bacteriophage. However, at 2 µg/ml *E. coli* strain B growth was less than at other concentration. The Mitomycin C method confirmed that isolate C5B was also lysogenic. This isolate was confirmed by UV and green plate method to be a possible phage-resistant strain, without a lysogenic phage. The Mitomycin C method proved to be reliable and more sensitive when compared to the other two methods (UV light and green plates) which did not show any sign of phage infection. This method proved to be reliable and reproducible compared to the other two methods.

CHAPTER 4

PREVENTION AND TREATMENT OF BACTERIOPHAGE INFECTION

4.1 Introduction

Production of many biotechnologically-important substances is based on cultivation of bacteria. Infection of bacterial cultures by phages is a serious problem in biotechnology laboratories and process plants (Callanan and Klaenhammer, 2002). Prevention strategies include good laboratory and factory hygiene and decontamination with different disinfection agents. However, bacteriophage infection occurs from time to time. Thus, the use of immunised or resistant bacterial strains against specific phages may be helpful, but the properties of genetically modified strains resistant to phages are often worse than those of the parent phage-sensitive strain (Los *et al.*, 2004). Decontamination of large factories is difficult and the phage propagated in a bioreactor can spread throughout the plant, and even survive for long periods in places that have been treated (Primrose 1990). Jones *et al.* (2000 – as cited by Los *et al.*, 2004) described the serious problems caused by bacteriophages in biotechnology industries. Apart from infection and lysis of bacterial cultures by phages, there is another problem caused by viruses: the insertion of phage genetic material into the host chromosome, forming prophages instead of lysing the bacterial cells. Although these lysogenic strains are resistant to phages, their growth is slower than nonlysogenic strains and the efficiency of synthesizing bioproducts can be decreased (Los *et al.*, 2004).

Some bacteriophages are capable of surviving drying for many years. Therefore, careful microbiological practice, especially during culture inoculations and sampling, is absolutely necessary. If a phage infection has occurred, it is important that everything that could have come into contact with the phage be cleaned and sterilised. Phage treatment methods include high temperature, UV irradiation, chemical treatment, and use of inhibitors (Los *et al.*, 2004).

UV irradiation is a powerful method for decontamination. Phage genetic material (enclosed only by capsid proteins) is sensitive to UV light. UV lamps are highly recommended in all biotechnological laboratories but may be impossible in large plants where installation of lamps can be expensive and technologically very difficult. Chemical disinfection remains a feasible alternative (Los *et al.*, 2004, Primrose, 1990).

Influence of temperature on phages has been closely studied. Most phages are sensitive to heat. At 100°C, they are almost instantly inactivated. Below 65°C, some phages can be inactivated but most bacteriophages are hardly inactivated. The medium in which the phages are inactivated plays a major role in inactivation. Inactivation in pure water is rapid, but addition of salts like calcium or magnesium and proteins reduces the rate of inactivation significantly (Primrose, 1990; Ogata, 1980).

Some surface disinfectants that are used against bacteria can also be effective in phage inactivation. Thus, compounds which react with proteins and nucleic acids should be effective in inactivation of phages. Under certain circumstances, phage particles exist as aggregates. Adding surface disinfectants to phage aggregates may fail to inactivate phages (Primrose, 1990). Also, dead microbial cells can shield the phage from chemical inactivation. A similar protective effect exerted on the phage was seen in the study by Stagg *et al.* (1977 - cited by Primrose *et al.*, 1982) when a phage was associated with inorganic clay. This indicated that phages temporarily inactivated by association with solids may be protected from other form of inactivation.

A number of authors have studied the virucidal activity of ascorbic acid (Gao *et al.*, 1999; Schwager and Schulze, 1998; Packer and Fuchs, 1997; Jariwala and Harakeh, 1996; Lho *et al.*, 1992; Wang and Ness, 1989; Samuni *et al.*, 1983; Murata *et al.*, 1971). Most, if not all, showed that the toxicity effect of ascorbic acid is dependant on the presence of copper or iron and oxygen. In the presence of hydrogen peroxide, the ascorbate-induced phage inactivation and damage is prevented by catalase (Jariwala and Harakeh, 1996; Wang and Ness, 1989). Chelating agents were also reported to inhibit the inactivation whereas salicylate only reduced the rate of phage inactivation (Gao *et al.*, 1999). In this inactivation, phage adsorption and DNA injection are impaired as a result of the exposure to ascorbate and copper. Failed expression of genetic information, as a result of a single or double strand breaks in the DNA, also contributed to the loss of plaque-

forming ability of phages. Fat soluble vitamins E and K were also shown to be capable of inactivating viruses (German and Dillard, 2006; Zhang *et al.*, 1998). Murata *et al.* (1985) studied inactivation of bacteriophages using fat soluble vitamins. They found that vitamin E and K were capable of inactivating a wide range of *E. coli* phages. A 10^{-5} M concentration of these vitamins provided greater than 90% inactivation.

This chapter describes the evaluation of methods used for treatment of bacteriophages affecting a threonine bioprocess. The methods evaluated in this study were heat, chemicals and addition of inhibitors such as ascorbic acid and fat soluble vitamins E and K. The effect of these inhibitor vitamins on threonine-producing *E. coli* strain B was also investigated.

4.2 Materials and Methods

4.2.1 Treatment using heat

Equal amounts of bacteriophage sample P1-P6 (section 2.2.5) were mixed together and exposed to different temperatures i.e., 80, 100 and 120°C for the following periods: 10, 20, 30, 40, 60, 90, and 120 min. Five ml of phage mix was decanted into a small vial. The vials were placed in a heating block at different temperatures and were removed at various intervals. DAL was carried out (section 2.2.3) using the heat-treated phage samples. DAL was also done using phage sample (mixture of P1-P6) and used as a control. The tests were performed in triplicate and the results were reported as an average.

4.2.2 Treatment using chemicals

Equal amounts of phage samples P1-P6 (section 2.2.5) were mixed and were exposed to different chemicals at various concentrations. The host bacterium (*E. coli* strain B) was treated with the same chemicals to determine the effectiveness of these chemicals against it. The chemicals used included sodium hydroxide (Merck), hydrogen peroxide (Merck), Albrom 100T disinfectant (Albemarle Corporation), ammonium persulphate (FMC Corporation), Virukill (ICA International Chemicals), and sodium hypochlorite. Bacteriophage and host bacterium (*E. coli*) samples were exposed to the chemicals for 10 minutes at 25°C. The treated phages were then used in DAL (section 2.2.3) to determine the effect of the chemicals on the phages. The treated bacterium was serially diluted and spread plated on plate count agar (PCA) and the resultant colonies counted.

The tests were performed in triplicate and the results were reported as an average.

4.2.3 Treatment using vitamins as inhibitors

According to literature some vitamins have an inhibitory effect on bacteriophages (German and Dillard, 2006; Gao *et al.*, 1999; Schwager and Schulze, 1998; Zhang *et al.*, 1998; Jariwala and Harakeh, 1996; Wang and Ness, 1989; Murata *et al.*, 1985; Samuni *et al.*, 1983; Shalitin and Katchalski, 1962). These vitamins were tested so that they could be added to fermentation broth as bacteriophage inhibitors. A mixture consisting of bacteriophage samples P1-P6 was used and exposed to vitamins for 30 minutes at 25°C. Vitamins used were vitamin C (50 mg/L - Kirsch)/FeSO₄ (250 mg/L -Merck), vitamin K1 (1 x 10⁻⁵M) (DSM nutritional products) and vitamin E (1 x 10⁻⁵M) (Sigma). The solutions were prepared separately by dissolving the vitamin in water and filter sterilising with a 0.1 µm filter. The vitamins were individually tested, with the exception of vitamin C because other researchers (Gao *et al.*, 1999; Jariwala and Harakeh, 1996; Wang and Ness, 1989; Murata *et al.*, 1985) proved that ascorbic acid antiviral activity was enhanced by addition of Fe²⁺ ions. The two vitamin solutions that had the highest bacteriophage inactivation level were mixed together to increase their efficiency of phage inactivation. Equal amounts of vitamin C and E were mixed. Bacteriophage mixture was then incubated in this solution (VitC / VitE) for 30 minutes at 25°C. A control was included which comprised the bacteriophage mixture incubated in diluent for 30 minutes at 25°C. At the end of

the incubation, all phage samples were used to perform DAL. Another experiment was conducted to determine if the inhibitor vitamins, at the concentration used, have any effect on the host bacterium. The host bacterium (*E. coli* strain B) was grown in the presence of the VitC/VitE solution in a flask. A flask with host bacterium grown without VitC/VitE solution served as a negative control. OD_{600nm} was measured hourly for 12 hours. The tests were performed in triplicate and the results were reported as an average.

4.3 Results

4.3.1 Treatment of bacteriophages using heat

A mixture of bacteriophages was exposed to temperature at different levels i.e., 70, 80, 100, and 120°C. The phage samples were removed from the heat and DAL was done to determine effects of heat on pfu counts. The results showed that pfu counts were decreased by exposure to heat indicating inactivation of bacteriophages. The pfu's counted decreased as the level of heat increased. At 70°C, the level of bacteriophages in the sample decreased by nearly 3 logs from 7.7×10^9 to 1×10^7 pfu/ml after 2 hours. At 80°C, bacteriophage counts decreased by more than 5 logs from 7.7×10^9 to 1.3×10^4 pfu/ml after 2 hours. At 100°C, the level of bacteriophages in the samples decreased by nearly 9 logs from 7.7×10^9 to 1×10^1 pfu/ml after 2 hours. At 120°C, DAL plated after 30 minutes did not have any plaques, only host bacterial growth. This indicated that complete inactivation of bacteriophages was achieved after 30 minutes. In the positive control sample, (untreated phage sample) the level of bacteriophage did not change (figure 4.1).

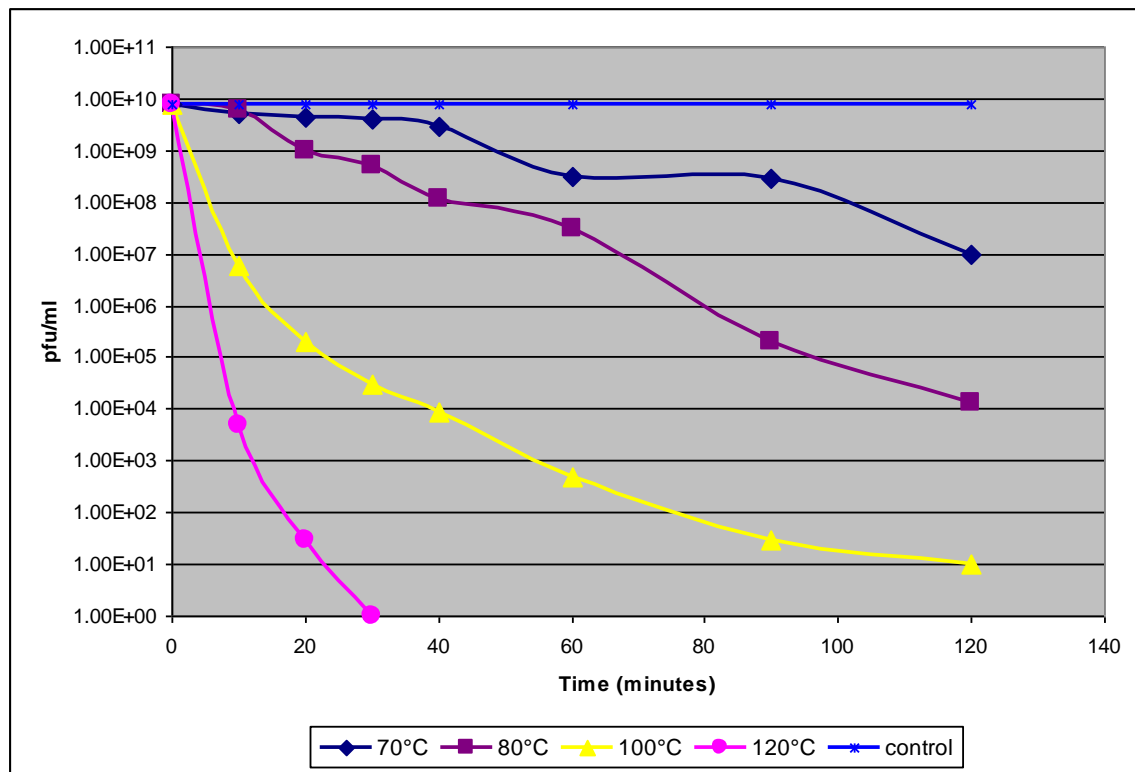


Figure 4.1 Treatment of bacteriophages using heat. Phage samples P1-P6 were mixed and exposed to different temperatures: 70, 80, 100, and 120°C using a heating block. Complete bacteriophage inactivation was achieved at 120°C after 30 minutes. Results were the average of tests done in triplicate.

4.3.2 Treatment of bacteriophages using chemicals

Bacteriophage samples P1-P6 were mixed and incubated with different chemicals for 10 minutes at room temperature. All the chemicals tested, with the exception of ammonium persulphate, were capable of inactivating the host bacterium *E. coli* strain B. Bacteriophages in the samples were completely inactivated by all lowest sodium hydroxide concentrations used as well as all sodium hypochlorite concentration used. Hydrogen peroxide was only capable of deactivating both the host bacteria and bacteriophages completely at concentration higher than 10%. Albrom 100T and Virukill achieved complete inactivation of both host bacteria and bacteriophages at 0.05% and 2% respectively. Ammonium persulphate did not have a major effect on bacteriophages at all concentrations tested, but the highest concentration used (1%) was able to completely inactivate host bacterial counts (Table 4.1).

Table 4.1 Treatment of bacteriophages and *E. coli* strain B using chemicals

Chemical (%)	Phage Counts (pfu/ml)	<i>E. coli</i> strain B counts (cfu/ml)
Sodium hydroxide		
0.1	0	0
0.5	0	0
1.0	0	0
Hydrogen peroxide		
0.5	3.4×10^3	0
5.5	5.9×10^6	0
10	0	0
Sodium hypochlorite		
0.5	0	0
1.0	0	0
3.0	0	0
Ammonium persulphate		
0.1	2.0×10^9	1.1×10^5
0.5	1.5×10^9	4.6×10^4
1.0	8.5×10^8	0
Albrom 100T		
1×10^{-2}	2.5×10^2	0
5×10^{-2}	0	0
0.1	0	0
Virukill		
0.5	5×10^3	0
1.0	2×10^2	0
2.0	0	0
Control (+ve)	3.8×10^9	5.9×10^7

4.3.3 Treatment of bacteriophages using inhibitors

This experiment was carried out by incubating bacteriophage sample (P1-P6) mixture with the inhibitors in a shaking incubator for 30 minutes at 37°C. A control experiment was carried out whereby the phage mixture was incubated in the diluent (5 g/l NaCl, 1 g/l nutrient broth) in the shaking incubator for 30 minutes at 37°C. After 30 minutes, aliquots were taken out of the incubator and DAL was done using both treated and untreated bacteriophage samples. The results (figure 4.2) showed that the control sample did not have any phage inactivation. The samples treated with VitC/Fe mixture had a greater than 4 log reduction in pfu counts from 7.7×10^9 to 2×10^5 pfu/ml, indicating that this mixture had a significant effect on the bacteriophages. VitE produced a 5 log reduction in pfu counts from 7.7×10^9 to 5×10^4 pfu/ml, indicating an even greater inactivation of bacteriophages than the VitC/Fe mixture. VitK1 had a lower effect on bacteriophages compared to the other two vitamins (VitC/Fe and VitE). It produced only a 2 log reduction in the pfu counts. VitC/Fe and VitE were mixed together to determine whether the mixture would have an even greater effect in deactivating bacteriophages. Results showed that when these two vitamins were mixed a greater than 9 log reduction on pfu counts from 7.7×10^9 to 3.0 pfu/ml was achieved. This vitamin solution had almost complete inactivation of bacteriophages in the samples.

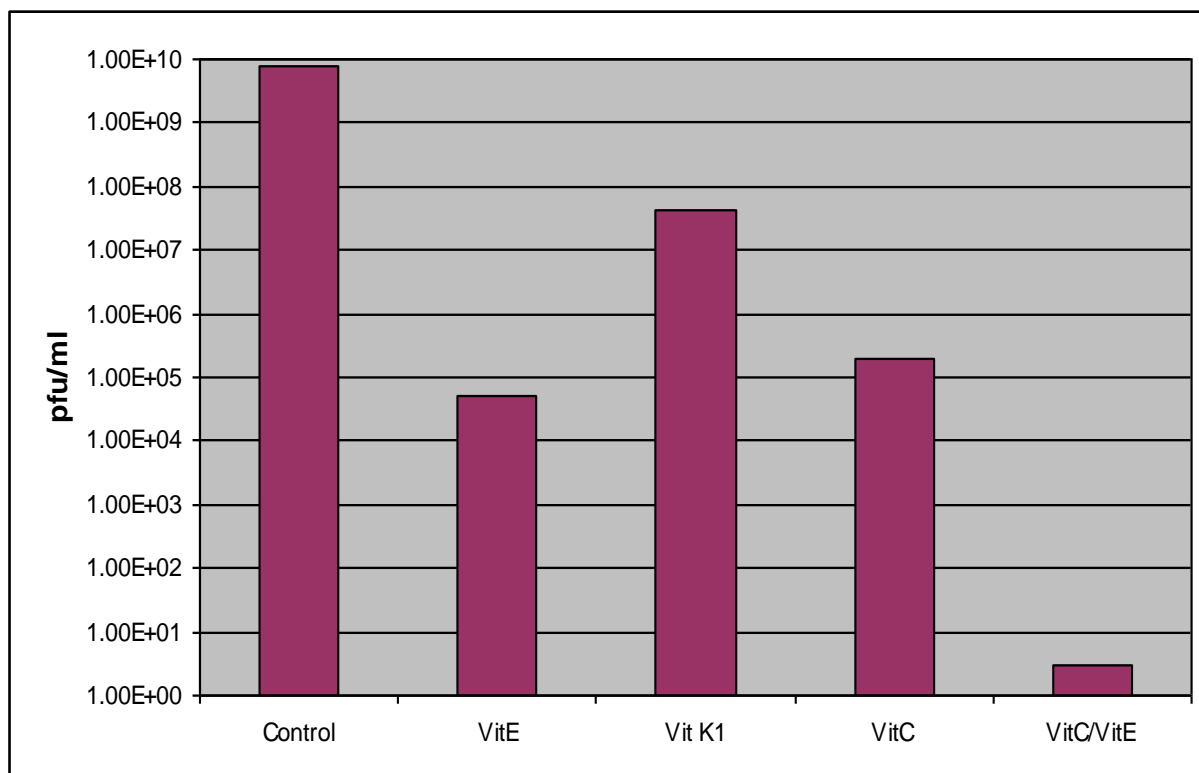


Figure 4.2 Bacteriophage inactivation using inhibitors. Different kinds of vitamins were used individually and then mixed. Phages were incubated with the vitamins at 37°C for 30 min and DAL was done. Results are an average of tests done in triplicate.

The host bacterium was grown in a flask that contained an inhibitor mixture (VitC/Fe/Vit E) and incubated at 37°C for 12 hours. A control experiment, which was a flask with a host bacterium grown in normal media, without the inhibitor mixture was grown parallel to the test bacteria. The bacterium grown in the presence of inhibitors followed the same trend as the control bacteria (figure 4.3). This indicated that the inhibitors did not have any effect on the host bacterium.

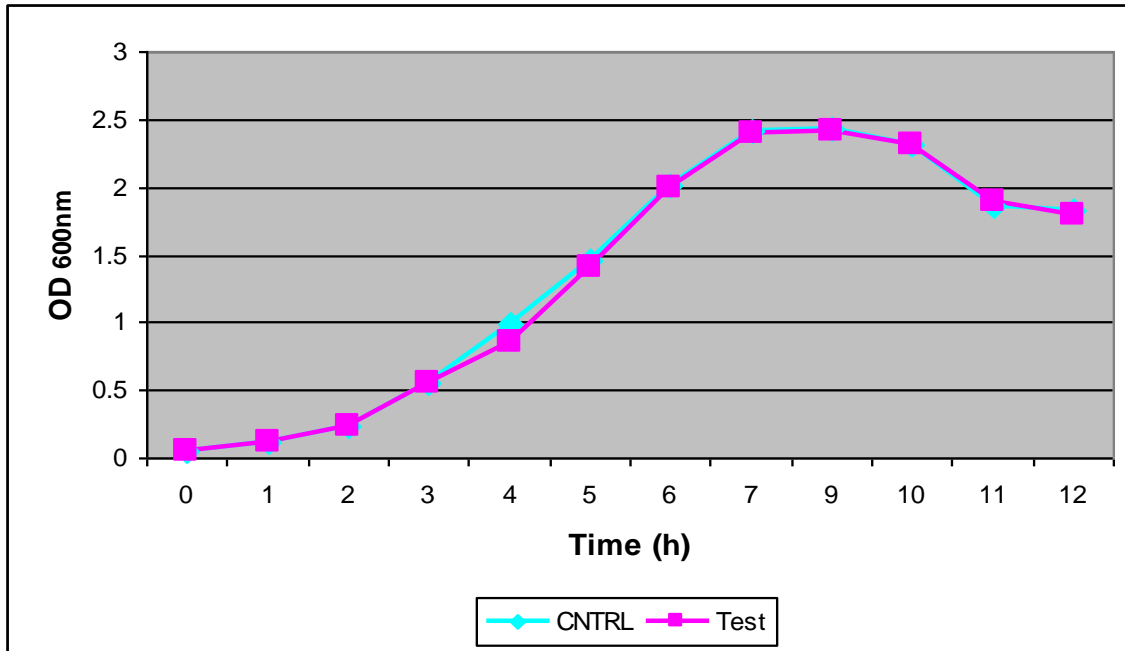


Figure 4.3 Effect of phage-inhibiting vitamins on the bacterial growth. Growth of the host bacterium showed no difference in the presence of inhibiting vitamins when compared to the control, host bacterium without the vitamins

4.4 Discussion

Bacteriophages have been known to have a detrimental effect on bioprocesses that use bacterial fermentations. When a bacteriophage infection is suspected, the area and the equipment have to be decontaminated. Decontamination of large bioprocessing plants has proven to be a very difficult process. Steam is still widely used in these plants to deactivate bacteriophages (Primrose, 1990). Thus, the influence of temperature on the inactivation of phages was studied in this chapter. Bacteriophage treatment was done at 70, 80, 100, and 120°C. Phage inactivation increased with an increase in temperature and exposure time. Temperatures used (70, 80, 100, and 120°C) were capable of decreasing the number of pfu counts by greater than 3, 5, 8 and 9 logs, respectively, after 2 hours. At 100°C, 50% of phage particles in the sample were deactivated after 20 minutes. This represented a major reduction in bacteriophages in the sample. At 120°C all bacteriophages were inactivated after 30 minutes. Therefore, temperatures greater than 120°C proved to be very efficient in decontaminating, among other things, equipments and bioreactors that have been infected by bacteriophages (figure 4.1).

The use of UV light has been widely used to decontaminate surfaces. But it can be technically difficult to install UV lights in large bioprocessing plants, therefore chemical disinfection remains an alternative (Lu *et al.*, 2003). Chemicals studied were chosen because they were already being used in the plant in other processes, therefore readily available, and also due to their cost effectiveness.

All the chemicals tested were efficient in inactivating phages and *E. coli* with the exception of ammonium persulphate. Hydrogen peroxide was able to inactivate all the phages but only when higher concentrations were used. Sodium hydroxide was capable of inactivating phages at low concentrations of 0.1%, which was the lowest concentration of NaOH used in this study. Concentrations of NaOH higher than the ones tested in this experiment are currently being used at S.A. Bioproducts (Pty) Ltd during cleaning of fermenters. Ammonium persulphate and Albrom 100T are disinfectants used for treating cooling water towers. It was shown that Albrom 100T was more effective than ammonium persulphate (table 4.1). Ammonium persulphate was able to reduce the concentration of phages significantly, but could not completely deactivate all the bacteriophages in the sample. Virukill is a disinfectant used to disinfect surfaces against all poultry viruses, bacteria, fungi, yeast, and algae. The supplier claimed that it would be effective in inactivating bacteriophages as well. This claim proved to be true, as 0.5% Virukill was capable of deactivating both bacteriophages and host bacterial cells (table 4.1).

The two methods mentioned above are mainly preventative and decontaminating methods. These methods can only be used to ensure that all the equipment and surfaces are clean and phage-free before it can be used in fermentation. But these methods could not be used in a case where bacteriophages have already gained entry into the fermenters. This is the reason the inhibitor vitamins were worth studying because they can be added directly to the fermentation broth and

inactivate bacteriophages without damaging the bacterial cells (Gao *et al.*, 1999; Murata *et al.*, 1971). Many authors have proved that ascorbic acid was capable of inactivating phages and even more efficiently when combined with copper or iron (Lho *et al.*, 1992; Wang and Ness, 1989; Samuni *et al.*, 1983; Murata *et al.*, 1971). This experiment was done in two stages. The first stage involved comparing vitamin C, E and K1 to determine the most effective in bacteriophage inactivation. Then second stage was to combine two vitamins that showed the highest inhibitory effect on bacteriophages. The results (figure 4.2) showed that vitamin C and E solutions had higher inhibitory effect (4 and 5 log reduction of pfu counts respectively) than vitamin K1 (2 log reduction of pfu counts). The ability of ascorbic acid to reduce inactivate bacteriophages has been proven by several authors (Schwager and Schulze, 1998, Packer and Fuchs, 1997; Lho *et al.*, 1992; Wang and Ness, 1989; Samuni *et al.*, 1983; Murata *et al.*, 1971). Lho *et al.* (1992) showed that Fe-Asc complex was capable of reducing 90% of phage concentration. A mixture of Vitamin C and E was also tested using a mixture of all bacteriophage samples (P1-P6). This VitC/VitE solution was capable of significantly reduce bacteriophages in the sample by greater than 9 log. This solution was then tested against host bacterium *E. coli* strain B to ensure that at the concentrations used it did not have any adverse effect on the host bacterium. The host bacterium was not affected by the inclusion of inhibiting vitamin solution in the growth medium. The same phenomenon was shown in the study by Murata *et al.* (1971).

It was hence shown in this chapter that a combination of strategies is required for the treatment of bacteriophages. This chapter showed that it is not feasible to use just one method to treat bacteriophage infections. Therefore, a combination of these methods can be used to prevent and then treat bacteriophages.

CHAPTER 5

GENERAL DISCUSSION

The infection of threonine-producing *E. coli* by bacteriophages has detrimental effects on the bioprocess. Consequently, the failure to detect bacteriophage infection signs early results in further dispersion of bacteriophages. This makes it difficult to execute post-infection cleaning procedures. Also, a bacteriophage infection halts production, which can range from a few days to many months.

This study was done since the *E. coli* strain B used by SA Bioproducts (Pty) LTD to produce threonine was suspected to be prone to bacteriophage infection. This phenomena was first seen on the pilot plant fermenters where oxygen uptake rate (OUR) in the fermenter would increase exponentially for about an hour and then it will suddenly drop to zero within an hour. Several theories were explored until it was decided to investigate for possible bacteriophage infection. These theories confirmed a bacteriophage infection. It was then decided that an assay for detection, isolation and enumeration of the bacteriophages should be developed. The source for the bacteriophages needed to be identified and treatment and prevention strategies had to be developed in order to effectively deal with this infection.

Areas in the plant were identified as potential sources of the infection. These areas included the sumps, the area outside the compressor house where the soil samples were taken, the odour scrubber, and the process water. Samples from

these areas were collected weekly and Adam's DAL method was used to detect and isolate bacteriophages in the samples. The sumps were shown to have the highest bacteriophage levels. The samples were pooled as there were too many to work with in the investigation. The samples were pooled according to their source, and were labelled P1-P6. It was noticed that some of the isolated bacteriophage samples had very low levels of bacteriophages and had to be enriched. The methods used to enrich bacteriophage samples were effective as they increased the amount of bacteriophages in the samples on average by about 5-fold. The samples were purified in order to be characterised by electron microscopy and for rate of infection studies. Results from both methods indicated that there was one type of bacteriophage that was prevalent in all samples. These results correlated with the findings of Theron (2006) who identified the prevalent bacteriophage using samples from infected fermenters. They were identified as T1-like particles. Bacteriophage sample preservation was investigated and it was concluded that both methods (storing bacteriophages in growth medium or Tris.HCl buffer) were effective for storing bacteriophages. The storage of bacteriophage samples at -20°C was more stable than storage at 4°C. It was concluded that both methods required that the samples be renewed every 8-10 weeks at 4°C and 3-4 months at -20°C.

A method was also developed to enumerate bacteriophages in the samples in order to monitor the levels of bacteriophages in the environment. This involved optimizing Adam's DAL method to the bacteriophage-host bacteria (BHB) system

used in this study. Optimal incubation time and temperature, bacterial concentration, and addition of bivalent ions were investigated to determine the optimal condition for our system. The optimal conditions for our BHB are listed in Table 5.1.

Table 5.1 Parameters for the bacteriophage-host bacteria system

Parameter	Condition
Incubation temperature	37°C
Incubation time	20-24 hours
Bacterial concentration	100 µl
Bivalent ion concentration	5 mM Ca-Mg

Adam's DAL method was improved (figure 3.2) by reducing the temperature for adding soft agar from 45°C to 40°C, by mixing phage sample with bacterial cells in the test tube and then decant into an agar plate and adding the soft agar into agar plate instead of into the test tube. This improved DAL method resulted in pfu counts about 2-fold higher than Adam's DAL.

Bacteriophages infect bacterial cells and either lyse the cells or integrate their genome into bacterial chromosomes (lysogenic cycle). Bacterial cells containing this integrated genome (lysogens) can confer phage resistance. Lysogens have been notorious for introducing new lytic cycles after post infection clean-up processes. Lysogens have also been reported by Coffey *et al.* (2001) to have

lowered production abilities. Therefore, an assay for detection of lysogenic phages was developed. Methods that were tested included UV light, green indicator plates, and the use of a mutagen. The mutagen (Mitomycin C) at 1 µg/ml proved to be optimal concentration and concentrations higher than 2 µg/ml proved to be toxic to the bacterial host. The use of the mutagen was the most reproducible and reliable method to be used for detection of lysogenic phages. It was concluded that this method should be integrated in quality control when preparing work cell banks to ensure that they do not contain any bacteriophages.

The bacteriophage samples P1-P6 were then used to develop a treatment method. Methods investigated for prevention and treatment included the use of heat, chemicals and vitamins as inhibitors. A temperature range of 70 to 120°C was used. At 100°C, pfu counts were drastically reduced after 120 minutes but at 120°C complete inactivation of bacteriophages was achieved after 30 minutes. This proved that autoclaving glassware and waste, and even sterilizing the fermenters at 121°C for 30 minutes is sufficient to inactivate the bacteriophages present. Chemicals that were used for treatment included hydrogen peroxide, sodium hypochlorite, ammonium persulphate, Albrom 100T, and Virukill. These chemicals were chosen as they were readily available and were being used for other processes in the plant, with the exception of Virukill. Virukill is a disinfectant and was selected because the supplier claimed that it was effective in virus inactivation. Complete inactivation of both host bacteria and bacteriophages was achieved using 0.1% sodium hydroxide and Albrom 100T. Sodium hydroxide is

currently being used at concentrations higher than 0.1% to wash the fermenters after every fermentation run. Therefore, sodium hydroxide can be dumped into the sumps after being used in the fermenters, to reduce the level of bacteriophages in the sumps. This could reduce the probability of further bacteriophage infection. Albrom 100T can be used in the odour scrubber to reduce the bacteriophages present here. It is of outmost importance that bacteriophage levels be kept very low in the odour scrubber as it is responsible for dispersing bacteriophage throughout the plant area. Virukill (2%) completely inactivated bacteriophages and could be used as disinfectant in the laboratories for surfaces and even hands, as it is not as harsh as the other chemicals. It is also important when selecting a chemical to use for cleaning the environment, to have a chemical that could also inactivate the host bacterium, because if the host bacterium is leaked into the environment then bacteriophages would flourish and increase. Methods mentioned previously were preventative methods for cleaning the environment and equipment. A method for combating bacteriophage infection in the fermenters during fermentation had to be developed because, if for some unforeseen reason, a bacteriophage found its way into the fermenter then the whole fermentation should not be lost. This was the reason vitamins were investigated as bacteriophage inhibitors. There is overwhelming literature that suggested that certain vitamins were capable of hindering bacteriophage infection (German and Dillard, 2006; Gao *et al*, 1999; Schwager and Schulze, 1998; Zhang *et al.*, 1998; Jariwala and Harakeh, 1996; Wang and Ness, 1989; Murata *et al.*, 1985; Samuni *et al.*, 1983; Shalitin and Katchalski, 1962). Vitamins

C, E, and K were tested. Vitamins C and K were mixed and then tested to determine whether their efficiency would increase. It was evident that combining these vitamins increased their efficiency. The mixture decreased pfu counts from 7.7×10^9 to 3.0 pfu/ml and could be used as a co-feed in the fermentation process. Vitamin E is not cost effective to be used as a co-feed in a 200 m³ fermenter. Therefore, it was concluded that only vitamin C could be used as co-feed in the fermenters.

Industrial bioprocesses using *E. coli* have a high risk of bacteriophage infection. Therefore, it is of great importance that the operators that work in these plants are trained to recognize bacteriophage infection early. This would impact on efficient cleaning up. Also, when an infection is suspected the bioprocess must be stopped immediately and the contents of the bioreactor must be sterilised. An assay should be available to ensure complete deactivation of the bacteriophages and bacterial cells in the reactor. Thereafter, a clean up procedure must be available and should be executed as soon as possible. Also, preventative measures should be adhered to at all times to ensure that the level of bacteriophages in the environment is kept low. Spillages that occur are cleaned and disinfected rapidly. Environmental monitoring should be done regularly to determine the levels of bacteriophages in the environment and detect the presence of bacteriophages capable of infecting the production strain. An increase in bacteriophages in the environment should be an indication that a plant clean-up is needed in order to avoid infecting the bioreactors. Another

factor to be considered is the plant design. There should be no pools of liquid containing live cultures anywhere in the vicinity of the bioprocessing plant. All sampling points should be equipped with funnels and any spillage must be piped into a deactivation tank. Where a new facility is being built, the location should be considered. The plant should be situated away from sources of coliphages and should not be close to cultivated land.

This study was successful in identifying bacteriophage infection as the cause of product losses. Bacteriophages isolated from the plant environment were identified and proven to be similar to those isolated from the fermenters, thus responsible for infecting production strains. Various treatment strategies were investigated and the most effective measures are currently being implemented at the plant. Finally, recommendations to prevent future infections were proposed and these included training of personnel to recognize phage infections timeously.

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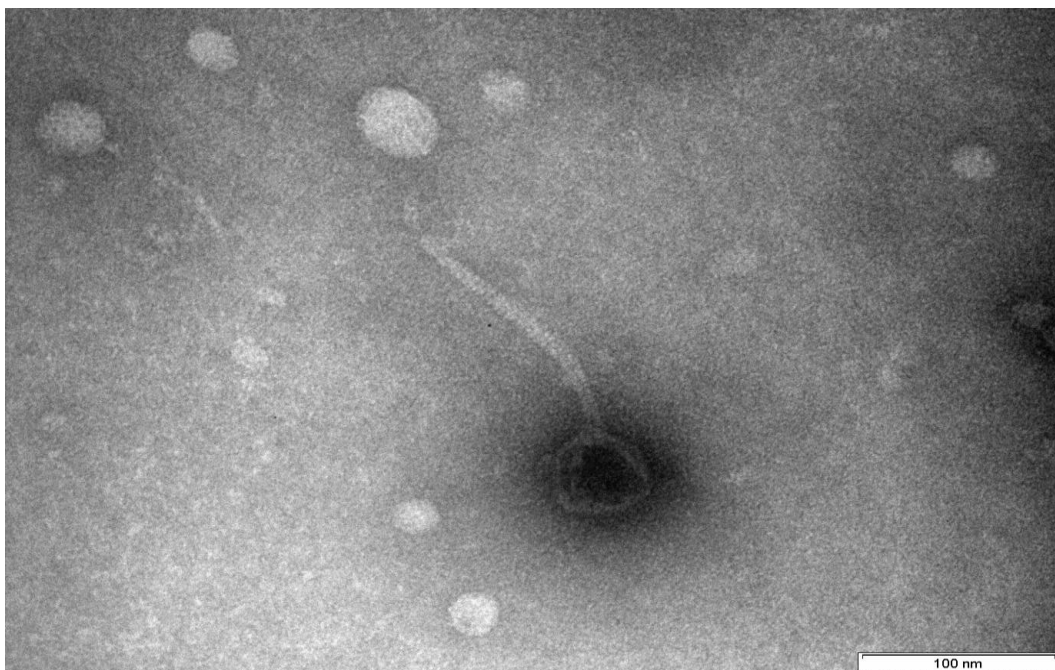
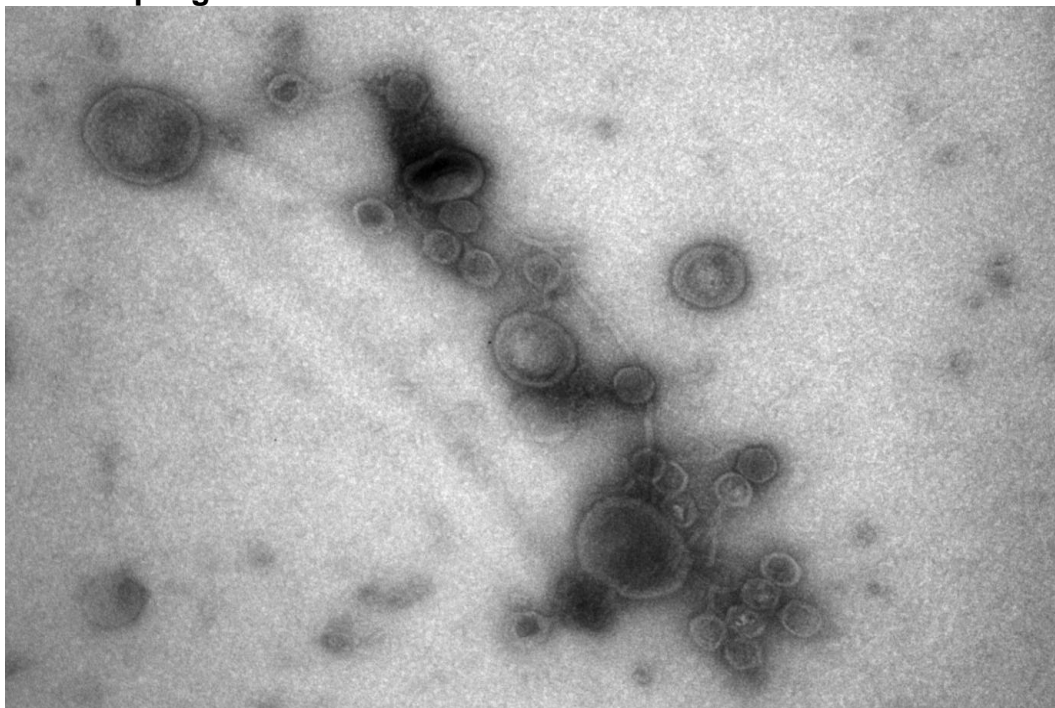
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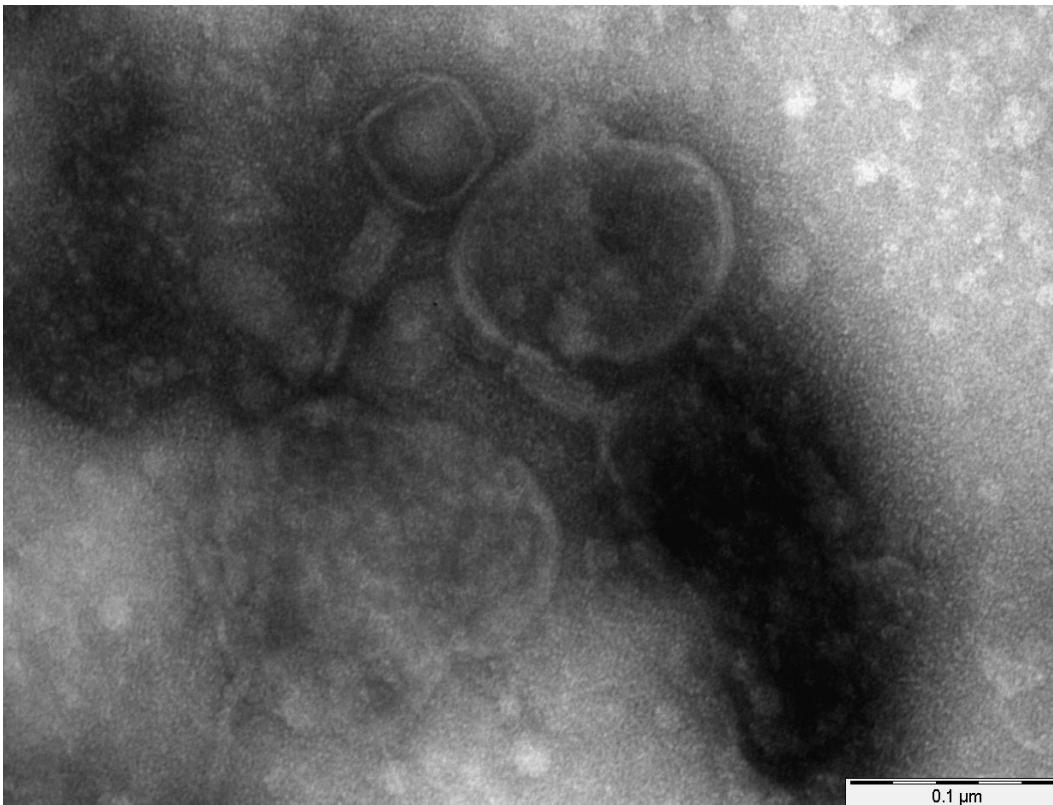
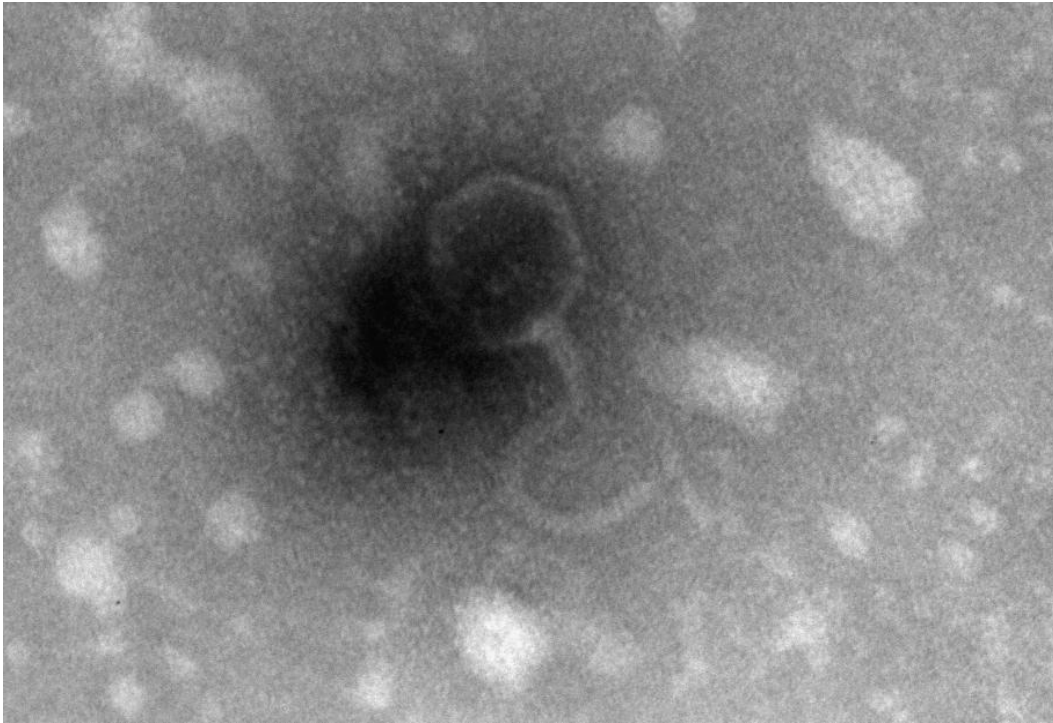
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APPENDIX

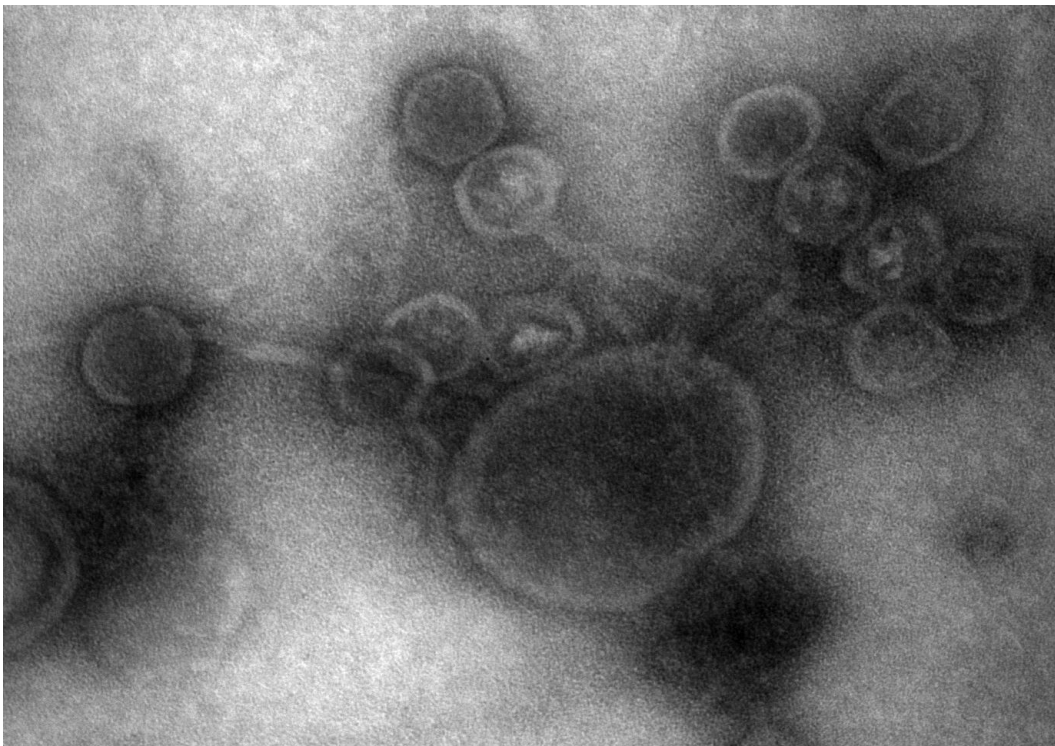
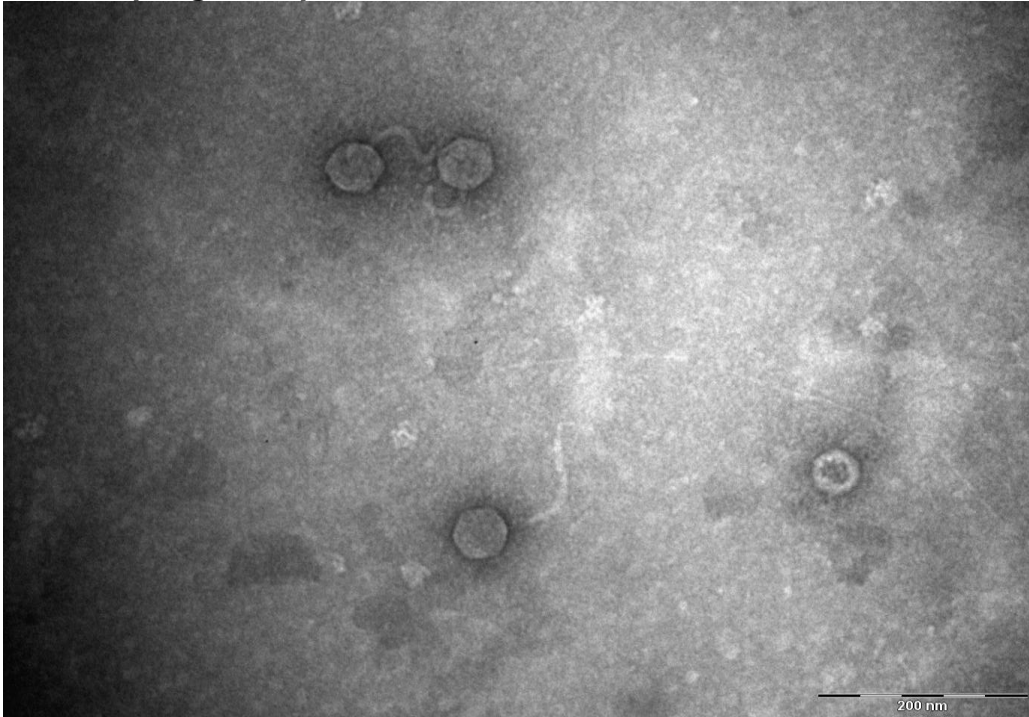
Bacteriophage samples micrographs for P1-P6, taken using a transmission electron microscope (Phillips CM120 Biotwin):

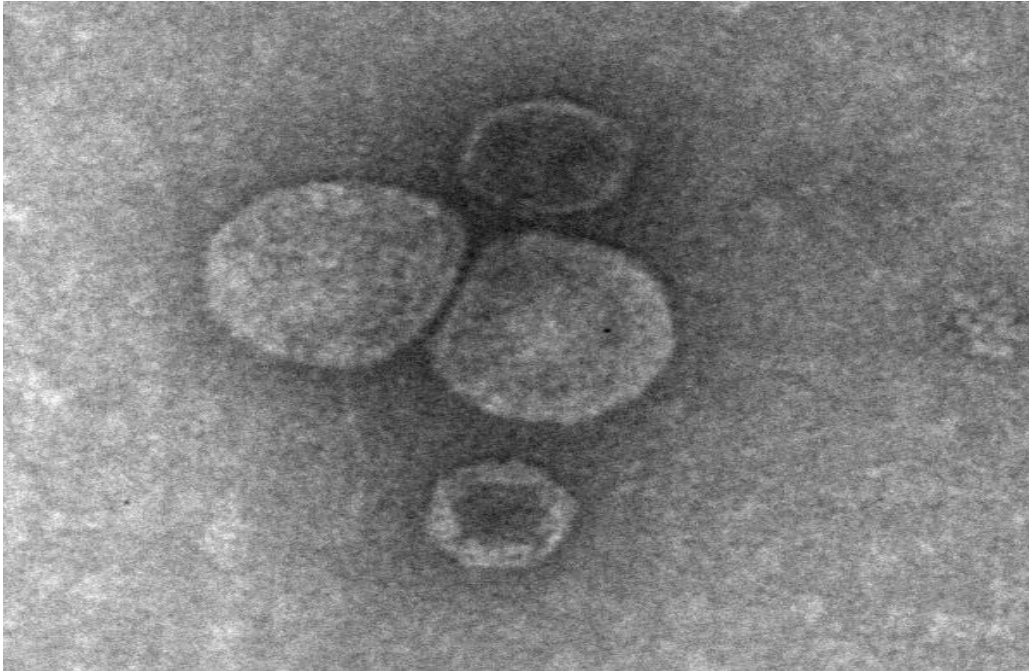
Bacteriophage P1



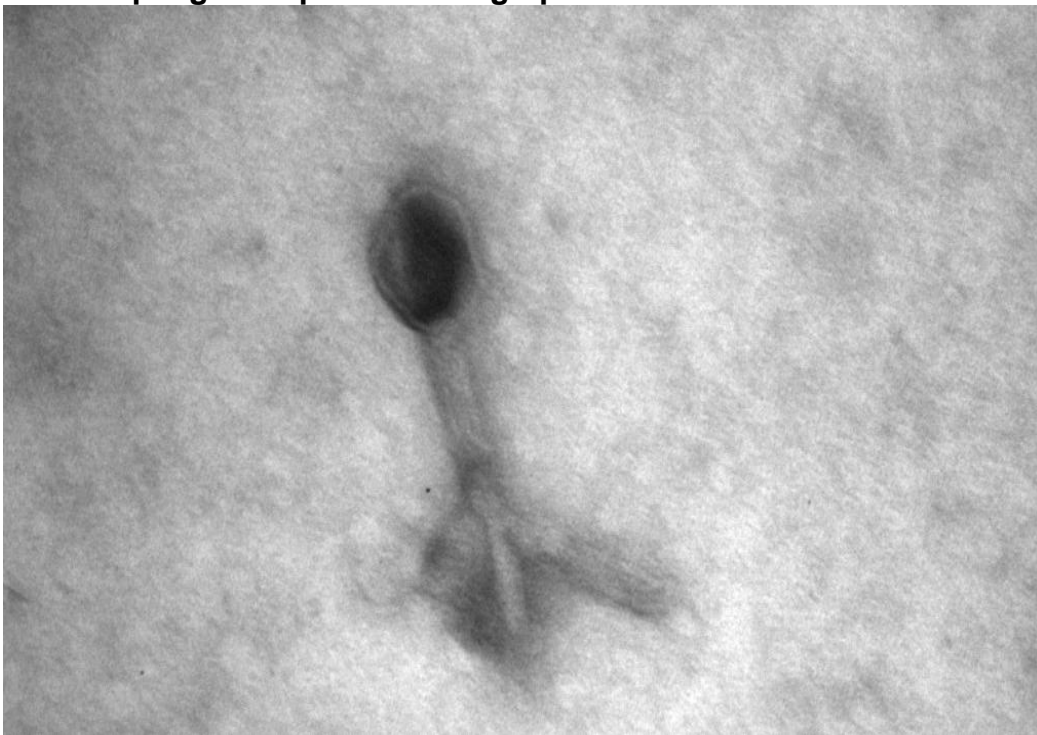


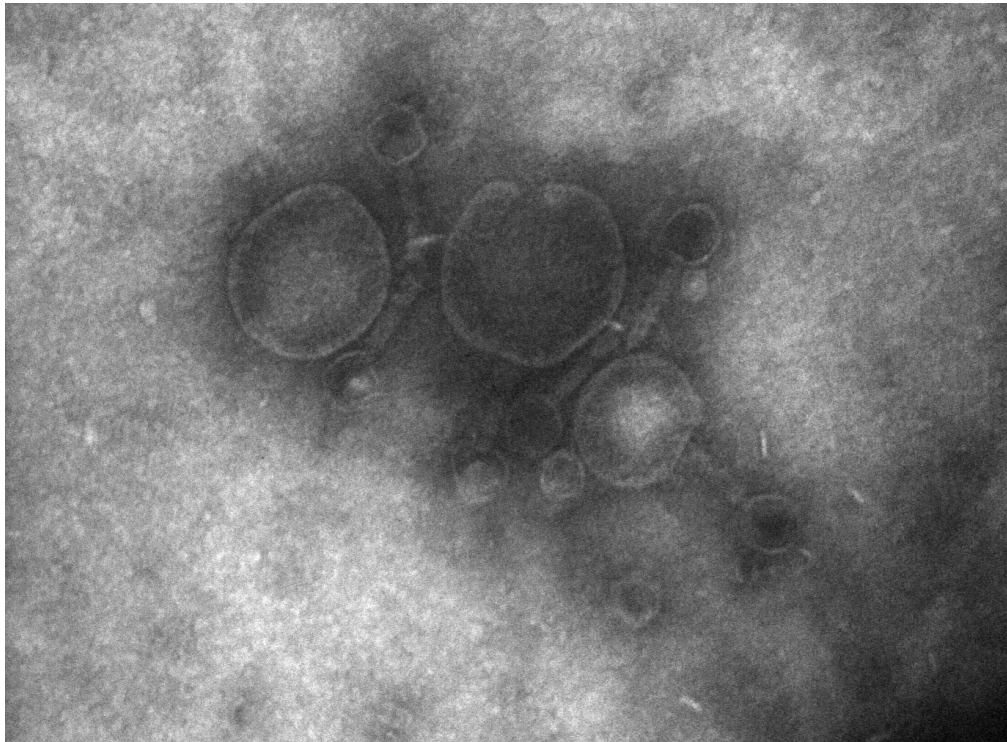
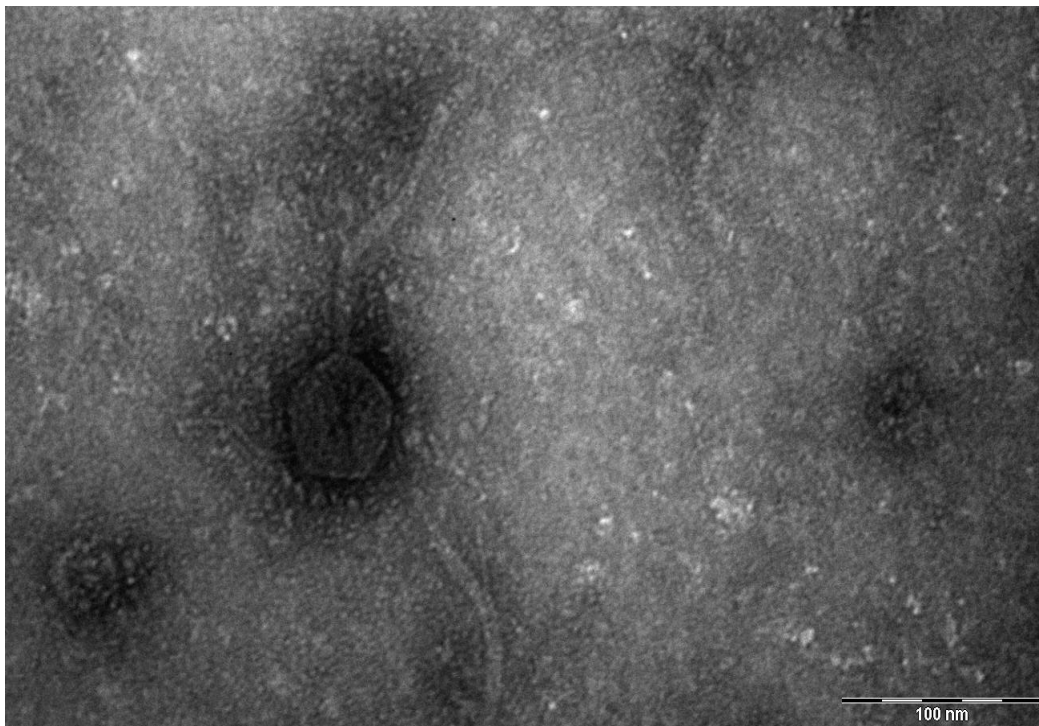
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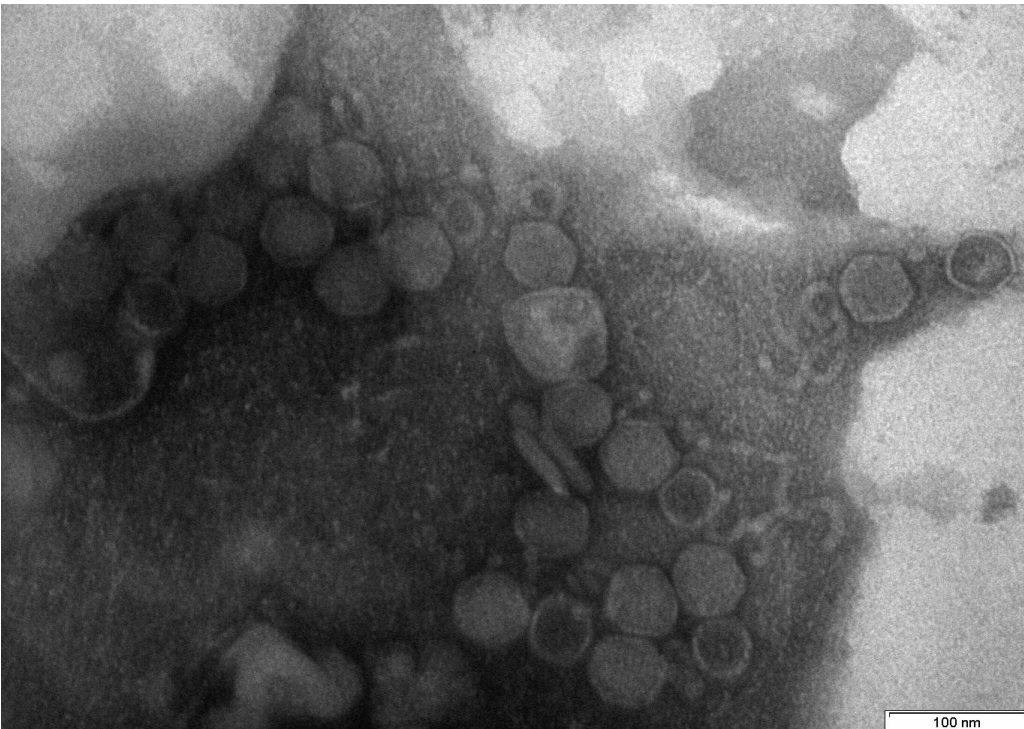
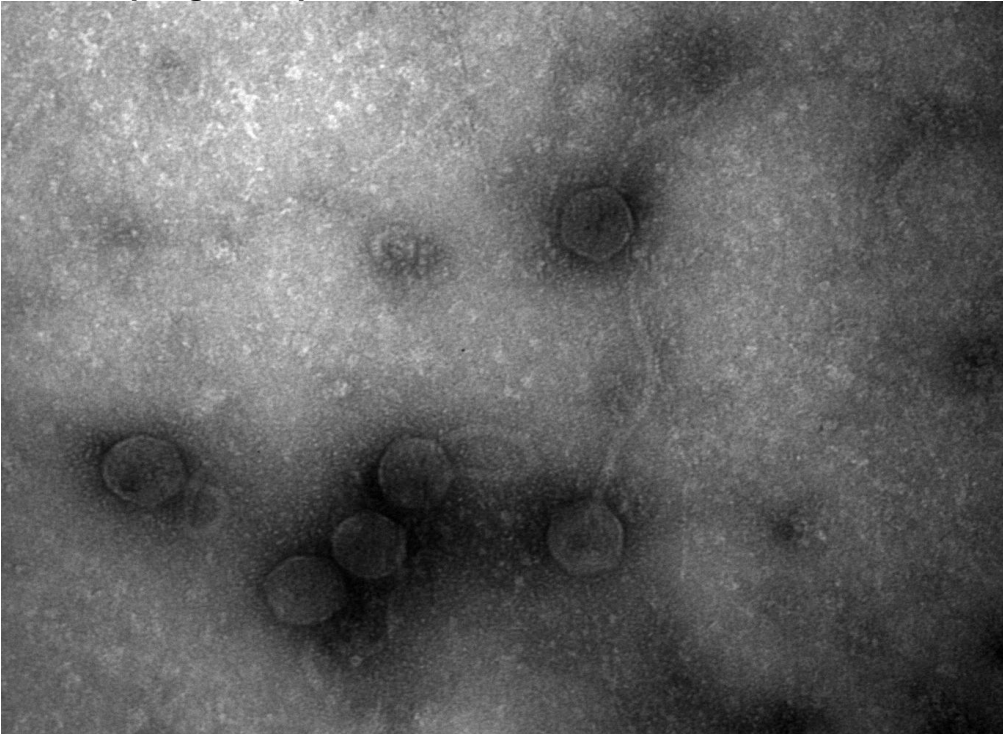


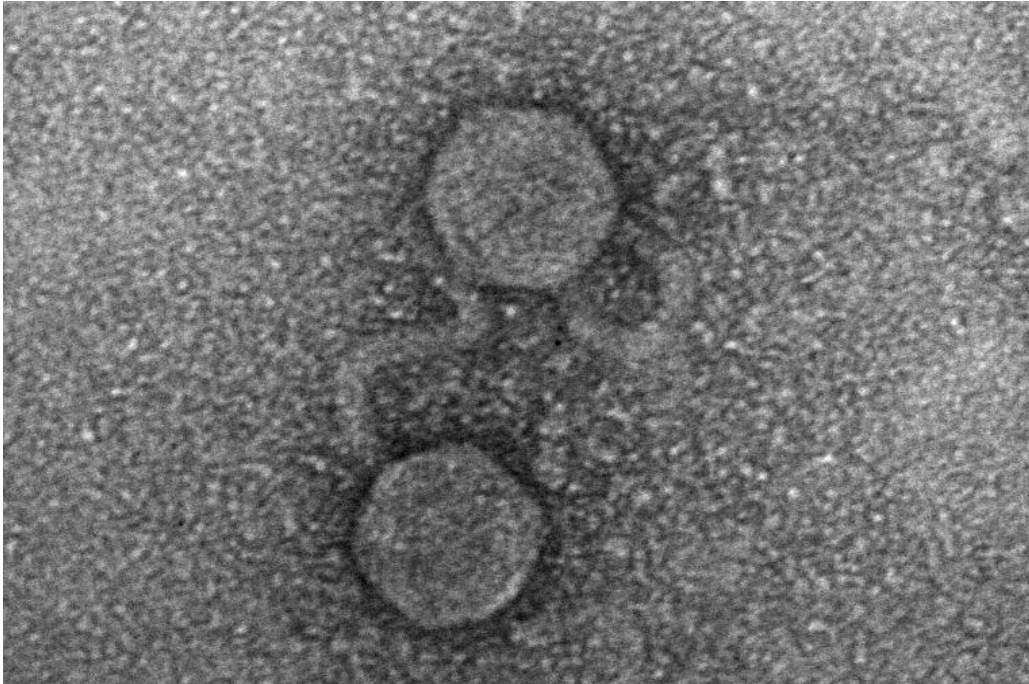
Bacteriophage sample P3 micrographs



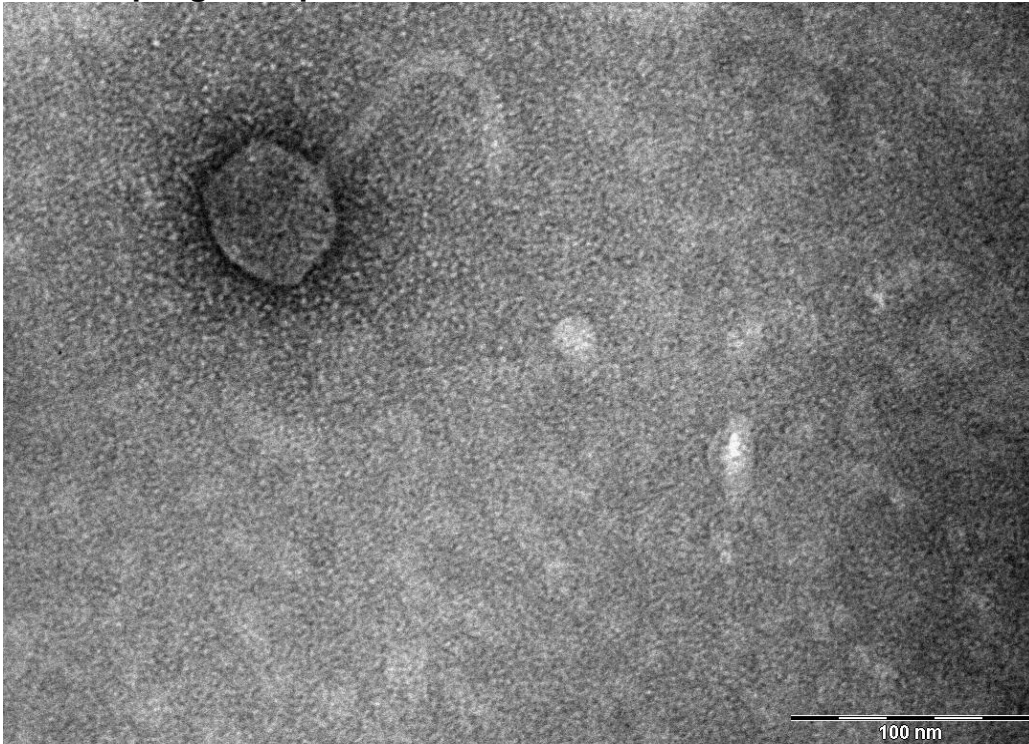


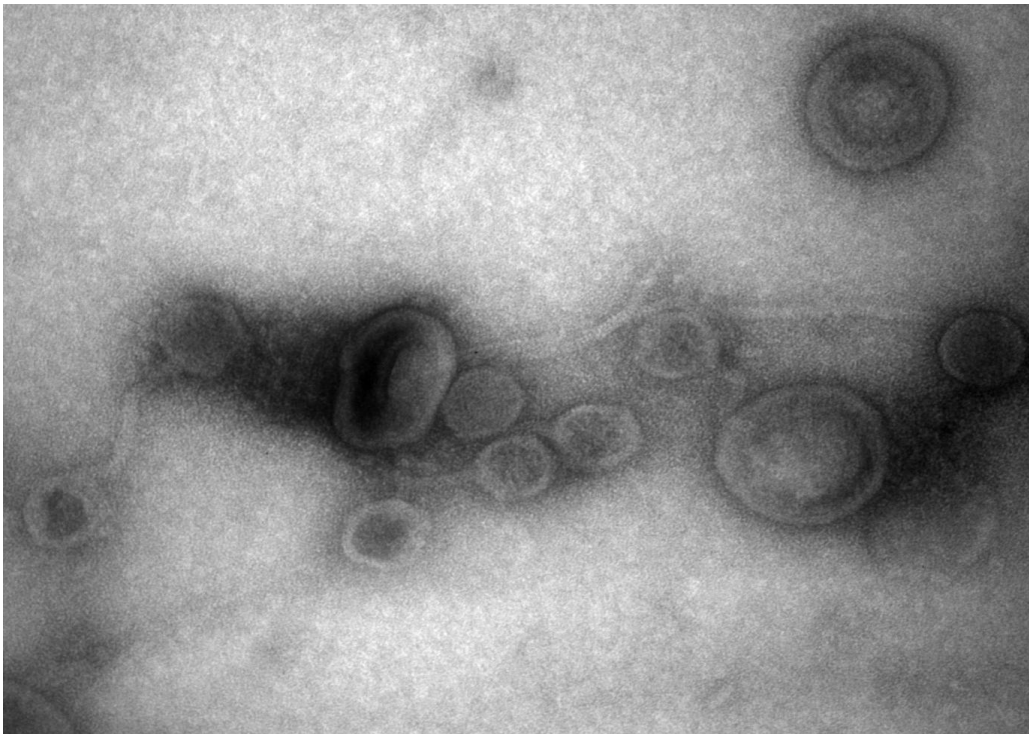
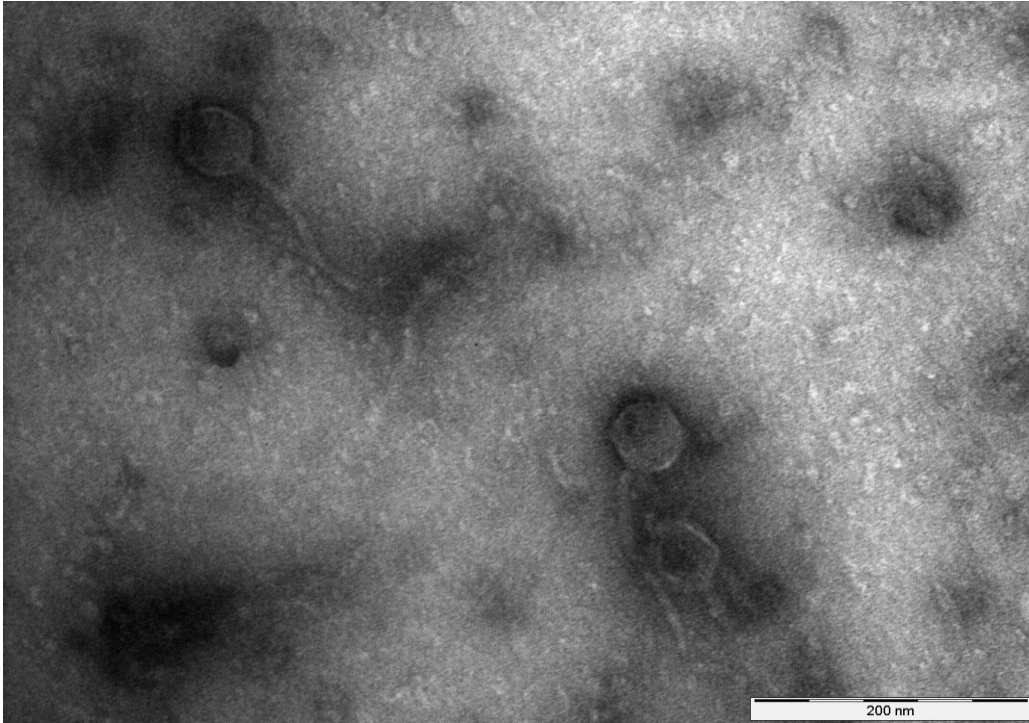
Bacteriophage sample P4





Bacteriophage sample P5





Bacteriophage sample P6

