Study of Bacteriophage Propagation on Biofilms of Cheese Starter Cultures

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A Thesis submitted to

Auckland University of Technology

in fulfillment of the requirements for the degree of

Master of Philosophy (M Phil)

2012

School of Applied Science

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LIST OF ABBREVATIONS

CBD	–	Calgary Biofilm Device
CDC	–	Centre for Disease Control
CDFF	–	Constant Depth Film Fermentor
CFR	–	Continuous Flow Reactor
CFU	–	Colony Forming Units
CIP	–	Clean-In-Place
CLSM	–	Confocal Laser Scanning Microscopy
CV	–	Crystal Violet
DNA	–	Deoxy Ribonucleic Acid
ds	–	Double Stranded
DT	–	Detection Time
DVI	–	Direct Vat Inoculation
EPS	–	Extracellular Polysaccharide Substances
GMP	–	Good Manufacturing Practice
ICTV	–	International Committee for Taxonomy of Viruses
LAB	–	Lactic Acid Bacteria
OD	–	Optical Density
PFU	–	Plaque Forming Units
PIM	–	Phage Inhibitory Medium
ss	–	Single stranded

ATTESTATION OF AUTHORSHIP

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

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Auckland

2012

ACKNOWLEDGEMENTS

I wish to convey my heartfelt gratitude to the following persons for their tremendous contribution and support both morally and financially towards the completion of this project.

I deem it my pleasure to thank Prof. John D. Brooks, my supervisor and mentor for his constant help and guidance throughout this project. His motivation and encouragement helped me to complete my research successfully.

My sincere gratitude goes out to the staff members of the School of Applied Sciences, for supporting me financially by paying my extension fees.

I would like to express my gratitude to Fonterra Research Centre, Palmerston North, for rendering me the starter culture and phage samples without which I would not have been able to commence my project.

I am grateful to my colleagues Isabel, Alina and Tanvi.S for their relentless support and assistance throughout my project.

I take this opportunity to thank all the WS Chemistry and Biology laboratory staff for aiding me with all necessary laboratory facilities.

I would like to convey my immense gratitude to Mrs. and Mr. Ganesh Ram for their moral and pecuniary support which facilitated me to complete this project.

I would like to thank my friends and family for their prayers and encouragement which motivated me to complete this project successfully.

Above all, I thank the Almighty God for helping me accomplish this venture with ease.

Dedicated to my loving family

“God always gives his best to those who leave the choice with him”- Jim Elliot

ABSTRACT

The main aim of this study was to investigate the potential propagation of bacteriophage in biofilms of cheese starter cultures. It was hypothesized that the biofilms of the starter cultures may form in cheese and casein plants, perhaps in whey drain lines and become a reservoir and site of propagation of phage particles. Phage can infect and destroy the bulk starter cultures used for cheese production and this might cause a severe economic loss in the cheese manufacturing industry. Bacteriophage attacks against thermophilic dairy starter bacteria are now recognized as the main cause of slow or faulty fermentative production in dairy plants. The objective of this study was to determine whether the starter cultures are capable of producing biofilms and to investigate the ability of phage to propagate inside them. The project was started by examination of attachment and biofilm formation by *S. thermophilus*, a common starter bacterium used for the manufacture of cheese.

The initial experiments showed that these starter cultures have the capacity to attach and produce biofilms on stainless steel coupons at a temperature of 30ºC, which showed that they were capable of attaching to the whey effluent lines in a cheese factory and thereby become potential sites for phage propagation. An experiment was conducted by infecting an existing biofilm with phage. The results showed that phage have the capacity to penetrate an existing biofilm matrix and produce an infection, causing the number of cells to decrease sharply. The results of this study has confirmed the fact that phage not only attack planktonic cultures, but have the ability to propagate in biofilms.

The experiment was repeated, observing the fate of the cells in the biofilm over a prolonged duration. It was observed that the number of cells in the biofilm decreased with concomitant increase of phage numbers. However, over the period of observation, there remained viable cells in the biofilm. Therefore it is evident from this study that phage particles replicate within a biofilm, and thus biofilms are a potential reservoir of infection in cheese and casein plants. It is suggested that the incidence of phage in the cheese industry can be reduced by preventing the growth of biofilms in whey and casein transfer pipes.

CHAPTER 1. INTRODUCTION

Bacteriophage infection of cheese starter cultures has always been considered a persistent threat to the dairy industry (Sylvain & Cline, 2004). Bacteriophage are viruses that attack bacteria and kill them to replicate (Ackermann, 2003). Phage are responsible for the malfunction of starter culture and it subsequently leads to the failure of the manufacture of cheese (Everson, 1991). The destruction of the starter culture causes a substantial loss of revenue to the company and thus to the New Zealand Dairy Industry. Howard Heap, the Manager of Starters and Microbiology at the New Zealand Dairy Research Institute (NZDRI) had earlier estimated that it would cost the industry about $100,000 to isolate a new starter strain. If the starter strain is destroyed, the loss of production of a few units to complete loss of a day's make in the production of cheese has been estimated to cost up to $45,000 per day.

Lactic acid bacteria are predominantly used as starters in the manufacture of cheese as they not only enable milk fermentation, but also bring about biochemical changes during the ripening of the cheese, which helps it to develop a significant flavour, (Parente & Cogan, 2004). *Streptococcus thermophilus* is a thermophilic lactic acid bacterium that is of great significance to cheese manufacturers (Annick, 1990). Amongst the other lactic acid bacteria used for the fermentation of cheese, *S. thermophilus* is considered as the dominant strain due to its various traits such as flavor and texture enhancement (Parente & Cogan, 2004). These starter bacteria are often attacked by bacteriophage, which is a major problem for the cheese manufacturing industries (Accolas & Spillmann, 1979). The enzymatic and proteolytic activity of starters is drastically affected by infection of the starter culture with phage, and this results in slow or incomplete fermentation (Whitehead & Cox, 1936). Slow fermentation eventually results in cheese with defects in flavor, texture and quality. (Coffey & Ross, 2002). Therefore, the phage is considered as a crucial factor and meticulous precautions are taken to

avoid phage infection in the dairy plants (Mc Grath et al., 2007). Despite the rigorous efforts, the complete removal of phage seems to be an impossible task, as phage are found ubiquitously in the manufacturing plant and find all possible means of access to the bulk starters. In some cases, the starter culture itself may become a source of phage when it forms biofilms on the surface of stainless steel pipes carrying effluent whey. Biofilms may harbour bacteriophage and may become sites of phage propagation. The release of the phage particles into the bulk fluid (whey) is liable to contaminate the factory and hence threaten starter culture in the vats, being carried in aerosols.

The aim of this study was to determine whether a cheese starter culture was capable of forming a biofilm and if phage were capable of penetrating in the biofilm and initiating an infection. The process followed in this study was first to attempt to isolate starter cultures and phage particles from cheese bought in the market place. This proved difficult and two starter culture/phage pairs were obtained from Fonterra. The ability of the cheese starter *S. thermophilus* to attach to stainless steel and form biofilms was then assessed. This was followed by infecting a 24 h grown biofilm with phage and finally examining the ability of the phage to proliferate within the biofilm. The reduction of viable cells in the biofilm was an indication of phage infection in the biofilm and the decrease in the cell numbers was be monitored at every 6, 12, 18 and 24 hours respectively. The amount of phage particles in the bulk fluid was be monitored over time, showing whether the biofilm could be a constant source of phage in the cheese plant. Understanding the process could lead to a better control of phage infection in the plant and thus reduce starter culture breakdown and monetary loss in the cheese industry.

CHAPTER 2. REVIEW OF LITERATURE

2.1 OVERVIEW OF THE CHEESE MAKING PROCESS

The process of cheese making started about 8000 years ago and now there are about 1000 cheese varieties produced worldwide, each unique in its flavor and texture (Sandine & Elliker, 1970). The process of cheese making involves the combination of four important ingredients such as milk, rennet, starter culture and salt. These ingredients are processed by a series of steps, including gel formation, whey expulsion, acid production, salt addition and a period of ripening (Beresford et al., 2001). While the processing factors such as temperature and handling procedures play a major role in the cheese manufacture, starter cultures play a critical role in the fermentation and ripening process (Nichols & Ineson, 1947).

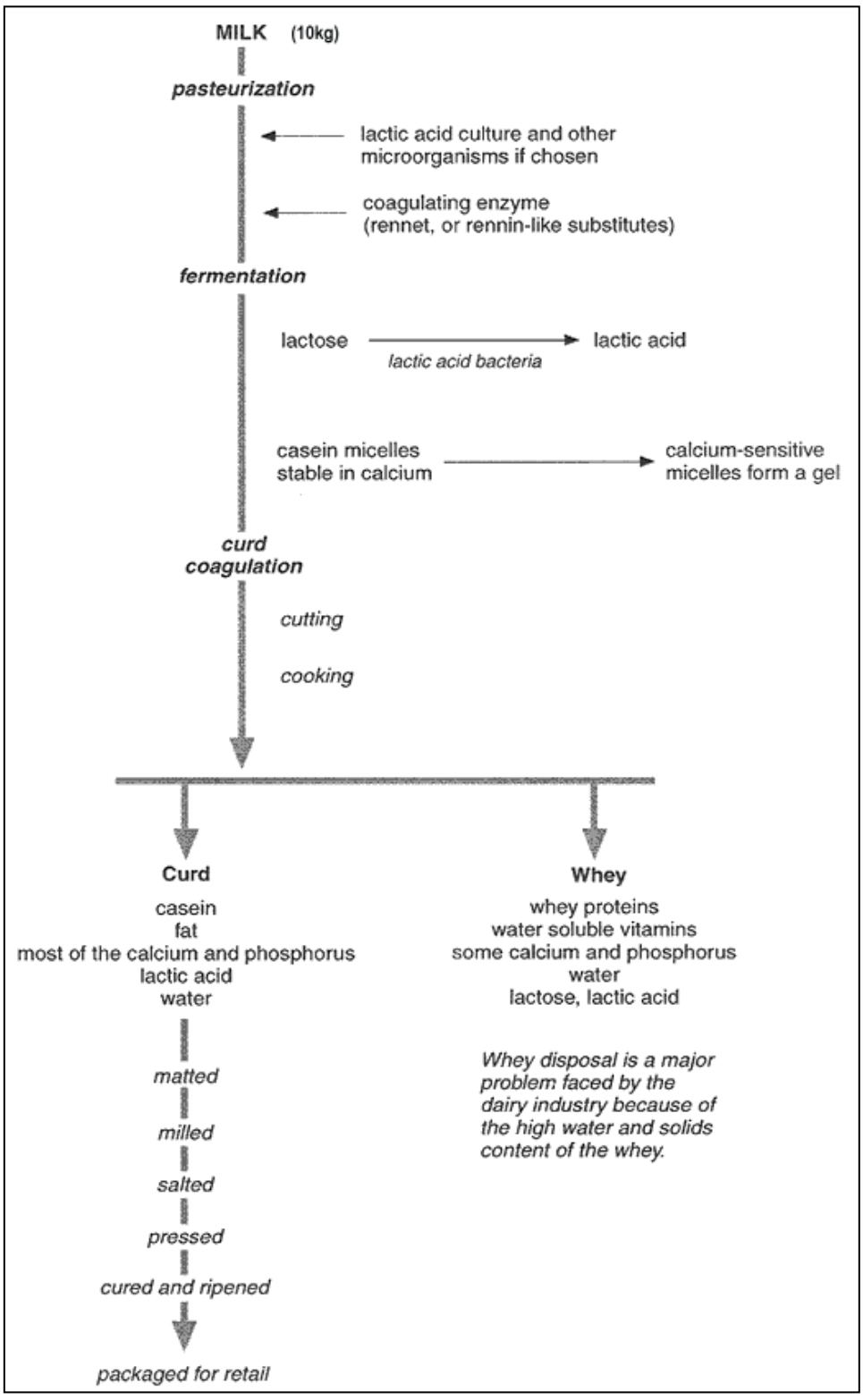


Figure 1: Schematic representation of cheese making process modified from (Mallatou et al., 2003).

2.2 ROLE OF LACTIC ACID BACERIA IN CHEESE PRODUCTION:

Lactic Acid bacteria (LAB) are used extensively in the dairy industry for the production of food products derived from wholesome milk, like cheese, butter, yoghurt, buttermilk and quark (Venema & Kok, 1987). The essential function of the starter bacteria is to produce lactic acid from the lactose present in the milk and hence they are called 'starters' or primary cultures (Parente & Cogan, 2004). During the manufacture of cheese, the milk is inoculated with starter culture and a clotting enzyme (Chymosin and Protease), (Venema & Kok, 1987). After the production of a sufficient amount of acid by the starter bacteria and action of the clotting enzyme, the milk proteins coagulate to produce curd. The function of the clotting enzyme Chymosin is to cleave the milk protein *k*-casein, enabling the formation of curd (Ross et al., 2000). The milk separates into two fractions, the curds and whey, which is expelled from the coagulated protein. The effluent whey is then removed and the curds are salted, causing further shrinkage and expulsion of whey, and finally allowed to mature (Venema & Kok, 1987).

Lactic acid bacteria play an essential role in many food fermentations. Besides carrying out the fermentation, they are also accountable for preservation, flavour, texture and increasing the nutritional attributes of the product (Daly et al., 1996). Some of their well-known traits include proteolysis, production of miscellaneous compounds like diacetyl, which is responsible for flavour and aroma, production of antagonistic compounds which inhibit the growth of taxonomically related or unrelated groups of Gram positive organisms, and autolysing ability, which helps in the ripening of the cheese (Crow et al., 1995). Besides these properties, the lactic acid bacteria have also been claimed to possess therapeutic characteristics, like decreased lactose intolerance, reduction of blood cholesterol, increased

immune response and prevention of cancer (Daly et al., 1998). The important species involved in cheese making include *Lactococcus lactis, Leuconostoc sp*., *Streptococcus thermophilus, Lactobacillus delbrueckii subsp. lactis, L. delbrueckii subsp. bulgaricus* and *L. helveticus,* but not all of them are used in every cheese variety. The first two organisms are used in most cheese varieties, while the rest are used in cheeses like Emmental, Parmigiano, Reggiano and Pizza/Mozzarella cheese varieties, which are heated to a high temperature during manufacture. In many artisanal cheeses, especially those produced in Mediterranean countries, other LAB, including *L. casei, L. plantarum, E. faecalis, E. faecium, L. salivarius,* and *Staphylococcus* species are also used (Parente & Cogan, 2004).

2.3 STREPTOCOCCUS THERMOPHILUS:

The cheese starter culture used in this study is *S. thermophilus.* The genus *Streptococcus* comprises of Gram positive, spherical or ovoid shaped cells arranged in chains or pairs. They are facultative anaerobes, non spore-forming, catalase negative organisms with complex nutritional requirement (Flint et al., 1999). They fall under the category of thermophilic lactic starters. They are used for the preparation of the so-called 'cooked cheeses' i.e. Emmental, Gruyere, Swiss cheese, Sbrinz and also Italian cheese like Gorgonzola or hard cheese called Grana, (Auclair & Accolas, 1983). *S. thermophilus* has a number of properties that distinguish it from other LAB. They are highly thermo-resistant and can tolerate pasteurization up to 72°C for 15 seconds and can grow at temperatures up to 52 $^{\circ}$C (Flint et al., 1999). Another important trait of this organism is the production of extracellular polymeric substances (EPS) which are used to improve the viscosity and texture of the product (Mora et al., 2002). The production of the EPS tends to increase the functionality of Mozzarella cheese by imparting a 'ropy' or viscous texture to the fermented milk (Broadbent et al., 2003).

2.4 CAUSES OF STARTER FAILURE:

Despite all these beneficial properties, one principle problem faced by the cheese manufacturers is that the starter bacteria are mostly susceptible to bacteriophage infection (Brussow et al., 1994). Phage attack has been of great concern in the dairy industry as this results in the lysis of the starter strains in the vats used for cheese production (Binetti et al., 2005). The lysis of the starters causes the delay in milk acidification or sometimes incomplete fermentation leading to partial or total loss of the product (Coffey & Ross, 2002). Interruption of dairy fermentations due to phage have been recognised since the mid-1930s (Whitehead & Cox, 1936). When the starter culture is lysed by a phage, continued incubation of the culture may bring about the growth of a phage resistant form of bacteria, but such resistant or mutant strains have a tendency to produce undesirable results in the cheese, (Whitehead & Hunter, 1941). Due to these implications rigorous methods have been implemented to control phage attack on starters, as the development of a new starter strain is costly (Quiberoni et al., 2006; Whitehead & Hunter, 1941).

2.5 BACTERIOPHAGE:

Bacteriophage (phage) are viruses that infect bacteria (Ackermann et al., 1984). Bacteriophage was independently discovered by Frederick Twort (1915) in England and by Felix d'Herelle (1917) at the Pasteur Institute in France. Felix d'Herelle coined the term "Bacteriophage", "from 'bacteria" and the Greek "phagein" – to eat, and are so called as they can cause the complete lysis of a susceptible bacterial culture. They are commonly referred as 'phage' (Ackermann, 2003). Phage are intracellular obligate parasites that multiply inside the bacterial cell by making use of some or all of the host biosynthetic machinery (Adams, 1953). Most phage are members of the *Siphoviridae* group, and their morphology is composed of a head which is packed with the genetic material and a connected tail structure which enables it to recognize its host. The International Committee for Taxonomy of Viruses (ICTV) classifies viruses into different families of the Order *Caudovirales*. The classification is based mainly on the morphological characteristics, type of nucleic acid and other physic-chemical characteristics.

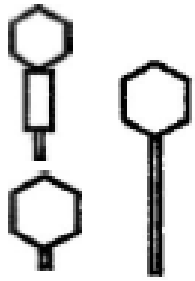
FAMILY	NUCLEIC ACID	CHARACTERISTICS	MORPHOLOGY
Myoviridae	Linear ds DNA	Non-enveloped, contractile tail	
Siphoviridae	Linear ds DNA	Non-enveloped, long non-contractile tail	
Podoviridae	Linear ds DNA	Non-enveloped, short non-contractile tail	
Corticoviridae	Circular ds DNA	Non-enveloped, isometric	
Tectiviridae	Linear ds DNA	Non-enveloped, isometric	
Lipothrixviridae	Linear ds DNA	Enveloped, rod-shaped	
Plasmaviridae	Circular ds DNA	Enveloped, pleomorphic	
Rudiviridae	Linear ds DNA	Non-enveloped, rod-shaped	
Fuselloviridae	Circular ds DNA	Non-enveloped, lemon shaped	
Inoviridae	Circular ss DNA	Non-enveloped, filamentous	
Microviridae	Circular ss DNA	Non-enveloped, isometric	
Levivirdae	Linear ss DNA	Non-enveloped, isometric	
Cystoviridae	Segmented ds RNA	Enveloped, spherical	

Table 1: ICTV classification of viruses (Van Regenmortel et al., 2000).

2.5.1 LIFE CYCLES OF BACTERIOPHAGE

Phage are host specific and can attach only to receptor sites of specific host cells, (Neve et al., 1989). In order to propagate they must attach to specific host receptors on the surface of the host bacterial cell and inject the DNA into the host cytoplasm. The replication of phage depends on various factors like durability of the virus particle, breadth of the host range and details of the infection strategy. Accordingly phage fall under two categories based on their mode of replication, they are lytic (or virulent phage) and temperate (or lysogenic) phage (Sharp, 2001). For a better understanding of the phage implications on the starter bacteria, it is first necessary to have a basic knowledge of the phage life cycles, which are as follows

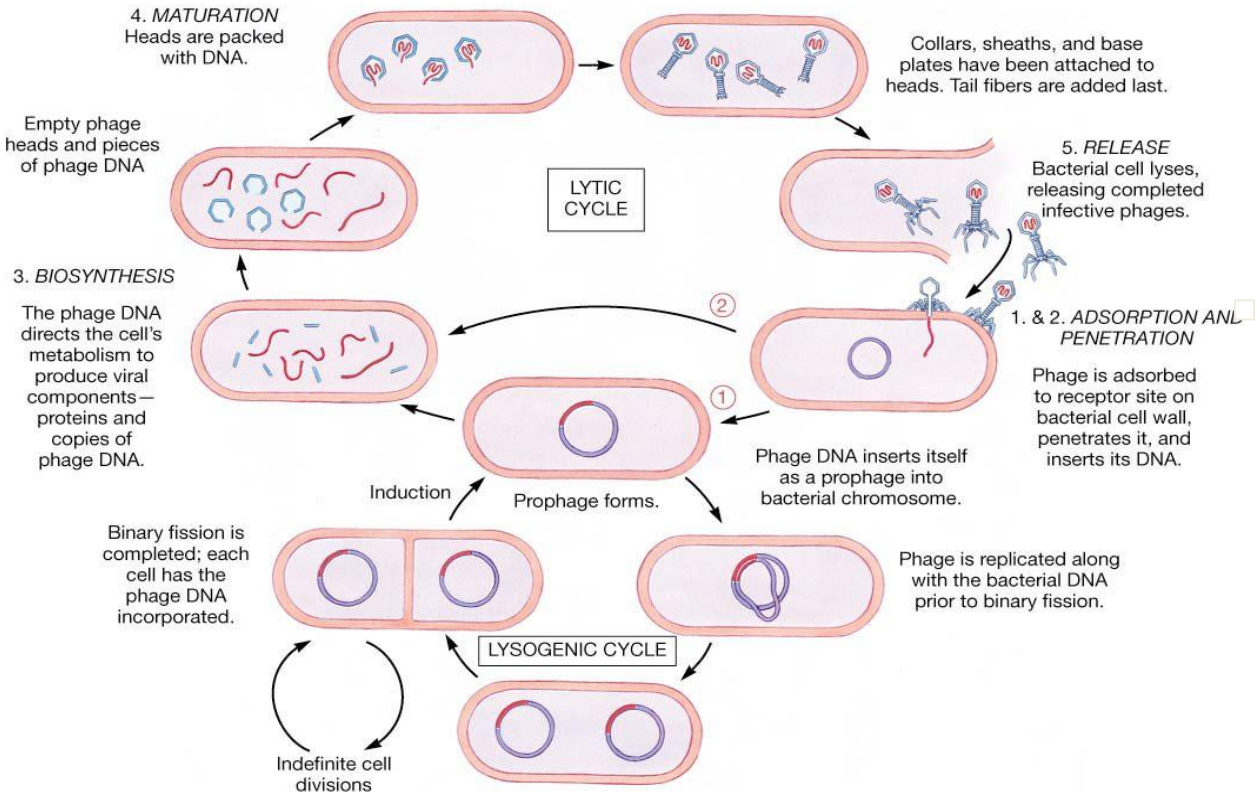


Figure 2: Life cycles of bacteriophage (Blair, 1959)

2.5.1.1. LYTIC CYCLE

Lytic phages are those that undergo the lytic life cycle. These are so called, as they replicate inside the host bacterium and eventually cause destruction of the cell by lysis, which occurs at the end of the phage reproductive cycle (Adams, 1953). During the onset of infection, the phage adsorbs onto the surface of the host bacterial cell and injects its DNA into the cytoplasm of the host cell in a linear form. Once the phage DNA enters the host cell, it directs the host's biosynthetic machinery to produce phage components, such as phage proteins and nucleic acids (Sharp, 2001). During this process, many copies of phage DNA are synthesized. This is then followed by formation of head and tail components. Once the heads are formed, the phage genome is packaged into the head and the tail components such as collar sheath, base plate and the tail fibres are attached to form a complete virion particle. This process is called "maturation". The mature phage particles are then released by lysis of the bacterial cell (Ackermann et al., 1984).

2.5.1.2. LYSOGENIC CYCLE

During the lysogenic cycle, the phage enters a period of dormancy inside the host bacterial cell, and in most cases, the phage genome integrates into the bacterial chromosome. The phage DNA is replicated along with the host chromosome to produce a dormant bacteriophage called a 'Prophage' (Kutter, 2001). At this stage, the phage genome exists in a repressed state i.e. the phage genes are not transcribed. This is enabled by a phage genome coded repressor. Once the repressor is relieved through the process of induction, the phage genome is excised from the host chromosome and enters the lytic cycle, resulting in phage replication and ultimate destruction of the host. The host bacterial cell concealing a prophage is not adversely affected by the presence of the prophage and the lysogenic state may persist indefinitely. The cell harbouring a prophage is termed a lysogenic strain. Phage are also capable of transferring genetic material from one host to another during replication. This process is referred to as 'Transduction' Some phages represent their prophages as plasmids, which are replicated independently and passed on to the bacterial daughter cells during bacterial division (Blair, 1959).

2.5.2 SOURCE OF PHAGE IN THE DAIRY INDUSTRY

The problem of phage was first noted during the 1930's when the cheese industry of New Zealand experienced starter failure as the bulk starter in the vats used for making cheese failed to clot, or if clotted, failed to produce the required amount of acid. This phenomenon was referred to as 'pack up' by Whitehead (Whitehead & Cox, 1936). The reason for starter failure was airborne infection of the bulk starter by bacteriophage and the source of the phage infection was probably spray from the whey separators. When the required amount of protection from phage was provided, the slowness in cheese making disappeared, indicating that phage were the probable source of starter failure (Whitehead & Hunter, 1941). Phage are omnipresent in the dairy industry. One important source of phage is the presence of phage in bulk raw milk used for curd manufacture. The phage particles may also be present at high levels in the effluent whey (Anderson & Meanwell, 1942). Of course, this is an old reference and modern control techniques have reduced the risk of contamination from whey handling. However, if phage infection occurs in the starter tanks or cheese vats, whey is still a likely route for spreading infection in the plant. Phage particles diffuse in the air as aerosols and infect the bulk starter cultures that are used for milk fermentation (Everson, 1991). The reasons for the occurrence of high levels of phage in whey and curd are stated below.

Causes of high level of phage in whey and curd, modified from (Everson, 1991)
➢ **The utilization of one centrifuge for whey and milk separation**
➢ **The presence of pumps and fine savers carrying whey and casein in the areas of raw milk silos, pasteurization equipment and milk vats.**
➢ **The location of starter tanks in the cheese vat room and the entrance of phage into tanks through air replacement during cooling and recycling.**
➢ **Separate facilities for starter cans that have not been pasteurized, movement of air from whey handling areas to raw milk storage or cheese vat areas.**
➢ **Storage of whey in the lines connecting the vats, i.e. failure to drain whey lines**
➢ **The floor drains in the starter room and drains in the cheese making room improperly flushed and sanitized during specific intervals of the day.**
➢ **Storage of starter cultures in the lines connecting the vats or the lines from the pasteurizer and starter room. The lines should be kept to minimum length and flushed properly**
➢ **Improper cleaning of cheese vats between the refills and lack of sanitizer usage**

Table 2: Causes of high level of phage in whey and curd

2.6 BIOFILM FORMATION: A POTENTIAL SOURCE OF PHAGE CONTAMINATION:

Researchers have identified several possible points of entry of phage into dairy plants and several studies have been performed on the planktonic forms of starter cultures. Isolations of *S. thermophilus* and lactococcal phages from dairy environments have been conducted to study their morphological and genetic characteristics (Bruttin et al., 1997). Through this work, several genetically modified and phage resistant strains of starters have been synthesized in order to reduce phage infections (Accolas & Spillmann, 1979; Annick, 1990; Batt et al., 1995; Binetti et al., 2005; Brüssow et al., 1998; Deane et al., 1953). Despite of all these attempts, phage attack on starter cultures seems to be inevitable. One possible source of phage is biofilm formed by the starter culture in the whey-carrying lines in cheese and casein plants. Protracted storage of the whey in the lines and the capacity of the starter bacteria to attach to the stainless steel surfaces may be considered as the reason for biofilm formation (Flint,Brooks, et al., 1997). Bacterial biofilm formation in cheese and food processing plants have been the focus of some reviews (Amy C, 1998; Brooks & Flint, 2008; Flint,Brooks, et al., 1997; Kumar & Anand, 1998) but still has not received much attention to the aspect of being a potential reservoir of phage, thus creating a gap in the knowledge.

2.6.1 BIOFILMS- INTRODUCTION

There exist different definitions for biofilms. In general, biofilms can be defined as "agglomerations of microbial cells and their excreted products attached to inert surfaces" (Sutherland et al., 2004) though this is by no means an exclusive definition. According to Donlan et al., (Donlan & Costerton, 2002), a biofilm is a sessile community of cells that are irreversibly attached to a substratum or interface, embedded in a matrix of extracellular polymeric substances produced by them, and, as a result, exhibit a change in their phenotypic characteristics with respect to growth rate and genetic transcription. Bacterial cells adopt the biofilm mode of growth as a survival strategy to resist factors such as environmental stress, antibacterial agents, heat and attack of bacteriophage (Carpentier & Cerf, 1993). The formation of these sessile communities and their inherent nature to resist antimicrobial agents are the root of many persistent and chronic infections (Hall-Stoodley et al., 2004). The advantages of living in a sessile community include increased availability of nutrients, efficient binding of water molecules, reduced level of dehydration and enhanced multiplication (Costerton et al., 1987).

2.6.2 DEVELOPMENT OF BIOFILMS

Biofilm formation begins with the adsorption of macromolecules (proteins, polysaccharides and humic acids) and smaller molecules such as fatty acids and lipids to the surface. The adsorption of the molecules causes the development of a conditioning film, which alters the physiochemical characteristics of the surface, including surface hydrophobicity and electrical charge (Brooks & Flint, 2008). However, it is possible for cells to colonise a surface in the absence of a conditioning film (Flint et al., 2001). This is followed by adherence of the cells to the surface. Once the cells adhere to the surface, they modify their planktonic gene expression to biofilm phenotype according to the growth environment (Busscher & van der Mei, 2012).

The adhesion of cells is followed by the secretion of extracellular polymeric substances and the organisms start to multiply within the matrix. This is called colonization. The process of colonization results in the formation of a biofilm (Carpentier & Cerf, 1993). This matrix protects the cells within it and facilitates their communication through biochemical signals known as quorum sensing molecules. These molecules help to synchronize the activities of large groups of cells. This process is called quorum sensing (Waters & Bassler, 2005). The spider web-like nature of the EPS matrix has the capacity to trap virus, bacteria and other antimicrobial agents before they start to act on the film (Costerton et al., 1987). However, the biofilm formed in the dairy lines are only a few cells thick and therefore can easily succumb to the action of phage (Brooks & Flint, 2008). The negatively charged molecules present inside the EPS matrix are capable of attaching to the biocide particles and there-by confer upon the film resistance to antibiotics and other antimicrobial agents (Mah & O'Toole, 2001).

The presence of some resistant cells blocks the particles from further penetrating into the biofilm matrix (Hall-Stoodley et al., 2004). Several factors, such as surface roughness, texture and cell surface properties of the starter culture bacteria greatly influence the formation of biofilms on stainless steel. Among these, the surface roughness is likely to play an important role in enhancing biofilm attachment and growth (Boulangé-Petermann et al., 1997). (Flint,Brooks, et al., 1997) have proposed that the surface properties of the bacterial cells also play a major part in influencing attachment and film formation. Another important factor aiding biofilm growth of thermophilic streptococci is their thermoduric nature. The ability of the bacteria to withstand high temperatures enables them to produce biofilms in heat exchanger plates of the milk pasteurisation unit in a cheese plant (Driessen, 1980).

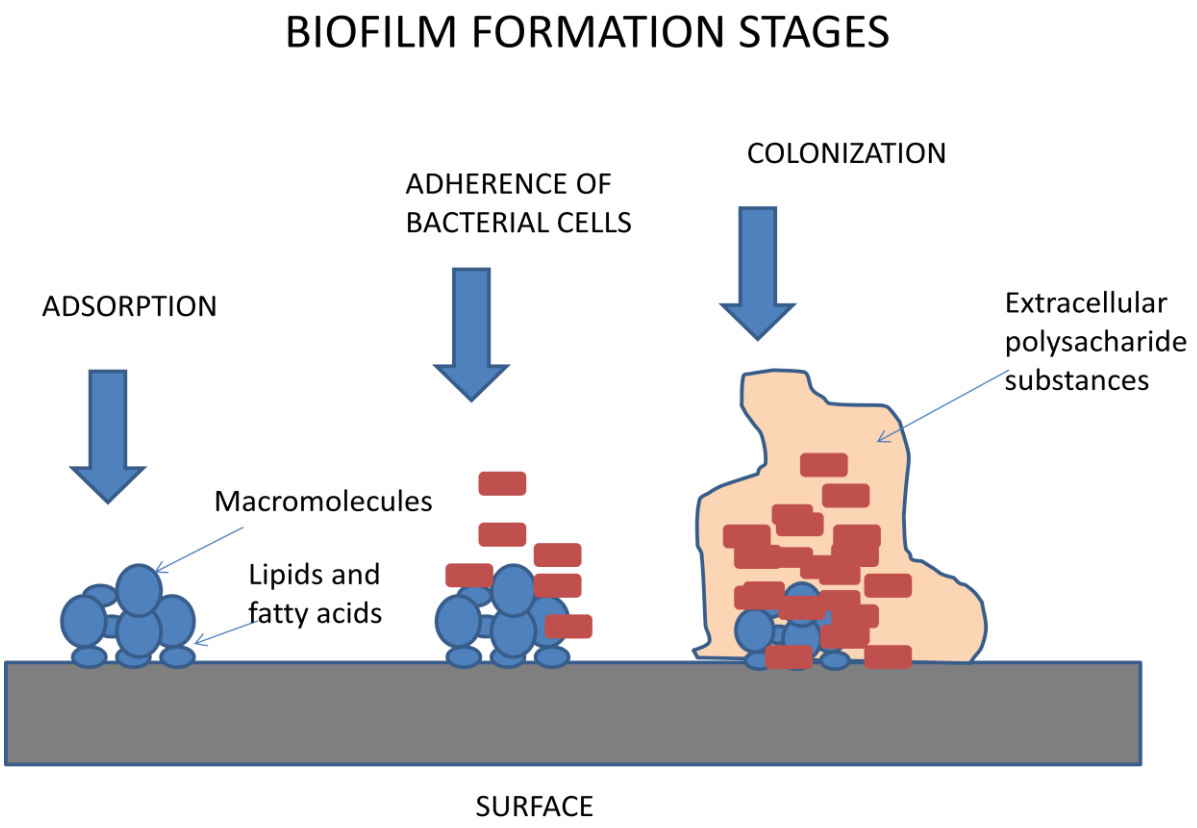


Figure 3: Stages of biofilm development. (Biofilms formed in the dairy may be only a few cells thick).

2.6.3 IMPACT OF BIOFILM FORMATION ON DAIRY MANUFACTURE

Bacterial adhesion and biofilm growth on surfaces in industrial settings, such as whey lines, can cause deterioration of cheese production and great economic loss to the dairy industry (Flint et al., 1999). In the case of lactic acid bacteria (LAB), their worldwide use in the food industry raises concerns about their capacity to adhere to stainless steel surfaces and to form biofilms, which may not only affect their performance in fermentations but may tend to become a source of phage in the cheese industry (De Jong et al., 2002). Besides causing contamination of cheese, biofilm formation causes blockage of the whey carrying lines, corrosion of stainless steel, reduction in plant run time and heat transfer in the plate heat exchangers (Parkar et al., 2003). In addition to stainless and Teflon®, which are common equipment materials in the industries, biofilms are found colonising packaging and other equipment surfaces like plastic, rubber, glass, wood etc, and can also be found in food stuffs (Somers et al., 2001). Furthermore biofilms might become reservoirs of bacteriophage, and the release of progeny virus particles from the film may cause contamination of the bulk starter culture in the tanks during spillage and handling. Wiggins et al., (1985) have reported that biofilm are preferred sites of propagation for less accessible bacteria (planktonic forms) present in liquid culture (Wiggins & Alexander, 1985). During industrial processing, it is often difficult to detect the location of biofilm colonization, which makes the elimination process a challenging one. Therefore it is first vital to understand the relationship of the film and the surfaces on which they are found in order to implement effective measures of biofilm removal (Lee Wong, 1998). One of the most significant methods followed by dairy manufactures to control biofilm formation is the clean-in-place (CIP) method which was

followed originally in the cheese plants. A typical cleaning programme included a fresh water rinse, followed by treating with 1.5% sodium hydroxide solution at 70°-80°C, then another rinse with water, then treated with 0.5% nitric acid at 60°C, again rinsed with water and finally treated with 2 ppm sodium hypochlorite at 10-20°C (Flint,Brooks, et al., 1997)

2.6.4 CULTURING BIOFILMS IN VITRO:

There are several in vitro systems for cultivation of biofilms. One of the traditional methods was the development of the Robbins Biofilm Sampler, which is a modified form of the Robbins device. The sampler was designed with evenly spaced ports through which the test surfaces may be inserted during biofilm growth (Nickel et al., 1985). A Constant Depth Film Fermentor (CDFF) was also developed to study the viability and acidogenicity of biofilm cells (Deng & ten Cate, 2004). The developments of perfused disc reactors, annular reactors and systems that can be operated under continuous flow environments such as the rotating disc reactor were also effective. Though these methods were useful for cultivating biofilms, there were several drawbacks, such as time consumption and lack of standardisation. Therefore to overcome this, a Calgary Biofilm Device (CBD) was constructed. The CBD comprised of a two-part device. The top component contains the lid containing 96 pegs. The top is sealed so that the pegs can be removed without opening the lid. The pegs were designed in such a way that they fit into the wells of the 96-well plate. The bottom component allows the flow of medium through the pegs and produces a shear force to enable the formation of biofilms in each peg (Ceri et al., 1999). The CDC biofilm reactor (CBR) was developed to study the structure of biofilms under conditions of moderate to high shear in batch or continuous flow systems. The CDC biofilm reactor can be used for growing repeatable biofilms under constant shear using different organisms (Goeres et al., 2005)

TECHNIQUE	PRIMARY USE	LIMITATIONS
Capillary reactor	Biofilm is formed on a glass capillary. Direct microscopic visualization can be done	Biofilm growth was limited to a single surface
Flow cell reactor	Biofilm structure can be studied under either laminar or turbulent flow to stimulate the changes in fluid velocity that take place during industrial operations	The design of the reactor did not solve the purpose
Robbin's device	Used in industrial biofouling. A brass pipe is used to remove sections of the wall of the reactor and tested for biofilm growth	Only one type of material could be tested at a time
Modified Robbin's device	Allows several materials to be tested at a time	Was not suited for performing genetic investigations
Calgary biofilm device	Used for performing rapid and reproducible assays in biofilm susceptibility to antibiotics	None for the purpose the method was designed
Rotating disc reactor	Different biomaterials could be used for colonization and the shear forces can be controlled	High variation in biofilm formation between samples
Rotating annular reactor	Uses a well-defined shear field	Did not solve the purpose
Batch and batch-fed growth reactor	Used for a wide range of biofilm experiments	High variation in biofilm formation between the samples
Constant depth film Fermentor	Used in the study of biofilm susceptibility to antimicrobials in multispecies biofilms	To maintain a steady state, the biofilm has to be grown in a chemostat
CDC biofilm reactor	Used to study biofilms under controlled shear conditions, characterize biofilm structure and examine the susceptibility to antibiotics	The baffle speed has to be controlled carefully

Table 3: The different biofilm growth techniques and their limitations, (McLandsborough et al., 2006).

2.6.5 ENUMERATION OF CELLS IN THE BIOFILM

Impedance microbiology is considered as a convenient and reproducible method of estimating the number of viable cells colonising on the surface (Flint et al., 2001; Kozáková et al., 2005). The basic principle of impedance microbiology is the measurement of changes in the molecular nature and electrical conductivity of the culture medium produced due to the growth of bacteria (Silley & Forsythe, 1996). The growth of bacteria in the culture medium causes the modification of the chemical composition of the medium due to the production metabolic products that alter the ionic content of the medium. This results in conductivity and capacitance changes in the growth medium. The rates of change of conductivity and capacitance are proportional to the concentration of viable organisms. The increase in the change of conductivity and capacitance causes a decrease in the electrical impedance as the organisms multiply. These changes are detected by impedance monitors such as the BacTrac®4300 system.

When the microbial population reaches a threshold level of 10^6 to 10^7 cells per millilitre, an exponential change is observed in the conductance and capacitance signals. The time required by the bacteria to produce this exponential change is inversely proportional to the initial cell concentration and the growth kinetics of the bacteria in the given medium. This is called the impedance detection time (DT). The impedance system needs to be calibrated to the specific organisms used in the study.

2.7 INTERACTION OF PHAGE AND BIOFILM

Studies on phage and biofilm interaction would provide an insight as to whether biofilms may become sites of phage propagation in the manufacturing areas (Abedon, 2011; Doolittle et al., 1996; Hughes et al., 1998; Meng et al., 2011; Tait et al., 2002). These studies confirm the fact that phage are capable of reducing the bacterial populations. Tait et al., (Tait et al., 2002) have reported the fact that after prolonged exposure of bacterial cells to phage, the bacteria and phage started to co-exist in biofilm communities. The propagation of phage in the film takes place through a series of steps. The first step is the adsorption of the phage to the specific receptor of the target bacterial cell. In order for the phage to reach the target site the phage must penetrate through the EPS matrix of the film. Several studies show that phage are capable of diffusing through the EPS matrix by producing several degrading enzymes.

Phage with polysaccharide depolymerase activity was able to degrade the polysaccharide produced by the film and reach the underlying cells (Tait et al., 2002). On the other hand, an experiment conducted on *Lactococcus lactis* by (Deveau et al., 2002) reveals the fact that phage particles do not always produce depolymerase enzymes and the EPS matrix does not confer upon the bacterial cells full resistance to phage entrance. The phage particles may also gain access to the interior of the film through water filled fluid channels and pores which are used for the transportation of nutrients (Sutherland et al., 2004). The presence of the fluid channels and pores were demonstrated using Confocal laser scanning microscopy (CLSM), (Wood et al., 2000), and moreover, the ability of the phage particles to gain access to the interior of the film depends solely on factors such as host bacterial cell, physiological characteristics, the growth environment such as turbulent and linear flow conditions and surface where the film is formed (Sutherland et al., 2004). Once the adsorption

step has occurred, the phage particles direct the hosts' biosynthetic machinery to produce hundreds of progeny phage particles. The progeny virus particles are released by lysing the host cells.

2.8 PHAGE DEFENCE STRATEGIES

Since the time of detection of phage as a major cause of dairy fermentation failure, scientists have been making strenuous attempts to develop strategies to eliminate phage from the dairy environment. The technological advancements in the plant design, starter strain development, use of media that inhibit phage action, maintenance of hygiene and good manufacturing practices (GMP) has led to a decrease in the level of detrimental effects caused by bacteriophage. Despite these efforts, phage are considered to be a persistent threat to the dairy industry, as the starter cultures used for dairy fermentations succumb easily to phage attack making the process of phage elimination a laborious one (Mc Grath et al., 2007). The most recent inventions in phage therapy include genetic modification of starter strains by incorporating resistance mechanisms, production of novel cultures for cheese manufacture, and utilization of phage inhibitory media (Coffey & Ross, 2002; Lucchini et al., 2000; Mc Grath et al., 2007; Ross et al., 2000; Sylvain & Cline, 2004; Venema & Kok, 1987)

2.8.1 TRADITIONAL STRATEGIES

A) STARTER STRAIN ROTATION

Phage infecting the starter culture *S. thermophilus* was detected and isolated from the cheese manufacturing areas and was tested for its host range and serological properties in order to develop rapid methods for the detection of phage in dairy manufacture, (Brüssow et al., 1998). The starter strains for dairy fermentations are usually selected by utilizing strains which are unrelated with respect to phage type and possessing most defined fermentation characteristics. During strain rotation, starter cultures are replaced with more non-lysogenic and phage unrelated strains possessing similar fermentation qualities. Although strain rotation is considered as an important means of defence from phage infection, it is documented that the frequent use of large numbers of phage unrelated strains at one time would increase the size of the available gene pool and might stimulate the evolution of new virulent phage by mutation or recombination (Ackermann, 2003). The process of 'starter recovery' was used where a multiple strain starter was used in the process of cheese making; though the main starter strain was lysed by phage leading to retarded acidification in the cheese vat, the starter soon recovered owing to the increase in acid production by other strains, (Nichols & Ineson, 1947). In recent years, the use of multiple-strain starters has been introduced as an alternative to traditional rotation programs. Multiple-strain starters are composed of three to six selected strains that are used continuously in the plant as part of the phage-monitoring program. The success of the multiple-strain starter is reliant on the selection of phage-unrelated strains that are resistant to attack by phage present in plants, (Sylvain & Cline, 2004).

B) PHAGE INHIBITORY MEDIUM (PIM)

The use of phage inhibitory medium (PIM) was widely adopted in the late 1970's to limit the entrance of phage in the bacterial cell. The medium was composed of phosphates and citrates, in addition to bacterial growth promoting ingredients. The phosphates and citrates bind to calcium ions which are essential for phage proliferation. The absence of calcium prevents the binding of phage to the bacterial cell. In the late 1990's Sandine and co-workers at the Oregon State University developed a most advanced form of PIM called 'Economy 6'. This medium incorporated with phage inhibitory systems and an internal buffering system that controlled the pH of the bulk culture and ensured that the pH was always maintained at 5.0 (Coffey & Ross, 2002).

C) IMPROVING SANITATION AND MANUFACTURING PRACTICES

Several manufacturing plants practice the open cheese vat system. Therefore, during the processing of milk and whey, there are several opportunities for spillage. The phage particles form aerosols by air displacement on the surface of the contaminated fluids (Everson, 1991). Hence, the virus particles are transferred throughout the manufacturing plant. Therefore, sterilizing the air used for cooling and sampling is one way of controlling phage infection (Sylvain & Cline, 2004). The implementation of good manufacturing practices (GMP) is an essential method of controlling phage infections in the manufacturing plants. Improved plant design such as using enclosed cheese vat systems, segregation of starter and cheese processing rooms, location of whey storage tanks and whey processing systems away from the cheese vats can reduce the entrance of phage in the cheese vat room.

Direct vat inoculation (DVI) is also an effective method of preventing phage attack of the starter culture. This is done by inoculating the cheese milk directly using starter culture concentrates rather than using the bulk starters used for cheese manufacture. This method is used by those industries which do not have the resource to produce phage-free bulk starters (Sturino & Klaenhammer, 2004). Other methods of phage control in the cheese plant include cleaning of vats between refill intervals, segregation of starter room and cheese processing equipment, superior sanitization procedures, removal of deposits on the starter vessel, and, careful handling and disposal of whey have also been effective phage control measures (Daly et al., 1996).

2.8.2 MOLECULAR STRATEGIES

A) ADSORPTION INHIBITION

The advances in the areas of phage genetics and molecular biology have provided a more sound evolutionary basis for the detection of phages (Jarvis, 1989). One of the important factors to prevent phage attack is inhibiting the adsorption of the phage to the surface of the host bacterium. This restricted the phage from entering into the cell but as the phage was unaffected, it could subsequently attack a sensitive host (Colin, 1993). The development of bacteriophage insensitive mutants (BIM) due to spontaneous mutations enables the bacterium to resist phage adsorption. The resistance mechanism was also transferable from one organism to another through multiple plasmid encoded systems. (Colin, 1993). Coffey et al., (2002) reported the presence of genetic material on the surface of the host bacterium was also responsible for the adsorption of the phage on the surface of the host bacterium. This problem was overcome by introducing an anti-receptor gene in the host bacterium using the anti-receptor gene sequencing method, to prevent the adsorption of the phage to the bacterial cell (Binetti et al., 2005).

B) PHAGE INGECTION BLOCKING

In this method the injection of the phage DNA is blocked by cloning the host cell plasmid with fragments from insensitive variants. The fragment encoded plasmids prevented the entry of phage DNA into the host cytoplasm and therefore resulted in abortive infection. (Colin, 1993). The presence of the proteins encoded by PIP gene prevented the injection of Phage DNA causing the rapid ejection of the phage genome.

C) RESTRICTION MODIFICATION SYSTEMS

This is an effective method of phage defence where the phage entrance into the host cell is restricted by the action of host encoded endonuclease enzyme. Therefore, the host cell remains unaffected. The drawback of this method is that under rare circumstances, the methylation of the recognition sites of the phage before being subjected to restriction systems may allow the proliferation of the phage inside the bacterial cell (Sturino & Klaenhammer, 2004).

D) ABORTIVE INFECTION

In this method the phage infection abortive systems are incorporated intracellular in the host cells to prevent the proliferation of phage inside the cell. This system is not very effective as it results in cell death due to phage attack but it limits the number of progeny phage particles released from the host cell (Allison & Klaenhammer, 1998).

2.8.3 GENETICALLY ENGINEERED STRATEGIES

Researchers have isolated a number of phage strains infecting starter cultures to deepen their knowledge about the phage activity in order to invent innovative phage control measures using genetic tools (Accolas & Spillmann, 1979; Brussow, 2001; Brüssow et al., 1998; Brussow et al., 1994; Bruttin et al., 1997; Deane et al., 1953; Jarvis, 1989; Kivi et al., 1987; Neve et al., 1989; Quiberoni et al., 2010; Ross et al., 2000; Sturino & Klaenhammer, 2004; Ventura et al., 2002). The various control measures by incorporation of genetic engineering include:

A) PHAGE ENCODED RESISTANCE (PER)

The origin of replication site (*ori*) of phage is placed in a multi-copy plasmid inside the host cell. When phage infects the cell, the phage replication factors are titrated by the multi-copy *ori* of the plasmid leading to plasmid induction. This causes the production of several copies of plasmid as well as an increase in the resistance, resulting in reduced phage DNA proliferation

B) ANTISENSE RNA SYSTEMS

This is also considered as an effective method of phage resistance. Antisense RNA is produced when a promoter is directed to transcribe the non-coding strand of a gene. This antisense RNA binds to the sense RNA strand and produces a highly unstable hybrid RNA which is rapidly degraded. This system is used to control plasmid copy number, regulate transposition and control bacteriophage development.

2.9 OUTLOOK OF THESIS

Bacteriophage infection of the starter cultures has always been a problem to the dairy industry, as it results in fermentation failure. Biofilm formation on the other hand compromises the hygiene standards of the industry as biofilms might act as sites for phage propagation. Much research has been focussed only on phage attack on planktonic forms of the starter cultures. This study focuses on the propagation of phage in a biofilm of a lactic acid bacterium, *S.thermophilus*, thus providing evidence as to whether phage can propagate in a biofilm and thus potentially threaten the cheese making process.

CHAPTER 3. MATERIALS AND METHODS

3.1 STARTER CULTURE AND PHAGE

S. thermophilus (ST45) culture and its phage ϕ5051 were obtained from Fonterra Research Centre (Palmerston North).

3.2 CULTURE MEDIUM

The bacterial culture was sub-cultured in M17 medium (Terzaghi & Sandine, 1975). M17 agar composition (5.0 g of Phytone peptone; 5.0 g of polypeptone; 2.5 g of yeast extract; 5.0 g of beef extract; 5.0 g of lactose; 0.5 g of ascorbic acid; 19 g of β-disodium glycerophosphate; 1 ml of 1.014 Mg $SO_4.7H_2O$; 1 litre of deionised water) M17 broth (42.5 g in 1000 ml deionised water; pH 6.8–7.0) M17 soft agar (42.5 g of M17 broth; 5.0 g of M17 agar; 1000 ml deionised water) Autoclaved for 20 minutes at 121°C.

3.3 CULTURE MANAGEMENT

The cultures obtained were maintained as museum, stock and working cultures. Museum cultures are the cultures which were received at the start of the project. They were preserved at -80°C immediately after receiving them. The museum cultures were treated with 15% glycerol before storage. The stock cultures were prepared from the museum cultures. They were kept in mid-term storage at -20ºC with 15% glycerol. The stock cultures were prepared in 50 vials (1 ml capacity) containing 1mL aliquots. The working cultures were sub-cultured from the stock cultures and stored in cold rooms at 4°C for daily use.

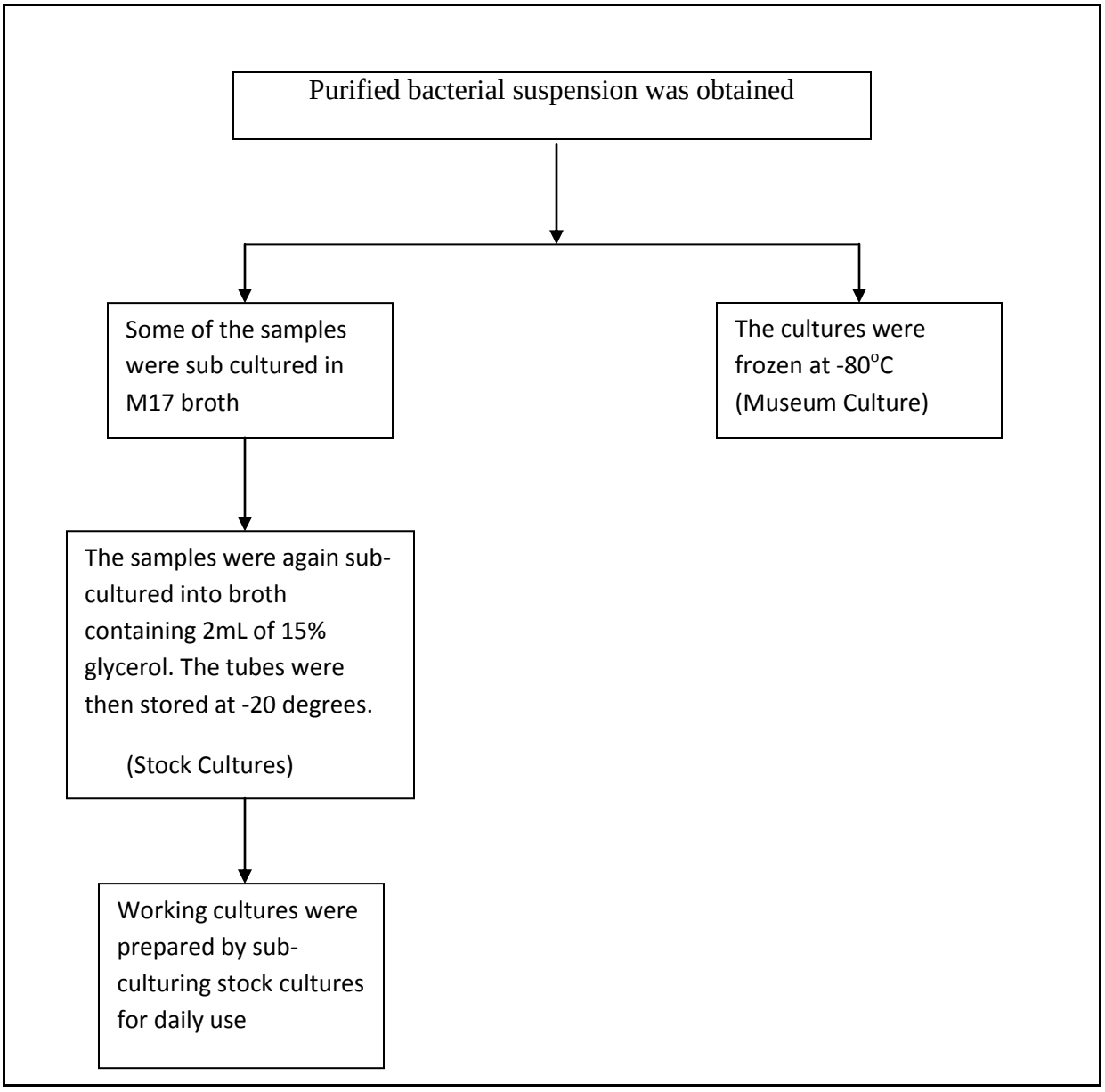


Figure 4: Culture Management. The museum cultures were stored at -80°C, the stock cultures were preserved at -20°C and the working cultures were kept in cold rooms at 4°C for daily use. All the cultures were treated with 15% glycerol before storage.

3.4 MICROTITRE PLATE ASSAY

The microtitre plate assay was based on the method described by (Oh et al., 2007) using 96 well flat-bottomed polystyrene microtitre plates (Falcon Microtest^{TM} 96) and polystyrene tissue culture treated by (vacuum gas plasma) microtitre plates (Falcon Microtest^{TM} 96). The wells were filled with 184µl of M17 broth (Terzaghi & Sandine, 1975). Uninoculated M17 broth (200µl) was included as the negative control. A 48 h culture of *S. thermophilus* ST45 (16µl) was added to the each well and incubated at 30°C for 24 hours. The contents of the plates were removed after incubation and the wells washed three times with 250µl of distilled water. The attached cells were fixed with 200µl of methanol per well for fifteen minutes. The methanol was discarded and the plates were left to air-dry. The drying process was accelerated by placing the plates in a 35°C incubator. The wells were stained with 200µl of 0.05% (w/v) crystal violet for five minutes. The stain was rinsed off under a gentle stream of running deionised water and the plates air-dried. The dye in the attached cells was resolubilized with 200µl of 33% (v/v) glacial acetic acid per well. The absorbance of each well was measured at 550nm and 595nm using a microtitre plate reader (BMG Labtech^{TM}, FLUOstar omega Microplate reader, Imgen Technologies, USA).

3.5 ENUMERATION OF S. THERMOPHILUS (ST45) USING IMPEDANCE DETECTION WITH BACTRAC® 4000:

Impedance microbiology was selected to enumerate numbers of cheese starter culture *S. thermophilus* ST45 using a BacTrac® 4000 (Sy-Lab, Austria). It was operated by setting the temperature of incubation and the threshold level for detection prior to each experiment, (Flint & Brooks, 2001)

3.5.1 Cleaning procedure:

A standard cleaning procedure for the BacTrac® 4000 tubes was developed and tested to determine the reliability of cleaning the tubes, based on the stability of the baseline reading. Twenty tubes were soaked in 95% ethanol for 24 h and the surfaces of the electrodes were swabbed several times with 95% ethanol before being rinsed with distilled water five times. The cleaned tubes were autoclaved at 121°C for 15 minutes. The cleaned tubes were filled (9ml) with M17 broth and incubated for 24 h at 30°C. The objective was to produce a stable baseline impedance reading with which to compare the test samples.

3.5.2 BacTrac® cell standard washing procedure:

The cells were autoclaved after the experimental run to kill all the bacteria. Each individual cell was brushed gently in hot soapy water with detergent. The detergent residue was washed off completely and the cells were filled with 5ml absolute ethanol (sufficient to cover electrodes) and soaked overnight. The cells were washed again with deionised water and autoclaved again before carrying out the experiments.

3.5.3 Cleaning and passivation of coupons

Circular stainless steel coupons (316 grade, 2b finish, 12.7mm diameter x 4mm thickness were obtained. The coupons were soaked in Trigene solution overnight then followed with a dairy industry standard caustic-acid wash procedure: rinsed coupons were heated at 75ºC in 2% NaOH for 30 minutes. Then the coupons were rinsed in deionised water before the acid cycle. The rinsed coupons were heated at 75ºC in 1.8% nitric acid for 30 minutes. The coupons were then washed 5 times in deionised water and autoclaved at 121ºC before being used for experiments.

3.5.4 Plate count:

Plate counts were performed using the spread plate technique, by inoculating 0.1 ml aliquot of the required dilution to the plate and spreading with a sterile glass spreader. Where possible, counts were done using plates containing between 30 and 300 colony forming units.

3.5.5 Calibration of BacTrac® growth analyser

A single colony of the starter culture (*S. thermophilus*) ST45 was inoculated into 10 ml M17 broth and incubated at 30°C for 48h. After incubation, the culture was agitated on a vortex mixer to make the suspension homogeneous before taking 1 mL of the culture and transferring to another bottle containing 9 ml M17 broth to make a dilution of 10^{-1} dilution. The process was repeated to provide a series of 10-fold dilutions down to 10^{-7}. A 0.1 ml aliquot of each dilution was surface plated in duplicate onto M17 agar and the plates were incubated at 30°C for 24 hours. Simultaneously, 1ml from each dilution was transferred in duplicate into BacTrac® 4000 cells containing 9ml M17 broth and incubated at 30°C for 24 h in the analyser to determine the detection time for the growth of the cells. Using the software

incorporated into the BacTrac® 4000, conductance/ capacitance changes were calculated to obtain calibration curves; so that the initial microbial loads in future samples could be deduced. The calibration curve was determined manually by plotting the $\log_{10}$ CFU/ml versus detection time in hours, (Flint & Brooks, 2001).

3.5.6 BacTrac®4300 calibration curve

A calibration curve was obtained for the ST45 strain using impedance microbiology. The calibration curve obtained enabled the estimation of the numbers of bacteria on the coupons in the biofilm formation experiments. The bacterial count on the coupons were calculated by using the equation

$$\text{Log}_{10}\ \text{CFU} = -0.4042 \times (\text{Detection time})\ \text{h} + 13.19$$

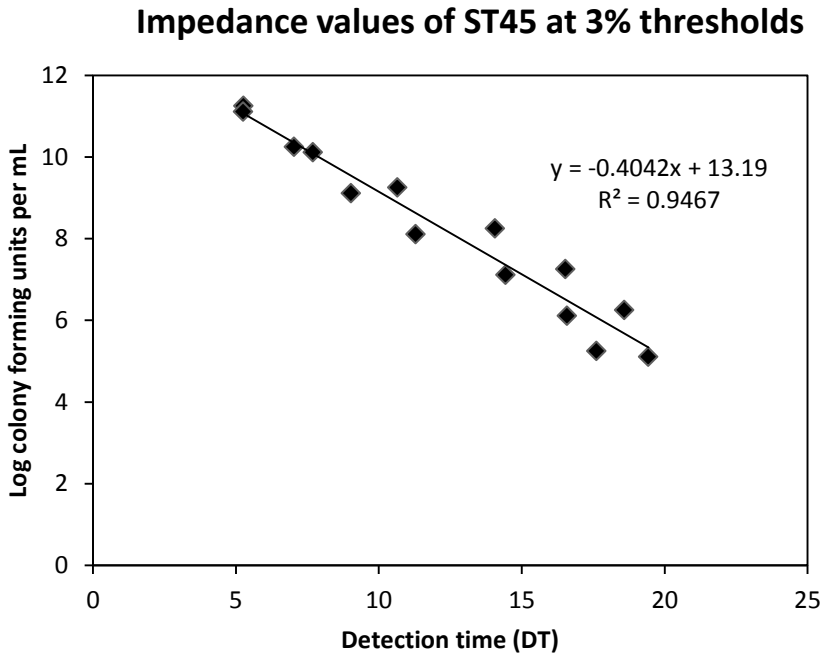


Figure 5: The calibration curve for ST45 strain was generated by BacTrac®4000 impedance monitor using 3% thresholds for the E-value to measure the detection time. The figure shows strain ST45 regression calibration curve (r^2=0.946).

3.5.7 Preparation of phage lysates

Purified suspensions of phage ϕ5051 were obtained from Fonterra as frozen samples. High titre lysates were prepared by inoculating 10 ml of the bacterial suspension in 100 ml of M17 broth and incubating at 30ºC overnight. When the medium turned turbid, 5 mM calcium chloride was added, followed by inoculation of 2% of the purified phage lysate. The medium was then incubated at 30ºC for a further 4-5 hours until the medium had cleared. The unlysed cells were removed by centrifugation at 4000 x g for 10 minutes. The culture was then filtered through a sterile 0.45µm pore, Whatmann nitrocellulose filter to remove the cell debris. The phage lysate suspensions were stored at 4ºC for long-term usage. Precautions were taken during storage of phage lysates and bacterial culture samples to avoid infection of the culture with phage.

3.5.8 Phage titration

Phage titration was conducted using a plating technique. About 4 ml of M17 broth was inoculated with the sensitive host *S. thermophilus* strain ST45 and incubated at 30°C overnight. A dilution series of the phage was prepared, such that there were about 10^3 particles per ml, in one of the dilutions. The seeded top agar (M17 soft agar) was prepared up by pipetting about 2 ml of melted agar into sterile capped test tubes in a 45°C water bath. About 0.1 ml of the bacterial suspension was inoculated in the melted agar. 100 µl of 10^{-3} phage was then added. The contents of the tube were mixed well using a vortex mixer. The contents of the tube were then distributed over a pre-warmed agar plate. Immediately, the plate was tilted back and forth for the contents to spread evenly before it gelled. Once the plate was gelled, it was inverted and incubated at 37ºC for 24 hours. As the culture grew, plaques or cleared areas appeared in the top agar layer. This represented the original bacteria

that had been infected and destroyed by phage. The plaques were counted and the log value of the plaque forming units (PFU)/ ml was calculated using the formula:

Number of plaques formed x Amount inoculated x Dilution factor

Example $230 \times 100 \times 10 = 2.3 \times 10^5$/ ml

3.6 CDC BIOFILM REACTOR SET UP

Twenty four, 2b surface finish, 316 stainless steel coupons. The coupons were cleaned and passivated before being fitted into the coupon holders of a CDC biofilm reactor (Biosurface Technologies, Bozeman). The reactor containing the coupons was filled with 300ml M17 broth and was autoclaved at 121ºC. The speed of the stirrer was set to 140 rpm and the temperature to 30ºC to enable the growth of the bacteria. Every 6 hours, a coupon holder was gently removed from the reactor without disturbing the stirrer. One coupon at a time was removed from the holder and placed in a BacTrac® cell. The BacTrac cell was filled with 9 ml M17 broth and placed in the BacTrac® incubator to produce an impedance curve to assess the number of viable cells present on the surface of the coupons. Simultaneously, a plate count was done by gently placing a coupon in a test tube containing 9mL sterile M17 broth and glass beads. The test tube was shaken on a vortex mixer for five minutes to detach the cells attached to the surface of the coupons. The resulting suspension was used to prepare decimal dilutions in M17 broth to $10^{-7}$and 0.1 ml aliquots of each dilution were surface plated in duplicate on M17 agar medium. The plates were incubated at 30° C in air and counted.

CHAPTER 4.INITIAL BIOFILM ATTACHMENT ASSAY AND BIOFILM FORMATION IN A CDC REACTOR

4.1 Introduction

Streptococcus thermophilus strains are commonly used as starters for the manufacturing of cheese. Knight et al., (2004) have stated that these organisms are capable of attaching to the stainless steel pipes that are used to carry the effluent whey released during the process of cheese production. A preliminary experiment, using a microtitre plate assay modified from (Oh et al., 2007) was performed to test the ability of the organism to attach and initiate biofilm formation. The purpose of this experiment was to monitor the ability of the starter bacteria to adhere to the surface of the wells of the microtitre plate within 4 hours of incubation. This technique indicates that the organism is capable of producing a biofilm and is useful in studying the early stages of biofilm formation including initial attachment and microbial colonisation. This was followed by a study of biofilm growth in a Centres For Disease Control (CDC) biofilm reactor, (Goeres et al., 2005). This experiment was done to determine whether the starter culture had the ability to attach to stainless steel and to produce a biofilm and, if produced, what was the extent of growth after 24 hours. Impedance microbiology, using a BacTrac® 4300 growth analyser, was selected to enumerate the biofilm cells on the stainless steel coupons.

4.2 Experimental Procedures

Biofilm production in a CDC reactor (Batch Culture):

S. thermophilus ST45 culture was grown in a CDC reactor (Goeres et al., 2005). The CDC reactor, fitted with 24 stainless steel coupons and containing 300 ml M17 broth was sterilized in an autoclave prior to the experimental run. The speed of the stirrer was set to 140 rpm and the temperature was set to 30ºC. 1ml of 48 hour *S. thermophilus* ST45 culture containing 10^5 CFU/ml was inoculated into the reactor. The reactor was run for 24 hrs. The extent of colonisation was assessed every 6, 12, 18 and 24 hour intervals by carefully removing the coupon holder from the reactor. The coupons were gently removed from the coupon holder and placed in individual BacTrac cells containing 9 ml M17 broth. The cells were positioned in the BacTrac®4000 incubator, set to a temperature of 30ºC. The detection time was measured using the BacTrac software.

4.3 Results and Discussion

4.3.1 Attachment assay

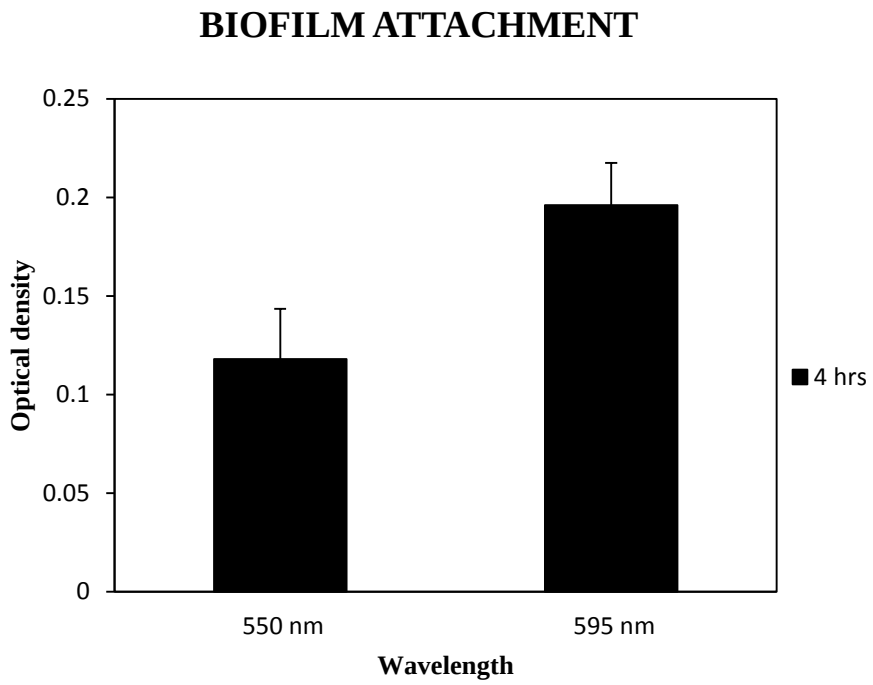


Fig 6. The results of the attachment assay performed using microtitre plate at 550 and 595 nm respectively. The experiment was carried out in duplicates and the results were averaged. The mean and standard deviation at 550nm were 0.118 and 0.025. The mean and standard deviation at 595 nm were 0.196 and 0.021.

4.3.2 Biofilm Formation

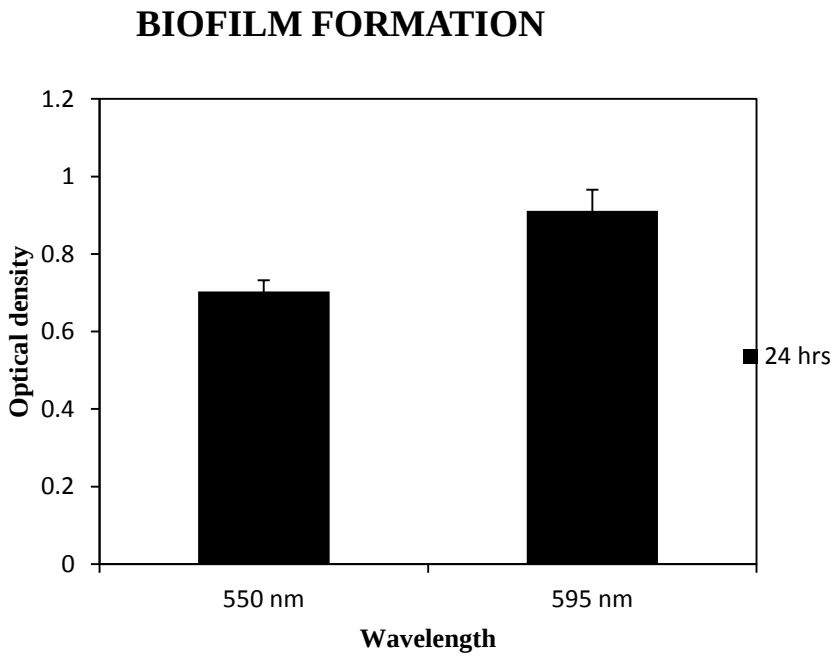


Figure 7: The O.D scores for biofilm formation assay. The experiment was carried out in duplicates and the plates were incubated at 30°C in air for 24 h. The results obtained were averaged. The mean and standard deviation at 550 nm were 0.703, 0.028 and at 595 nm were 0.911 and 0.054 respectively.

The crystal violet based microtitre plate biofilm assay is used widely to detect microbial attachment to an abiotic surface, (Oh et al., 2007). The crystal violet dye is used to visualize the microbial entities attached to the polystyrene surface by binding on to the negatively charged molecules present within the biofilm complex. The intensity of the dye attached in the wells corresponds directly to the density of the cells in the biofilm. The resulting O.D scores detect the density of the adherent cells. In this experiment the O.D. results read at 550 and 595 nm show that the starter culture was capable of attachment and biofilm formation. Though the organism was not capable of producing a very strong film formation but the results of the attachment and biofilm formation assay clearly indicate that the organism has a capacity to attach to abiotic surfaces and develop a biofilm in the course of time. The crystal violet assay was used as a preliminary test to detect the biofilm formation. Since the growth of biofilm was carried out in a CDC biofilm reactor using stainless steel coupons, the crystal violet assay was not useful and therefore the BacTrac® 4000 growth monitor was used.

4.3.3 Biofilm formation in a CDC reactor

The Centre for Disease Control biofilm reactor is considered as a most reliable tool for producing biofilms under shear conditions (Goeres et al., 2005). The reactor was run for 24 hrs and at 6h, 12h, 18 and 24h, two coupons were removed from the reactor and placed into BacTrac cells containing 9ml M17 broth and the impedance was measured. The detection time obtained was used to estimate the number of bacteria attached to the coupons and this value was then plotted against the run time.

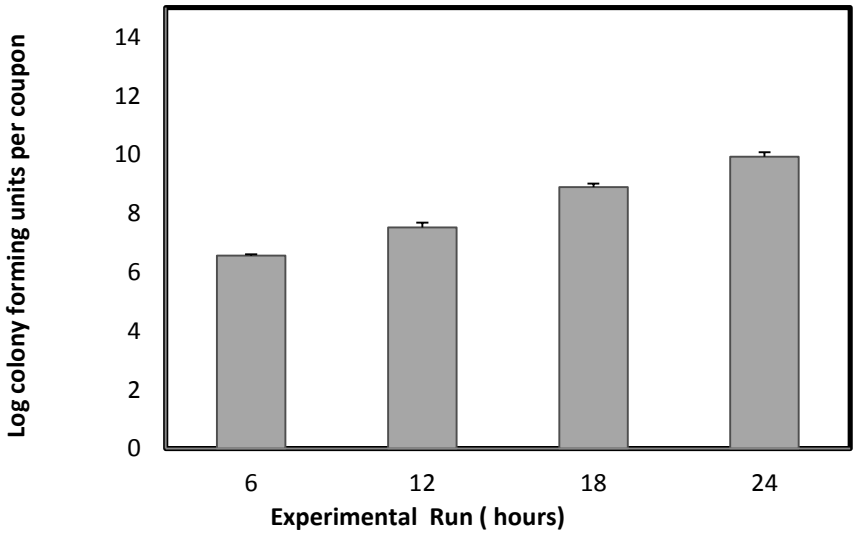


Figure 8: Biofilm growth in a CDC biofilm reactor. The biofilm cells colonising the coupon was estimated using the calibration curve obtained earlier. The Log_{10} number of cells per coupon was plotted against the time of incubation.

This experiment was conducted to examine the ability of the cheese starter culture *Streptococcus thermophilus* to attach to stainless steel coupons and produce biofilms. The reason for using stainless steel coupons was to check whether these starters had the capacity to attach to the whey carrying lines in the cheese plants. The whey lines are used for the transport of effluent whey from the cheese vats to the storage tanks. Whey is permitted to be in contact with the lines for long periods, possibly combined with improper cleaning practices, starter culture, which is found in abundance in the whey might attach to the stainless steel surfaces and produce biofilms. There have been several studies on the ability of the starter culture to produce biofilms in the cheese manufacturing industry, (Austin & Bergeron, 1995; Flint,Bremer, et al., 1997; Kives et al., 2005; Knight et al., 2004; Mercier et al., 2002). The result of this experiment shows a gradual increase of the cells in the biofilm with time, which reaches about 10^9 colony forming units/coupon at 24 hours. This indicates that the starter culture ST45 has the ability to form a biofilm. There are several factors which aid the attachment and formation of biofilms, like the growth phase of the starter bacteria, temperature of the suspending medium and surface, surface roughness and topography, the duration of contact of the bacteria with the surface, turbulence of the medium, properties of the cell surface such as hydrophobicity and surface charge. As the starter culture is used for the manufacture of several cheese varieties, the formation of biofilm in the industrial processing whey processing lines may cause detrimental effects. Biofilm formation may not only cause the blockage of the whey carrying lines but it might also become a reservoir of phage and lead to the destruction of starter cultures by contamination through air, etc. Therefore biofilm formation is a hazard to the cheese making process.

CHAPTER 5.BACTERIOPHAGE PROPAGATION ON BIOFILMS OF CHEESE STARTERS

5.1 Introduction

Bacteriophage propagation on cheese starter cultures is admittedly a serious problem in the dairy industry (Anderson & Meanwell, 1942). The proteolytic and enzymatic activities of the starter culture such as acidification and flavour production are potentially destroyed when a phage infection occurs (Batt et al., 1995). The contamination by a phage may lead to lysis of the starter culture strains in the vat, causing slow fermentations or complete failure of the starter culture, leading to a subsequent loss of the desired product (Cox, 1974). It is hypothesised that the starter bacteria can attach to stain steel surfaces in the whey processing lines and produce biofilms. The biofilm can eventually become a site for phage reproduction. In order to confirm the fact that biofilms do become reservoirs and sites of phage propagation, experiments were conducted by infecting an existing biofilm in a batch culture system with phage and determining the rate of decrease of viable cells by using the BacTrac® 4000 impedance monitor. In an attempt to demonstrate that a biofilm a reservoir of phage, an experiment was conducted using a CDC biofilm reactor in continuous flow, which was run for 72 hours, and the number of phage particles released was determined by a phage titration procedure.

5.2 Experimental Procedures

5.2.1 Phage infection in a batch culture

The reactor was run as before for 24 h and the biofilm density on coupons was assessed after 6, 12, 18 and 24 h. After 24 h, the reactor was infected with 0.3 ml of phage lysate containing 10^6 PFU/ml. The reactor was run for a further 6 h and cell concentration on the coupons was measured using the BacTrac® 4000 impedance monitor. The experiment was repeated, allowing the reactor to run for 12 h, estimating the cell numbers on the coupons after 6 hrs and 12 hrs respectively. 0.3 ml of the phage lysate was then added to the reactor and the cell numbers in the biofilms were recorded at 18 and 24 h i.e. 6 and 12 h after infection with phage lysates.

5.2.2 Phage infection in a continuous flow reactor

The reactor was inoculated as before and run for 12 h and the concentration of the cells on the coupons was measured by impedance. The reactor was then infected with 0.3 ml phage lysate containing 10^6 PFU/ml. The reactor was run for a further 2 hours and the biofilm concentration was again measured to check for phage activity. The considerable decrease in the biofilm cell numbers would be indicative of phage infection. Then the lid containing the coupon holder was gently lifted and the whole assembly was transferred in a beaker containing sterile distilled water to remove unattached cells and then finally placed into a new reactor containing 300 ml of sterile M17 broth. The reactor was run in continuous mode for a further 72 h and the cell number in the biofilm was measured using the BacTrac at 24, 48 and 72 h respectively. Sterile M17 broth was pumped continuously into the reactor at a flow rate of 30 ml/hr. The flow rate was adjusted by using a peristaltic pump to prevent the net increase in planktonic bacterial cell numbers in the medium. The required flow rate was calculated by multiplying the dilution rate with the working volume of the reactor. The growth rate was assessed by plate count method. The flow rate was calculated such that the dilution rate is greater than concentration of the bacterial cells in the medium. The effluent medium was collected in a sterile jar and the number of phage plaque forming units was counted at 24, 48 and 72 hrs respectively by taking samples from the effluent medium using the phage titration procedure.

<u>Calculation of required flow rate</u>

D= F/V (D is the dilution rate/h, F is the flow rate, ml/h and V is the volume)

D= 1/h and V= 300ml

Therefore, $F= 1h^{-1} \times 300ml$

$F= 300mlh^{-1}$

5.2.2.1. Measurement of maximum growth rate (μ_{max})

The maximum growth rate of the starter culture was determined prior to the continuous culture experiment in order to set the flow rate of reactor such that the dilution factor is greater than the maximum growth rate (μ_{max}). The maximum growth rate depends on factors such as adsorption rate, growth rate and rate of removal of cells within the biofilm.

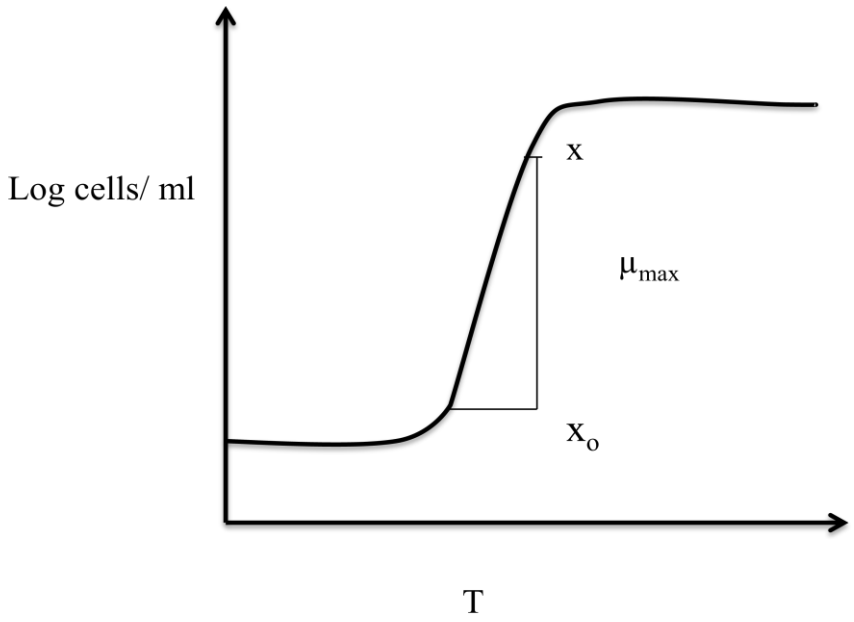


Figure 9. Measurement of maximum growth rate to determine the flow rate for continuous flow reactor set up.

Calculation of μ_{max}

$\mu_{max} = 2.3 (\log X - \log X_o) / \text{time}$

$2.3 \times 1.5 / 6 = 0.575 /h$

5.3 Results and Discussion

5.3.1 Bacteriophage infection in a 24 h grown biofilm:

The decrease in the growth of the cells in the biofilm was assessed 6 hours after phage infection.

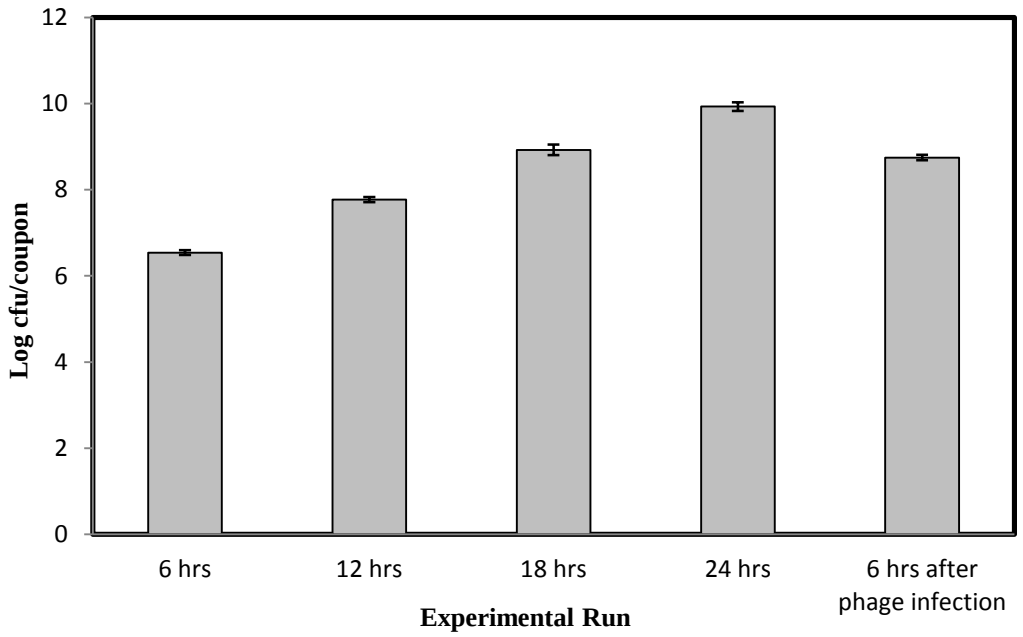


Figure 10: The growth of cells in the biofilm was monitored for every 6 hours for 24 hours after which the reactor was inoculated with phage. The decrease of cells after phage infection is apparent. The experiment was conducted with duplicates and the results were averaged.

5.3.2 Phage infection after 12 hours of biofilm growth

The experiment was repeated to show longer term effect of phage infection i.e. to answer the question "Does phage infection kill all the bacterial cells, or do some cells in the biofilm survive?" in this experiment, the biofilm was grown in the CDC biofilm reactor for 12 hours after inoculation, before the addition of phage lysate (0.3 µl containing 10^3 phage particles/ml). The reduction in the cell numbers of the biofilm was monitored using the BacTrac®4000 growth analyser.

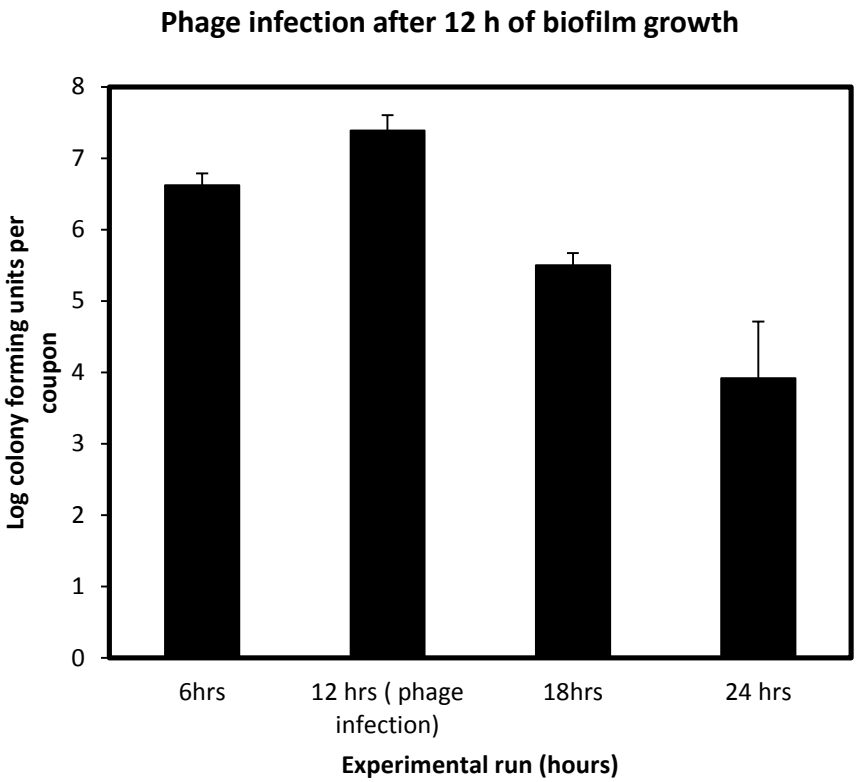


Figure 11: Phage infection after 12 hours of biofilm growth. The reduction in the cells of the biofilm was clearly observed due to phage infection reaching about 4 log cells in 24 hours.

The results of phage infection after 24 hours of biofilm growth show a slight decrease of 10^9 to 10^8 CFU per coupon, whereas the infection with phage after 12 hours of biofilm development showed a prominent decrease in the cell numbers of the biofilm. This indicates the fact that it is difficult for a phage to cause significant damage in a fully grown biofilm complex. The reason for this might be due to the difference in the density of the biofilm. When phage was infected after 12 hours biofilm growth, there was substantial decrease in cell numbers due to the fact that the biofilm was in its developmental stage and probably had a much thinner glycocalyx. The decrease in the cell numbers was approximately logarithmic from the time of infection to the point at which the experiment was terminated. This experiment was conducted in a batch mode, so as the experiment progressed the surviving cells were exposed to phage particles released by the burst cells.

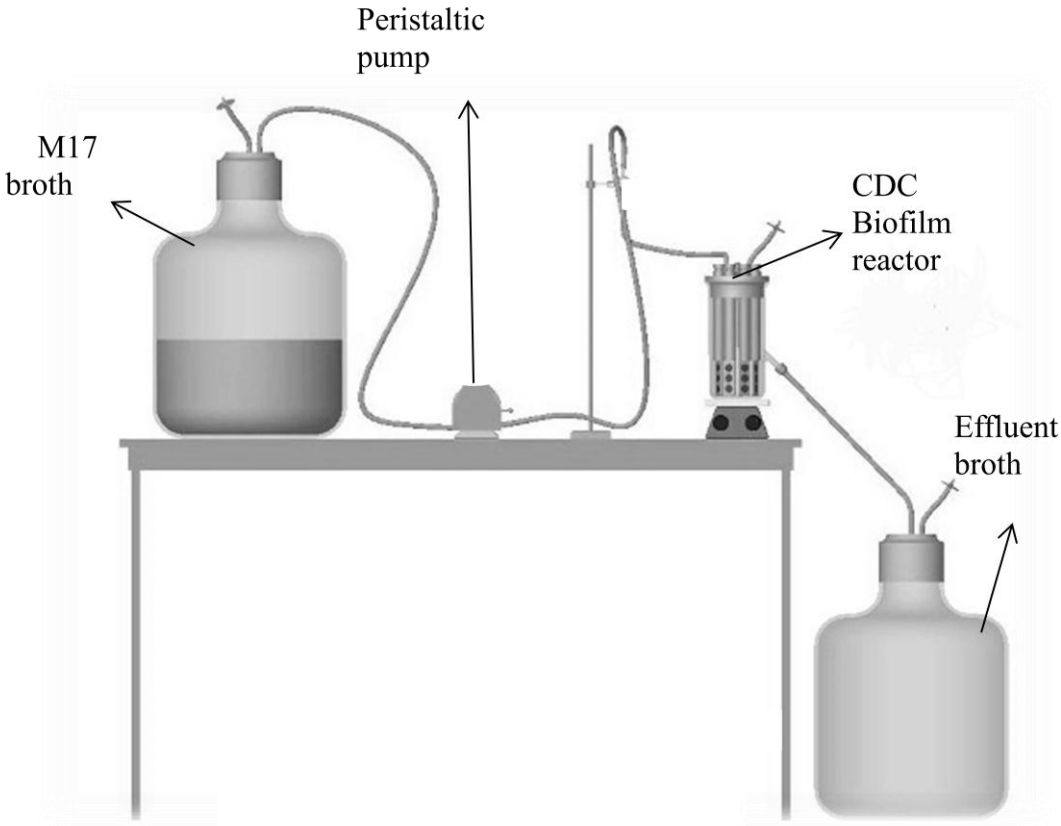


Figure 12: Schematic representation of a continuous flow reactor set up (Centre for biofilm engineering, Montana State University, Bozeman). The flow rate of the medium pumped into and out of the reactor is controlled by the peristaltic pump.

5.3.3 Bacteriophage infection in a continuous flow reactor for 72 h:

The conditions in a whey line might be better represented by a continuous flow, rather than a batch mode reactor. The experiment was repeated in a similar way as the previous experiment except that the period of exposure of the bacterial cells to phage was reduced to 20 minutes. The continuous flow through the reactor washed away planktonic bacteria and phage particles. The intention of the experiment was to determine whether the phage destroyed all the cells in the biofilm or if the bacteria and the phage levels reached equilibrium. The reactor was run for 12 hours and phage lysate was inoculated. After 20 minutes, the lid containing the coupon holder was gently removed from the reactor and rinsed in a sterile water bath. As in the earlier experiment, the reactor was then placed in a new reactor vessel containing 300 ml M17 broth. Sterile medium was allowed to flow into the reactor at a rate of 30ml/ hour. The number of bacterial cells on the coupons was measured at intervals up to 72 hours. The effluent medium was examined for the presence of phage particles by plaque counting.

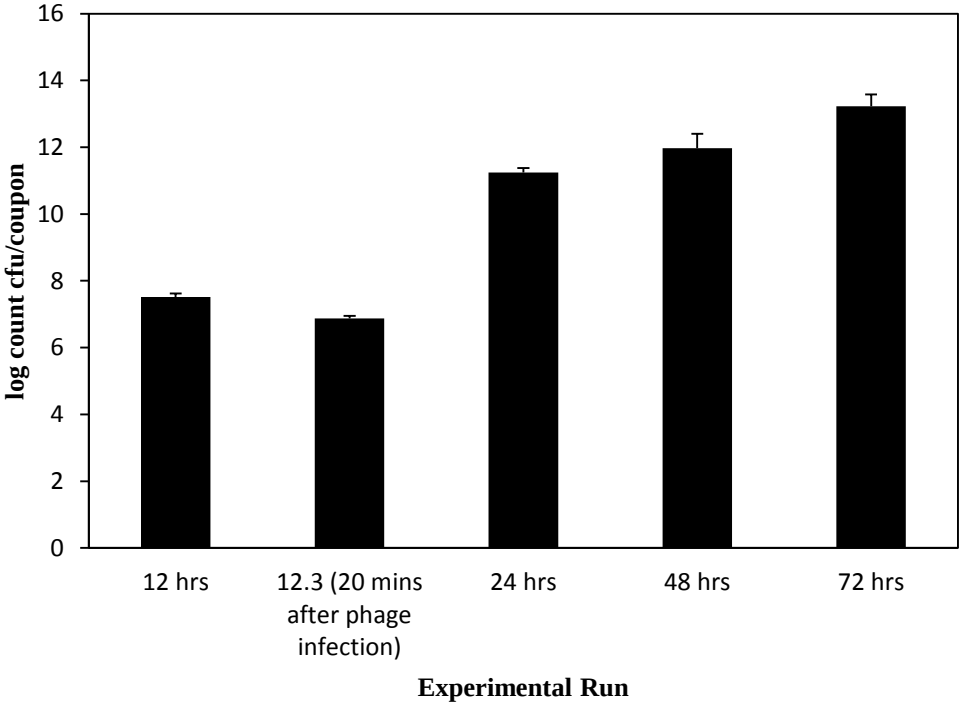


Figure 13: Time course of the biofilm growth of *S. thermophilus* in an experiment to investigate phage propagation. Biofilm concentration on the coupons was monitored at 12 h and the reactor was then subjected to phage infection for 20 min. Growth was again monitored up to 72 h in a continuous flow mode. The phage count data is shown in figure 13.

5.3.4 Repetition of the above experiment (5.3.3)

The absence of phage particles in the effluent medium with steady increase of the cells in the biofilm indicated that the 20 minutes incubation time was not sufficient for phage to penetrate inside the biofilm matrix and initiate infection. Therefore the experiment was repeated by prolonging the time of incubation post phage inoculation to 2 hours and then transferring the reactor head to another sterile reactor vessel. The biofilm on the coupons was again monitored at various periods up to 72 hours and the number of phage particles was counted in the effluent medium.

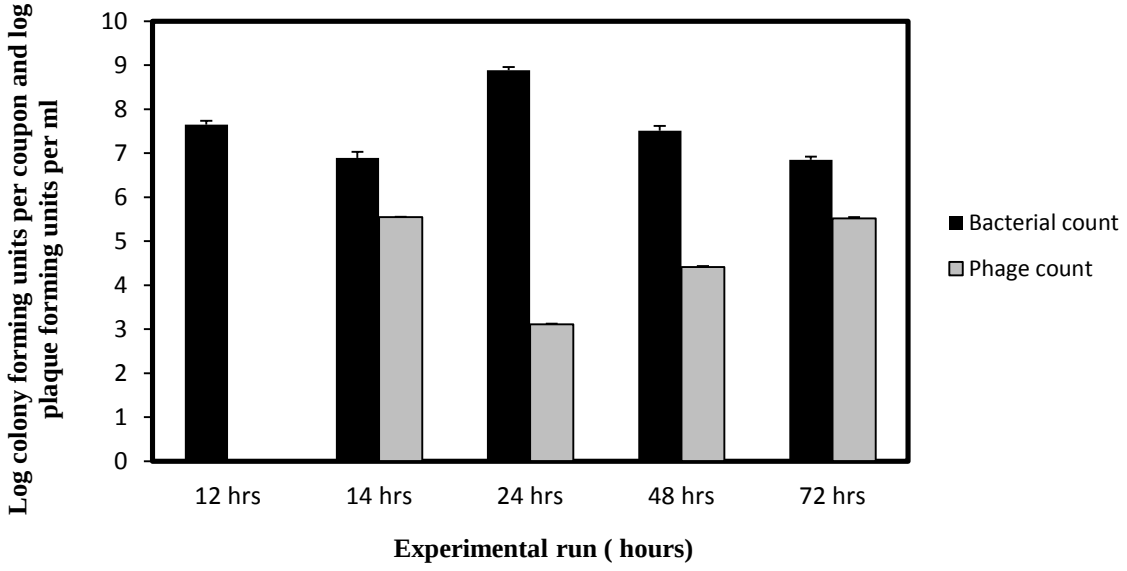


Figure 14: Repetition of the previous experiment (5.3.3) by prolonging the duration of phage infection. The fate of cells in the biofilm after 2 h of infection with phage was monitored. The presence of phage in the effluent medium was detected at 14, 24, 48 and 72 hours

This experiment was done to investigate the fate of the cells in the biofilm and the number of phage particles in a continuous flow reactor for duration of 72 hours. The infection by phage after 12 hours of biofilm growth caused a steady decrease in the number of cells in the biofilm. However, upon removal of the reactor head from the reactor after 20 min and transfer to fresh sterile medium, no phage particles were detected in the following 72 hours. The reason might be the incubation time was not sufficient for the phage particles to degrade the EPS matrix and penetrate the biofilm (Hughes et al., 1998). Therefore the cells in the biofilm continued to increase until the run was terminated. It is also possible that the phage particles were trapped in the biofilm matrix, but were unable to infect the cells and therefore the phage particles were not detected in the liquid phase. The experiment was repeated with incubation period post phage inoculation extended to 2 hours. The cells in the biofilm complex continued to grow, causing an increase in the cell number per coupon at 24 hours. Over the next 48 hours, increasing numbers of phage particles were detected in the liquid phase with a concomitant steady decline of cells in the biofilm. It is apparent from this result that, phage particles require a longer time to infect and destroy the cells in the biofilm, when compared with planktonic cells. This suggests that the biofilm cells are protected by the EPS matrix and it takes a considerable amount of time for the phage to penetrate into the matrix and cause the depletion of the cells in the film. It is also evident from the experiment that the phage particles cause a visible decrease in the cells of the film, but do not completely destroy it. This experiment demonstrates that it is possible for the starter culture to attach to whey handling lines and form a biofilm that could be infected by phage. If the film were allowed to remain in the lines, this could become a reservoir of phage that could result in contamination

throughout the manufacturing plant, gaining access to the bulk starter tanks and cheese vats following spillage and aerosol generation.

5.3.5 Bacteriophage propagation in a continuous flow reactor for 144 hours:

In the phage propagation experiment run for 72 hours, the number of cells in the biofilm was decreasing and the number of plaque forming units was increasing when the run was terminated. The experiment was repeated again, prolonging the experiment run time up to 144 hours. All the details of the experimental method were as in the previous run. The intention was to determine whether the number of bacterial cells in the biofilm and the phage numbers stabilised.

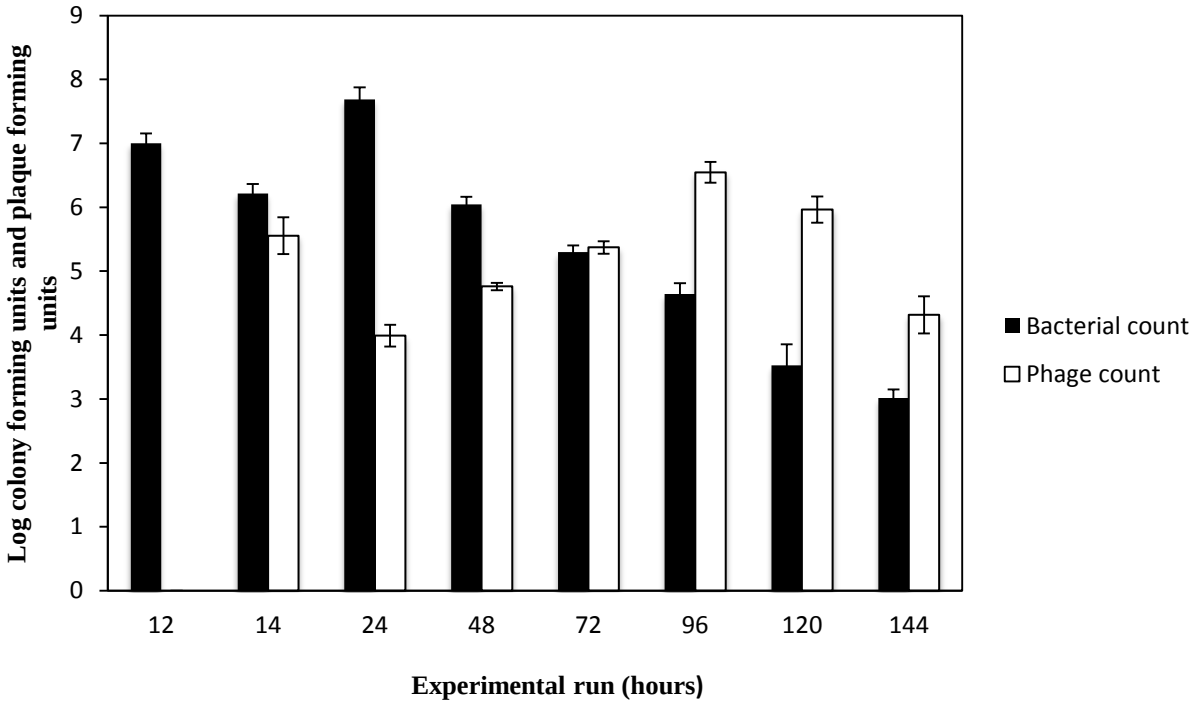


Figure 15: Phage activity was observed for 144 hours. There is an initial increase in the cells of the biofilm reaching a maximum of about 8 log cells with a low titre of phage. By the course of time, the phage titre increases with a steady decrease in the cells of the biofilm. By 144 hours the phage activity also starts to drop with the decrease of biofilm cells.

As in the earlier runs, the reactor head holding the coupons was transferred to fresh sterile medium in a new reactor at 14 hours. At this time, coupons were sampled to estimate the concentration of biofilm cells and the phage titre of the culture medium was measured. The transfer process involved rinsing the coupons to remove phage-containing medium. As a result, the number of phage particles measured at 24 hours decreased and biofilm cell numbers were higher than at any other time during the run, and probably because not all cells were infected in the first cycle, so the uninfected cells were able to continue growing. From this point onward, the remaining attached phage particles started to multiply rapidly and the bacterial cell count decreased, continuing to do so until the experiment was terminated at 144 hours. However, the number of phage particles did not continue to increase, peaking at 96 hours and about 100 particles per cell was released. The experiment showed again that a complete degradation of the biofilm was not achieved by the phage, though the number of bacterial cells in the film continued to decrease. In fact, the proportion of both communities appeared to stabilise. Using *Escherichia coli* and T4 bacteriophage for a prolonged period of up to 52 weeks, Horne observed that phage and bacteria can co-exist (Spanakis & Horne, 1987). Tait et al., (2002) also suggested that another reason for the failure in eradication may be due to the development of bacterial resistance to phage infection due to the prolonged period of incubation (Tait et al., 2002). This study shows that the formation of biofilm by starter organisms and subsequent infection by phage could eventually lead to phage contamination of the whole plant environment. During whey disposal or spillage, the phage may gain access to the bulk starters and cheese vats. Though this study was conducted in the laboratory in a biofilm reactor filled with artificial medium (M17), it shows that phage reproduction can occur for

a period of at least 144 hours. This underlines the importance of regular and complete draining; cleaning and sanitation of whey lines should to prevent biofilm growth.

CHAPTER 6.GENERAL DISCUSSION AND CONCLUSIONS

Bacteriophage are found ubiquitously in the dairy plant (Anderson & Meanwell, 1942). Phage attack of the starter culture used for cheese production has always been considered a serious problem in the dairy industry worldwide (Beresford et al., 2001). Despite incessant attempts like sanitary precautions, starter strain rotation and production of phage resistant strains, complete elimination of phage has not been achieved. The disruption of the starter by phage causes a great economic loss to the cheese manufacturers (Everson, 1991). Therefore the detection and prevention of phage in the dairy environment is a matter of concern.

The important sources of phage are air, raw milk, appliances, whey and workers. Once phage enters the dairy plant, it spreads readily throughout the plant. The elimination of phage becomes difficult because of their short latent period, relatively larger burst size and resistance to pasteurisation (Lewis, 1956).

S. thermophilus is a lactic acid bacterium used as a starter for the manufacture of several types of cheeses (Zirnstein & Hutkins, 1999). The effluent whey from the cheese plant sometimes remains in the lines for a considerable period before the plant is cleaned; creating an opportunity for the organisms to attach to the stainless steel surfaces and produces a film. The biofilm not only compromises the hygiene standards of the dairy environment but may become a site for phage reproduction (Flint,Bremer, et al., 1997).

The current project was undertaken to identify the biofilm forming capacity of the starter culture and to observe if the film may act as a reservoir and a phage propagation site. The results of the initial biofilm attachment and formation assay indicate that the starter culture S .*thermophilus* (ST45) has a good biofilm forming capacity. *S.thermophilus* biofilms were grown in a CDC reactor and the number of cells in the film

colonising the stainless steel coupons were analysed using BacTrac® 4300 growth monitor. The results showed that a maximum of up to 9 log cells per coupon was achieved in 24 hrs. The result demonstrates that *S. thermophilus* has the capacity to attach to stainless and form a biofilm as reported earlier by (Flint,Brooks, et al., 1997). There are several factors aiding the biofilm formation such as surface structure, topography, milk proteins, hydrophobicity and surface charge. Therefore it is very likely that the starter culture *S. thermophilus* my form biofilm in the cheese plants (Knight et al., 2004) reported that the thermo-resistant nature of the bacteria also aids in the biofilm formation as the organism is able to withstand pasteurisation temperature.

Biofilm formation in the cheese plants may become potential source of phage and in order to examine the ability of phage to propagate in the film, the biofilm was subjected to phage infection and the reduction of cell numbers in the film indicated that the phage was capable of penetrating into the film and initiating infection. A continuous flowing system was set up to observe the fate of the bacterial cells in the biofilm and phage numbers in the liquid phase. The reactor was run for a prolonged period to mimic the flow of whey in the dairy plant. It was observed that the cells in the biofilm were not completely eliminated by the phage. Horne (2007) had demonstrated phage and bacteria can co-exist for a prolonged period of up to 52 weeks (Spanakis & Horne, 1987). It was also reported by Tait et al., (200) that during a prolonged encounter of biofilm with phage, the cells in the biofilm tend to become resistant to phage. Therefore the reason for failure of the phage to completely degrade the cells in the biofilm may be due to the development of bacterial resistance to phage infection.

The whey lines in a cheese plant are less frequently CIP'd than the rest of the plant, particularly during the peak of the season and prolonged presence of whey in the lines

could facilitate attachment and biofilm formation by the starter bacteria present in the whey. It was hypothesised that the formation of biofilm would enable the phage particles to multiply in the film and be released in the whey. This was observed in the experiment when the effluent medium leaving reactor was tested for the presence of phage particles. If whey spillage occurs, the phage particles are distributed throughout the plant in aerosols and might contaminate the bulk starters used for cheese production.

Therefore this study may shed light on the potential reservoir of bacteriophage in the whey drainage and treatment areas in the cheese manufacturing plants. The use of cleaning regimes and following good manufacturing practices can prevent the formation of biofilms in the dairy environment. The pipes carrying effluent whey should be continually monitored and cleaned after every refill. This would probably reduce the formation of biofilms thereby lowering the risk of phage attack and thus preventing a severe economic loss to the industry. The limitations of this study are that the CDC reactor is a very artificial environment and cannot be completely compared to the flow of whey in the whey lines. M17 medium used in the study cannot be directly compared to the effluent whey removed during cheese production as it does not contain certain proteins and therefore the biofilm formed in the whey lines may be different from those that were formed *in vitro*.

This study can be further extended by observing the capacity of other dairy starter cultures to form biofilms and if bacteriophage are able to propagate inside them. The effect of phage on dual and mixed species biofilms can also be examined. As stated in the Literature Review, modern cheese manufacturers use mixed starters i.e. a ‘cocktail’ of unrelated phage types. It is therefore possible that mixed species biofilms could form in the whey handling lines. These could once again become a reservoir of phage infection

and could also be a breeding ground for mutant phage strains, leading to phage attack on previously resistant starter strains. Therefore the behaviour of the mixed species biofilms can be examined using the techniques described above.

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