Abstract

MUDGAL, PRASHANT. Modeling the dynamics of bacteriophage-host interaction in sauerkraut fermentation. (Under the direction of Drs. Fred Breidt and K. P. Sandeep)

The main objective of this study was to investigate the possibility of using starter cultures in sauerkraut fermentation and thereby reducing the quantity of salt used in the process. This in turn would reduce the amount of waste salt in our water resources. Phage, naturally present in sauerkraut fermentation, could potentially affect the starter cultures introduced. Thus, a mechanistic mathematical model was developed (using MATLAB) to quantify the growth kinetics of the phage and starter cultures. The model was validated based on the kinetics of *Leuconostoc mesenteroides* 1-A4, *Leuconostoc pseudomesenteroides* 3-B11 and their corresponding phage, Φ1-A4 and Φ3B-11, respectively, in MRS media.

The predictions of the model were found to be in close agreement with experimentally determined phage and bacterial counts with time. Based on the results from the model, it was seen that phage quickly kills the host cells even when they are present in low numbers of the order of 10^3 pfu/ml in the MRS media. A population of phage resistant cells was also found to be present, which replaced susceptible cells in the presence of phage. It was observed from the results of the model that the adsorption rate constant decreased with the time of phage-host interaction and was found to vary with initial cell densities. The effect of heterogeneity and other environmental factors such as temperature and pH should be considered to make the model applicable to commercial fermentations.

MODELING THE DYNAMICS OF BACTERIOPHAGE-HOST INTERACTION IN SAUERKRAUT FERMENTATION

By

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Dedicated to my respected and loving grandmother

‘Vidya Sharma’

BIOGRAPHY

Prashant Mudgal was born on 10th September, 1980, in Bharatpur, a small town in North-Western India. His interest in mathematics at an early stage of his education shaped his career as an engineer. He obtained his Bachelors degree in Agricultural and Food Engineering from the Indian Institute of technology (IIT) Kharagpur, India in 2002. Upon graduation, he came to North Carolina State University to pursue his Masters in Food Science. He joined the USDA-ARS laboratory in the Food Science Department and worked on mathematical modeling of bacteriophage-host interaction under the direction of Drs. Fred Breidt and K.P. Sandeep. Working on his project, he developed an interest in Biomathematics and also received a minor in this field.

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'Feeling gratitude and not expressing it
is like wrapping a present and not giving it.'
(William Arthur Ward)

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Chapter 1

Introduction

1.1 Sauerkraut Fermentation

Sauerkraut fermentation is carried out by a small population of lactic acid bacteria (LAB) indigenous to cabbage in the presence of 2-3% salt. Sauerkraut fermentation is a complex process, with various bacterial populations playing important roles at different times during fermentation. Fermentation is carried out in two stages, initial heterofermentative stage followed by a homofermentative stage. *Leuconostoc mesenteroides* initiates the early heterofermentative stage and is primarily responsible for the quality characteristics of sauerkraut (Mundt *et al*. 1967). The correct microbial succession during the fermentation is a critical factor to the quality of sauerkraut produced. Salt withdraws water and nutrients from the cabbage tissue, while nutrients serve as the substrate for the growth of LAB. Salt serves as a selecting agent for LAB, because they are more tolerant to salt than other natural micro flora initially present on the cabbage. Salt in conjunction with the acids produced by fermentation, inhibits the growth of undesirable bacteria. During fermentation, at the end of the heterolactic phase brine is removed from bottom of the tanks and is discharged in effluent from the plant. Also after the fermentation, sauerkraut is processed, and diluted brine is discharged in effluents from the processing plant before packaging of sauerkraut in cans into streams or into municipal waste systems. The U.S. environmental protection agency has proposed a limit of 230 parts per million (ppm) of chloride in freshwater bodies (Fleming *et al*., 1995). Many pickle and Sauerkraut companies in the U.S. have difficulty in meeting this

limit in the effluent from their processing plants. Due to the environmental concerns of waste salt disposal and the associated economic issues, it may be desirable to reduce salt concentration by 50% or more. Sauerkraut fermentation at low salt concentrations may be controlled by using starter cultures and the desired flavor and texture can be maintained. Use of starter cultures in sauerkraut fermentation has been attempted for several decades. *Leuconostoc* species have been used as starter cultures and were found to prolong the heterolactic fermentation in sauerkraut fermentation (Harris *et al.* 1992; Breidt *et al.* 1995). However, to date, commercial use of starter cultures has not been a widespread practice.

1.2 Bacteriophage

Bacteriophage (phage) are ubiquitous in nature, and they are an important component of the microflora on vegetables. Previous studies have shown that there are phage active against the LAB in sauerkraut fermentation (Yoon *et al.* 2002; Lu *et al*. 2003). Phage contribute to bacterial mortality and thus they can modify the microbial succession in sauerkraut fermentation. As the sauerkraut fermentation environment is not sterile, phage contamination could be an important factor especially in fermentations using starter cultures. *Leuconostoc* species are potential starter cultures and phage active against these have been isolated from commercial sauerkraut fermentations. It is necessary to understand the dynamics of a starter culture and its corresponding phage to efficiently control the sauerkraut fermentation. The objective of the present study was to develop a mathematical model for the population dynamics of phage and host and quantitatively determine the impact of phage on the starter culture.

Generally phage are host specific and infect a particular species of bacteria, however there are some phage that can infect several bacterial species and a particular host species may be susceptible to infection through different phage. Phage 1-A4 was found to infect two bacterial species, *L. mesenteroides* 1-A4, and *L. mesenteroides* 1-E10, both being distinct *Leuconostoc* species (Lu *et al*., 2002). Another phage, 3-B11, was found to be host specific and only infected *L. pseudomesenteroides 3-B11* (Lu *et al*., 2002). Kinetic parameters defining phage-host interaction such as bacterial growth rates, burst size, latent period, and adsorption rates are specific to a particular phage-host system.

1.3 Mechanistic and Empirical Models

Models are basically classified in to two types. One is the class of empirical models, and the other one of mechanistic models. Mechanistic models are developed from the theoretically or experimentally determined data describing the cause or mechanism behind the dynamic changes observed in an experimental system. Parameters used to define mechanistic models have biologically significant meaning, and can be measured in the laboratory most of the time. Empirical models include functions that are used to fit the data and approximate a particular process. Development of a mechanistic model requires a good theoretical knowledge of the system. In cases where good knowledge about a system is not available empirical models are used. Empirical models do not have a causal structure. Mechanistic models may be very complex, as they may require too many details to accurately predict the observed data.

A model can be made very complex and more predictable by increasing the number of parameters, and variables. Validation of a model requires measurement of parameters and variables, therefore more the number of parameters and variables, more will be the effort required to validate the model. Moreover, it may be very difficult to determine some of the parameters. Therefore, these models may be limited in their usefulness when applied in real situations due to associated time and cost issues.

1.4 Objective

The objective of the current study was to develop and validate a mechanistic model for the interactions between a host and its corresponding phage using *Leuconostoc* species. Our approach was to start with a simple model, based on the mechanistic assumptions, and then increasing the complexity of the model, depending on the kinetic studies in the laboratory, until there is an equilibrium between the complexity of the model and the predictability of the model.

Chapter 2

Literature Review

2.1 Sauerkraut Fermentation

Fermentation is a very ancient method of preserving plant materials. It not only serves as a preservation method, but also results in the development of desirable characteristic sensory properties such as texture, flavor, and aroma. As in most vegetable fermentations, sauerkraut fermentation is carried by a small population of lactic acid bacteria (LAB) that are naturally present on fresh vegetables. The correct sequence of different LAB species playing a dominant role in fermentation and their metabolic activities are necessary for the safety and quality of these products (Pederson and Albury, 1969). Sauerkraut fermentation is carried out in two stages, an initial heterofermentative stage followed by a homofermentative stage. In the homofermentative stage generally 1.8 mol of lactate is produced per mol of glucose fermented, while in heterofermentative stage 0.8 mol of lactate per mol of glucose is produced. In the heterofermentative stage, CO_2 and acetic acid are also produced along with lactate. Heterofermentative *Leuconostoc mesenteroides* initiates the fermentation and predominates the early stage of the fermentation as it is present at an initially higher numbers (Mundt *et al.*, 1967). *L. mesenteroides* grows more rapidly at a lower temperature than most other LAB found on plants (Pederson and Albury, 1969). As the fermentation proceeds, the pH of the brine solution drops from around 6 to 4.5, and *L. mesenteroides* being less tolerant to acid dies off and is replaced by other heterofermentative species and eventually the homofermentator, *Lactobacillus plantarum.* Ecology of commercial sauerkraut

fermentation is complex with several bacterial species playing important roles. Recently some of new bacterial strains were isolated from commercial sauerkraut fermentations these include *Leuconostoc citreum*, *Leuconostoc aregentinum*, *Lactobacillus parapalnatarum*, *Lactobacillus coryniformis*, *Lactobacillus plantarum*, and *Weisella* species (Plengvidhya, 2003). The microbial succession (sequence of microorganisms playing dominant role during fermentation) in sauerkraut fermentation is critical in achieving a stable product with typical texture, flavor, and aroma. Quality of sauerkraut is largely due to the initial microbial load on cabbage, the growth rates, salt and acid tolerance of different LAB species, acid concentration, pH, salt concentration, and temperature (Pederson and Albury, 1969; Fleming *et al.*, 1982). In its first definition of sauerkraut, the Federal Food and Drug Administration stated that "sauerkraut is made in presence of not less than 2 % and not more than 3 % salt"(Pederson and Albury, 1969). A salt concentration of about 2 % has been traditionally being used in sauerkraut fermentation. The presence of salt on shredded cabbage leaves withdraw water and nutrients from the cabbage tissue, while nutrients serve as the substrate for the growth of lactic acid bacteria. Salt serves as a selecting agent for LAB because they are more tolerant to salt than other natural micro flora initially present on cabbage. Salt in conjunction with the acids produced by fermentation, inhibits the growth of undesirable bacteria and delays softening of cabbage.

2.1.1 Waste Salt Disposal Problem

After fermentation, sauerkraut is often stored in fermentation tanks, which sometimes lead to problem of excessive acidity. Dilution of brine may be required to

deal with problem of excessive acidity (Breidt *et al*., 1995). Diluted brine is discharged in effluents from the processing plant before packaging of sauerkraut in cans. The U.S. environmental protection agency has proposed a limit of 230 parts per million (ppm) of chloride in freshwater bodies (Fleming *et al*., 1995). Many pickle and Sauerkraut companies in the U.S. have difficulty in meeting the 230 ppm limit in effluent from their plants. When a lower salt concentration is used in fermentation, other undesirable microorganisms that are the part of naturally present initial micro flora may influence the fermentation and thus microbial succession may change.

2.1.2 Starter Cultures in Sauerkraut Fermentation

Use of starter cultures in sauerkraut fermentation has been attempted for several decades. However to date, commercial use of starter cultures has not been a widespread practice. It has been suggested that sauerkraut fermentation at low salt concentrations may be controlled by using starter cultures so that the desired flavor and texture can be maintained. A paired starter culture system consisting of *Leuconostoc mesenteroides* and a nisin producing *Lactococcus lactis* was attempted in sauerkraut fermentation (Harris *et al.*, 1992). Nisin and nisin-resistant *Leuconostoc mesenteroides* were also used and found to prolong the heterofermentative stage in sauerkraut fermentation which is responsible for characteristic flavor and aroma of sauerkraut (Breidt *et al.*, 1995).

2.2 Bacteriophage

Bacteriophage (phage), are viruses that infect bacteria. They are classified in to two types, temperate and virulent bacteriophage based on the type of life cycle they follow in their hosts. They modify microbial communities by lysing hosts, transferring genetic material, and effecting lysogenic conversion of host strains. Phage are an important component of the micro flora on vegetables. Previous studies have shown that there are phage active against LAB in sauerkraut fermentation (Yoon *et al.,* 2002). Phage contribute to bacterial mortality and thus they can modify the microbial succession in sauerkraut fermentation. Diversity and ecology of phage active against LAB in commercial sauerkraut fermentations have been studied (Lu *et al.,* 2003). Several distinct phage and hosts were isolated from commercial sauerkraut fermentations (Lu *et al*., 2003). Phage may kill the starter culture and thus affect the fermentation. Therefore, it is imperative to understand how significant an effect these bacteriophage can have on the starter culture, and the quality of the sauerkraut produced at the end of the fermentation process. It is necessary to understand the dynamics of starter cultures and the corresponding phage to efficiently control sauerkraut fermentation. The following sections describe properties, life cycles and importance of phage.

2.2.1 Discovery of Bacteriophage

The first account of a bacteriophage was published by Twort (1915). Twort (1915) had noticed that some colonies of a bacterium, *Micrococcus*, had undergone what he termed a 'glassy transformation'. When Twort (1915) transferred a minute speck of

material from such a glassy colony to a normal colony, that too, in a day or so became glassy. The glassy appearance could be transmitted from colony to colony indefinitely. The author could find no bacterial cells in the glassy colonies and deduced that cells must have lysed, and called the phenomenon 'transmissible lysis'. Material from a glassy colony was able to pass through a filter whose pores were fine enough to restrain bacteria. Only colonies of living bacteria could be lysed with it and during lysis, it was shown that the lytic agent increased many hundred folds in quantity. On the basis of this evidence Twort (1915) cautiously suggested that the agent might be, among other possibilities, a virus similar to those that infect plants and animals, which had been discovered a few years previously. However the word 'bacteriophage' which means 'bacteria-eater' was coined by D'Herelle (1917) who rediscovered the phenomenon independently in 1917. D'Herelle with no doubt declared it a virus and succeeded in arousing world-wide interest in bacteriophage research. The author's ideas (D'Herelle, 1917) relating phage to immunity of disease attracted the general attention of medical scientists, indirectly yielding both practical and theoretical benefits (Douglas, 1975).

2.2.2 Bacteriophage (Phage) and Their Importance

Bacteriophage are important ecological components because of their impact on bacteria. They modify microbial communities by lysing hosts, transferring genetic materials, and cause lysogenic conversions. By lysing the prokaryotes at the base of the food webs, phage are capable of disrupting the flow of energy within ecosystems. Phage-host interaction have been a subject of interest in aquatic environments (Beretta *et al.*,

1998; Middleboe *et al*. 2000; and Middleboe *et al*., 2001), the rhizosphere (Ashelford *et al*. 1998; Ashelford *et al*., 2000; Burroughs *et al*., 2000; and Caldas *et al*., 1992), and in fermentations (Lu *et al*., 2003; Yoon *et al*., 2000). Recently some of the studies have shown an interest in the area of vegetable fermentations (Lu *et al*., 2003; Yoon *et al*., 2000). Phage are employed as a model of predator-prey interactions. Phage are of increasing interest as mediators treatment of bacterial diseases (Hadas *et al.*, 1997; Kasman *et al*., 2002; Levin and Bull, 1996; and Payne *et al*., 2000).

2.2.3 Classification of Bacteriophage Based on Their Life Cycle

Bacteriophage are categorized into temperate and virulent phage. This classification is based on the type of life cycle that a phage follows in its host. The two types of life cycles known for bacteriophage are the lytic life cycle and the lysogenic life cycle. In lytic life cycle phage are released in the environment as a result of the lysis of a bacterial cell. The lytic life cycle was recognized by D'Herelle (1917). In the lysogenic life cycle, bacteriophage and host cell enter a symbiotic relationship in which the host cell continues to multiply, carrying the virus for an indefinite number of cell divisions. D'Herelle (1917) referred to this phenomenon as "symbiose bacterie-bacteriophage". In the lysogenic life cycle, the phage genome integrates with the host cell's genome and makes a prophage. During bacterial growth, conversion of prophage to vegetative phage occurs spontaneously with a small but characteristic frequency, for example in one cell per million cells, as evidenced by the lysis of an occasional cell to liberate an infective phage particle. In some lysogenic cultures, the natural frequency of productive lysis may

be increased by exposure to UV light, X-rays, nitrogen mustard, peroxides and certain other agents (Adams, 1959; Campbell, 1961; and Ellis and Delbruck, 1939).

2.3 Phage-Host Interaction

Phage-host interaction can be divided in different steps. These steps are adsorption, penetration of genome, phage maturation and lysis of cells in a lytic life cycle. Following sections describe steps in phage-host interaction, parameters defining phage-host interaction, and history of kinetic studies on phage-host interaction.

2.3.1 Parameters Defining Phage-Host Interaction

Phage-host interactions begin by a highly specific attachment of phage to the bacterial cell envelope. The phage tail fibers then bind to specific receptors in the bacterial envelope (Goldberg *et al.*, 1994). This step is known as adsorption. Adsorption depends on environmental factors, and is followed by penetration of the phage DNA through the bacterial wall, which is weakened by the lysozymic activity of the tail baseplate. After infection, phage-encoded proteins are expressed and phage multiplication takes place inside the cell. Early proteins govern DNA replication and synthesis of coat components, and late proteins induce cell lysis thus releasing phage progeny. D'Herelle (1917) divided the infective process into arbitrary stages: 1) Adsorption of the phage particle to the host cell, 2) Penetration of the phage particle into the bacterium, 3) Intracellular growth of the phage particle in the bacterium, and 4) Lysis of the host cell and release of phage progeny. However, kinetic aspects of the infective

cycle are studied by means of one step growth experiment devised by Ellis and Delbruck (1939). Latent period (the time between adsorption of the phage to the cell and the lysis of the host cell with release of progeny phage) and burst size (mean yield of phage particles per bacterium) are determined from one step growth experiments. The eclipse period is also an important parameter especially used for studying intracellular growth of phage. It was defined as the delay time until the first phage is completed inside the bacterium from the time of adsorption. The eclipse period was determined by disrupting infected cells before their spontaneous lysis (Doermann, 1952).

2.3.2 History of Phage-Host Kinetics

After the discovery of bacteriophage, by D'Herelle (1917) and Twort (1915), there was a huge interest in the phage research due to the fact that phage attack and lyse bacterial cultures. Considering phage as a potential therapeutic agent against bacterial diseases, several studies focused on phage-host kinetics. Some of the initial studies dealing with phage –host kinetics include those performed by Kruegger and Northrop (1930), Kruegger (1931), Schlesinger (1932), Ellis and Delbruck (1939), and Delbruck (1940). These studies defined the phage-host interaction both qualitatively and quantitatively and served as a foundation to phage-host modeling studies. However, interest in bacteriophage waned due to mixed and unpredictable results from some studies pertaining to phage therapy and was further reduced after the advent of antibiotics. After reduced interest in phage-therapy, several studies on phage-host kinetics focused on ecological importance of phage. Recently there has been a renewed interest in phage-

host models because of potential in phage therapeutic treatment of bacterial diseases (Barrow and Soothill, 1997).

2.3.3 Adsorption of Phage to Host

The first step in the growth of bacteriophage is adsorption. It is defined as the attachment of phage to specific receptors on bacterial cell wall envelope, (also known as binding site). Since this is the first step in the phage-host interaction, it may control the growth of phage, (Ellis and Delbruck, 1939). "Phage can not multiply unless attached to the bacteria, therefore the rate of this attachment under certain conditions may limit the growth of the phage" (Ellis and Delbruck, 1939).

Various studies in the past have specifically focused on this step of the life cycle of a phage, especially for lytic bacteriophage (Kruegger, 1931; Schlesinger, 1932; Ellis and Delbruck, 1939; Delbruck, 1940; and Wang *et al*., 1997). Early studies on the kinetics of phage adsorption were those of Kruegger (1931), Schlesinger (1932) and Delbruck (1940). There have been two schools of thought to describe the adsorption process. The first approach is to measure attachment by exponential free phage decay and second approach is using mean free time for adsorption.

In the exponential free phage decay (free phage numbers decrease exponentially in the MRS broth), the rate of adsorption was found to be first order with respect to the bacterial as well as phage concentration (Kruegger, 1931; Schlesinger, 1932; Ellis and Delbruck, 1939; and Delbruck, 1940). In the determination of the adsorption rate constant, growth of bacteria was stopped so that there is no production of phage from lysis, which may interfere with the adsorption measurements (Kruegger, 1931; and

Schlesinger, 1932). Another method to avoid this problem was to conduct adsorption kinetics in a short time (latent period) so that no phage is produced from the lysis of cells (Ellis and Delbruck, 1939). To keep bacterial numbers constant, heat killed susceptible bacteria were used, or the growth of bacteria was stopped using very low temperatures (Kruegger, 1931; and Schlesinger, 1932). Schlesinger (1932) worked with living bacteria, and time of the adsorption reaction was kept relatively short (less than 12 minutes). In this time, the bacterial population changed little and could be assumed constant. It has been repeatedly found that the adsorption is a relatively fast process, involving 70-90 % of phage adsorption in the initial few minutes. Delbruck (1940) was the first to study the details of the adsorption process. Specifically Delbruck (1940) studied the dependence of the adsorption rate constant on the physiological state of the cell. The size of bacteria change considerably depending on the phase of growth in a given culture medium, and a larger cell surface may lead to an increase in adsorption rate. Rate of phage T4 adsorption to *E. coli* cells per unit surface area was calculated according to an experimental equation (Hadas *et al*., 1997). Adsorption rate was found to be higher with higher growth rates of bacteria which are correlated with increased cell surface area, as also noticed by Delbruck (1940).

Adsorption was found to depend on the motility of the bacteria and it was reported that adsorption rate constant may vary widely depending on the physiological state of the cell (Delbruck, 1940). Adsorption rate constants were found to vary with the physiological condition of the host or the growth phase of bacteria. Adsorption rate increases with increase in surface area of bacterial cell. Adsorption rate constants have been calculated using higher ($> 10^6$ cfu/ml) bacterial, and phage numbers. The

limitations in time for adsorption reaction require high bacterial concentrations (>10^6 cfu/ml) so that decrease in free phage is measurable (Delbruck, 1940).

Another approach to model adsorption is to use mean free time (MFT) (Wang *et al*., 1997). Wang (1997) defined a term called adsorption time or mean free time, denoted by $t_{A,}$ which is the time taken by a free phage to diffuse towards cell and adsorb to the cell envelope. The generation time for a phage was defined as the sum of the adsorption time and the latent period. Adsorption time was calculated by the following equation using mean free time approach:

$$t_A = \frac{1}{kN} \qquad (1)$$

Where,

k - Adsorption rate constant

N - Cell density

Mean free time represents the average length of a time a cohort of free phage requires to adsorb to host cells. Levin and Bull (1995) worked with heat killed susceptible bacteria, and used an initial concentration of phage and bacteria as 1.8 x 10^7 pfu/ml, and 3.55 x 10^7 cfu/ml respectively and the adsorption constant was found to be 6.24 x 10^{-8} ml/hr.

2.3.4 The Effect of Calcium Chloride on Phage-Host Kinetics

It has been found that certain phage require divalent cations to help in the process of adsorption. There are many bacteriophage that have been shown to require a higher concentration of calcium (divalent cations) at some stage in their growth cycle than the concentration required for the growth of the cells. The nature of these requirements also varies for a variety of bacteriophage. Rountree (1955) described phage which required calcium during multiplication of phage in staphylococcal cells. With a *Streptococcus lactis* phage (Cheery and Watson, 1949) and phage p465 of *Brevibacterium lactofermentum* (Oki and Ozaki, 1967) calcium was required for adsorption, and in some cases it was found for the stage of penetration. Watanabe *et al.* (1972) studied PL-I phage infection in *Lactobacillus casei*. They found that adsorption and intracellular multiplication of phage was independent of the $CaCl_2$ concentration. It was also found that for the penetration of genome inside the host cells Ca^{2+} ions were required by the phage; impairment of infection could not be reversed by later addition of calcium ions. The optimum pH for successful infection was 5.5 to 6.0 and optimum temperature was 30 ºC. Multiplicity of infection (MOI) is usually described as the ratio of number of virus particles to that of number of bacteria however Watanabe *et al*. (1972) used Poisson's law and the equation is same as given by Chimi (1958):

$$P(r) = [(P/B)^r / r!]\, e^{\frac{-P}{B}} \qquad (2)$$

Where,

P(r) - Proportion of bacteria (B) having MOI of r

P - Density of the phage

2.4 Mathematical Models for Phage-Host Interaction

Various studies have focused on the quantitative aspects of phage-host interaction. These studies are chronologically discussed in the following sections.

2.4.1 Introduction

Several previous studies have focused on the development of a mathematical model to depict the phage-host interaction. Most of these models were developed from the studies concerning with the potential of phage as a therapeutic agents against antibiotic resistant pathogens (Barrow and Soothill, 1997, Kasman *et al*. 2002, Levin *et al*. 1996, and Payne *et al*. 2000), especially studies dealing with infection of *E. Coli* cells by their specific phage (Hadas *et al*., 1997, Levin *et al*. 1977, Rabinovitch *et al*. 2002, and Rabinovitch *et al*. 1998). However some of the studies have been performed to model phage-host ecology in marine environment (Beretta *et al*. 1998, Beretta *et al*. 2000, Middleboe *et al*. 2000, and Middleboe *et al*., 2001) and rhizosphere (Ashelford *et al*., 2000, Ashelford *et al*., 2000, Burroughs *et al*., 2000, and Caldas *et al*., 1992). Models for environmental ecology, however, were more complex and include spatial heterogeneity. Most of the previous models for phage-host interaction can be broadly categorized in two different classes. First, there are models described by sets of continuous time ordinary differential equations (Beretta *et al*., 1998, Bremermann *et al*.,

1983). The other class of models describes the dynamics with the use of a set of delay differential equations (Beretta *et al*., 2000, Campbell, 1961, Lenski and Levin 1985, and Levin *et al*. 1977). Since infected cells lyse only after latent period, there is a delay in the emergence of progeny phage particles from infected hosts. The use of continuous time models, however, predict a continuous increase in the phage population irrespective of the latent period effect or delays present. In subsequent sections various studies of phage-host interactions are described and discussed in detail.

2.4.2 Models and Kinetic Studies

Accounts of one of the first kinetic studies of phage–host interaction come from the study performed by the Kruegger and Northrop (1930). They studied in detail an anti-stapylococcus bacteriophage acting upon a single strain of *S. aureus*.
They concluded that lysis is not a continuous process.

Kruegger and Northrop (1930) concluded that both bacteria and phage follow logarithmic growth rate, until lysis of cells starts. However, they found that the phage growth was faster and suggested the following model for the phage and bacterial growth. It was first mathematical description of a phage-host interaction.

$$\frac{P}{P_0} = \left(\frac{B}{B_0}\right)^n \qquad (3)$$

Where,

P - Density of phage at any time

P_0 - Initial density of phage

B - Density of bacteria at any time

B_0 - Initial density of phage

n – Parameter

It was also found from their study that bacterial growth is necessary for the production of the phage. They suggested that it was multiplicity of infection (MOI) that determined lysis. They found that when this ratio was between 125 and 200, instantaneous lysis takes place. It was later explained by Delbruck (1940) as lysis from without. Lysis from without takes place due to deformation in cell envelope by rapid adsorption of phage, and no progeny phage are formed. Kruegger and Northrop (1930) found that adsorption was very fast and after the lag period of about 0.5 hr, only 10-30 % of phage remains free in the solution. Kruegger (1930) found that the final titer of a lysate prepared by infecting bacteria with a varying number of phage falls in a relatively narrow range, with maximum differences of one log. The author found that there exists an equilibrium between extra-cellular and intracellular phage, and extra cellular phage forms a small fraction of total phage until the onset of the lysis. Kruegger (1931) studied the adsorption phenomenon quantitatively. He worked with heat killed susceptible bacteria, and live resting bacteria. Using heat killed susceptible bacteria the phage attached irreversibly to the dead bacteria. It was found that both live and dead bacteria did not differ in the rate of removing phage from the solution. The rates were calculated by the following formula:

$$k = \frac{1}{t[B]} \ln \frac{(P_0 - P_e)}{(P_t - P_e)} \qquad (4)$$

Where,

B - Bacterial concentration

P_0 - Initial phage concentration

P_e - Concentration of phage at the equilibrium

P_t - Concentration of phage at any time t

k - Adsorption rate constant which was found to be of the order 10^{-10} ml/min for both live and dead bacteria.

Schlesinger in 1932, studied the process of adsorption quantitatively, and calculated adsorption rate by the following equation:

$$k = \frac{2.3}{bt} \log \frac{n_0}{n_t} \qquad (5)$$

Where,

b - Bacterial concentration

n_0 - Initial phage concentration

n_t - Phage concentration at any time t

Schlesinger (1932) found an adsorption constant to be 1.2 x 10^{-9} ml/min at 15 °C and 1.9 x 10^{-9} ml/min at 25 °C while working on a coli phage. Schlesinger (1932) worked with heat killed susceptible bacteria, so the bacterial concentrations were same at all the times. Schlesinger (1932) measured adsorption rate constants with varying initial bacterial concentrations, reaction times, and phage concentrations. It was found that adsorption constant did not vary significantly for different conditions. The adsorption rate constant was found to be 1.3 x 10^{-11} ml/min for coli88 phage. He also worked with living bacteria, and times for the adsorption reaction were relatively low less than 12 minutes. In that

time bacterial population can be assumed constant. He worked with concentration of 10^8 cfu/ml while comparing adsorption rates for living and dead bacteria. It was found that, adsorption rate was 2.5 times higher for living cells than for dead cells. Schlesinger (1932) also estimated the size of phage particles from the laws of diffusion and adsorption rate constant values.

Ellis and Delbruck (1939) made significant contributions in the area of phage-host interaction studies by defining a classical one step growth experiment, which gives latent period and burst size simultaneously. This approach is still used to determine these parameters associated with any phage-host system. Ellis and Delbruck (1939) made measurements of adsorption up to the time of start of the first burst. It was found that for bacterial concentrations greater than 3 x 10^7 cfu/ml adsorption was very fast (70-90 % attachment in 10 min). Lag phase cultures were used to keep the bacterial concentration fairly constant. It was also found that rate of attachment was first order with respect to phage and bacterial concentration, over a wide range of concentrations, in the agreement with the results provided by Kruegger (1931). Ellis and Delbruck, 1939 found that concentration of free phage (P_f) followed the equation:

$$-\frac{dP_f}{dt} = k_a (P_f) (B) \qquad (6)$$

Where,

k_a - Adsorption rate constant

B - Density of susceptible bacteria.

k_a was found to be 1.2 x 10^{-9} cm^3/min at 15 ºC and 1.9 X10^{-9} cm^3/min at 25 ºC.

The equation they used for describing adsorption is based on simple law of mass action and is often used in epidemiological studies or predator-prey interaction studies. They found that the adsorption followed the equation accurately until more than 90 % attachment was accomplished, then attachment rate was reduced. Ellis and Delbruck (1939) defined the latent period, rise time and burst size. The eclipse period was discovered by a procedure devised to disrupt infected bacteria before spontaneous lysis and without damage to the mature phage, using chloroform. Ellis and Delbruck (1939) studied the effect of temperature on the latent period of cells. They showed that latent period depends on the growth rate of bacteria. Another interesting observation was that individual bursts varied in size from a few particles to several hundreds. Ellis and Delbruck (1939) found that a difference was seen between plaque assay's results obtained by plating free phage to that of already adsorbed phage. In one step growth experiment they found the initial increase in phage titer was not due to lysis. The initial increase was 1.6 times and was explained by the efficiency of plating, which they defined as the increase in probability of plaque formation. This probability increased if phage were already adsorbed to cells. Delbruck, (1940) also found that the adsorbed phage will result in a higher titer than the free phage, because adsorption of phage is the first step in the process of lysis.

Delbruck (1940) for the first time paid attention to the details of the adsorption process, especially the dependence of the adsorption rate constant on the physiological state of the cell. The size of bacteria changes considerably depending on the phase of growth in a given culture medium, and an increased cell surface will lead to an increase in adsorption rate. Adsorption rate constants was found to vary between wide limits

depending on the physiological condition of the host. Delbruck (1940) introduced the concept of lysis from within and lysis from without. Lysis from without occurred by the rapid adsorption of phage to cells at a threshold limit, which is also equal to the adsorption capacity of bacteria, and resulted in deformation in the cell envelope. No phage were produced in this case, and adsorbed phage were lost. Lysis from within occurred by a single or few phage, which multiply inside the cell and then cell is lysed liberating mature phage particles.

Campbell (1961) developed a simple model for phage- host dynamics considering the effect of delay in the production of new phage by lysis. However he considered total bacterial density, and did not separate cells into susceptible and infected categories. He found a steady state solution for the coexistence of phage and host. The model developed by Campbell (1961) is described below:

$$\frac{dS(t)}{dt} = \alpha S(t)[1 - \frac{S(t)}{C}] - KS(t)P(t)$$
$$\frac{dP(t)}{dt} = bKS(t-T)P(t-T) - \mu_p P(t) - KS(t)P(t) \qquad (7)$$
$$I(t) = \int_{t-T}^{t} KS(\theta)P(\theta)d\theta$$

Where,

S (t) - Susceptible bacterial density at time t

P (t) - Phage density at time t

b - Burst size

T - Latent period

μ_p - Phage mortality rate

K - Adsorption constant

α - Growth rate of bacteria

I (t) – Infected cell density at any time t

In this model infected bacteria are not competing in the logistic equation with bacterial growth. Bacterial growth models are an important part of phage-host models. The Logistic model is used here to describe bacterial growth. However there are growth models such as developed by Baranyi *et al*., (1992), and Baranyi *et al*., (1994) which take into account the effect of environmental variations, and also describe lag period as an adjustment period prior to bacterial growth. In these models the pre-inoculation environment of cells is denoted by E1 and the post inoculation environment is denoted by E2. Considering E1 as significantly different from the growth environment, (generally more favorable from the actual environment E2) a model was developed that described lag as an adjustment of the cells to the new environment. These kinds of models may be useful to describe bacterial growth where the environmental conditions or habitat is not homogeneous throughout. A non-autonomous differential equation model is employed for the purpose.

It has been observed that many of the models of predator-prey interactions used by ecologists have solutions specifying stable equilibrium points or stable limit cycles. Levin *et al.* (1977) presented a model of phage – host interaction which is based on specific assumptions about the habitat, the use of primary resources, the population growth, and the nature of interaction between bacteria and phage. Levin *et al*., 1977 found conditions for coexistence in a one resource, one host and one phage system. They also found conditions for coexistence in one resource, two host, and one phage system.

In their model they assumed that only one phage attaches to bacterium, and they also considered the delay effect in the development of progeny phage from "consumption" of one host. The law of mass action was used to describe the phenomenon of adsorption and adsorption constant was found to be 6.24 x 10^{-8} ml/h. Levin *et al.* (1977*)* used delay differential equations to model the dynamics and found an equilibrium for phage and host. In their study they emphasized the equilibrium and steady state solutions for a chemostat model, which indicated the coexistence of phage T2 and its corresponding prey *E. coli*. They also considered two bacteria, one of which was phage sensitive, and other phage resistant, and found coexistence. Coexistence between *E. coli* strains and their corresponding phage T2, T3, T4 etc. has also been found by other studies (Paynter and Bungay, 1969, and Horne, 1970).

Lenski and Levin (1985) presented a model that was the extension of the model by Levin *et al.* (1977), and Campbell (1961). The model is quite similar to that of Levin *et al.* (1977), except for few changes, and is described below:

$$\begin{aligned}
\frac{dr}{dt} &= \rho(C - r) - \varphi(r)\varepsilon n \\
\frac{dn}{dt} &= \varphi(r)n - \rho n - Knp \\
\frac{dm}{dt} &= Knp - \rho m - e^{-\rho L}Kn(t - L)p(t - L) \\
\frac{dp}{dt} &= Be^{-\rho L}Kn(t - L)p(t - L) - Knp - \rho p
\end{aligned} \tag{8}$$

Where,

B - Burst size

K - Adsorption constant

ρ - Rate of entry of resources in the habitat, also equal to washout rate from the habitat

φ(r) - Multiplication rate of bacteria which is increasing function of resource concentration.

C – Concentration of resource entering the habitat

r – Concentration of resource within the habitat

n – Density of bacteria

m – Density of infected bacteria

p – Density of phage

L – Latent period

ε – Amount of resource used for production of each bacterium

These equations differ from the Lotka-Volterra predator-prey equations in two respects:

1) The prey's growth rate is a function of a potentially limiting resource;
2) There exists a time lag associated with the predator's numerical response.

Equilibrium usually occurs at low cell densities of the order 10^4 to 10^5 cfu/ml.

Lenski and Levin (1985) expanded the formal theory of interactions between virulent phage and bacteria by incorporating mutational events into the dynamics. Theoretical considerations lead to specific predictions about the time course of evolutionary changes in the relationship between bacteria and virulent phage, and to more general predictions about the consequences of evolutionary constraints for their coexistence.

Beretta and Kuang (2001) proposed a mathematical model for marine bacteriophage infection with a latency period. This model was developed as an extension of their previous model (Beretta *et al.,* 1998), in which they did not consider the delay

effect during the latent period of the model. However introduction of the delay term during latency was not a new approach and had been used by several researchers in previous studies (Campbell, 1961, Lenski and Levin, 1985, and Levin and Bull, 1996). Levin *et al.* (1977) also included a delay term in their model. The model presented in Beretta *et al.* (2001) is more or less similar in all respects to the models previously developed (Levin *et al*., 1977). However one difference in the model presented by Beretta *et al*. (2001) was that it included a mortality rate for infected bacteria. This was needed for the marine bacteriophage infection case and may not be significant for other models developed. It also allowed for a supply of free viruses from the environment. Beretta *et al.* (1998, 2001) have studied these models from a mathematical point of view and have described different behaviors for different conditions. The model proposed by Beretta and Kuang (2001) is described below:

$$\begin{aligned}
\frac{dS(t)}{dt} &= \alpha S(t)[1-\frac{S(t)+I(t)}{C}]-KS(t)P(t)\\
\frac{dI(t)}{dt} &= -\mu_i I(t)+KS(t)P(t)-e^{-\mu_i T}KS(t-T)P(t-T) \qquad (9)\\
\frac{dP(t)}{dt} &= \beta-\mu_p P(t)-KS(t)P(t)+be^{-\mu_i T}KS(t-T)P(t-T)
\end{aligned}$$

S - Susceptible cell density

I - Density of infected cells

P - Density of free phage

α - Growth rate of bacteria

T - Latent period

b - Burst size

C - Carrying capacity

β - Parameter for entry of free viruses from environment

μ_I - Additional mortality rate of infected cells due to protozoan grazing

Beretta and Kuang (1998) in their previous paper described a model for phage-host interaction with no delay terms included. The model developed is described below:

$$\begin{aligned} \frac{dS}{dt} &= \alpha S(1-\frac{N}{C}) - KSP \\ \frac{dI}{dt} &= KSP - \lambda I \\ \frac{dP}{dt} &= -KSP - \mu P + b\lambda I \end{aligned} \qquad (10)$$

Where,

S – Density of susceptible bacteria

I – Density of infected bacteria

P – Density of phage

C - Carrying capacity of bacteria

α - Growth rate

b - Burst size

λ - Lysis rate.

The model is described by three nonlinear differential equations, and the sophisticated features such as modeling of the latent period using the delay terms were omitted. The model is quite similar to the susceptible-infected-recovered (SIR) model used frequently in epidemiology (Beretta *et al*., 1998). Bacteriophage infection causes bacterial lysis, which releases an average of b viruses per cell (Burst size). In the model developed b

was taken as the main parameter on which the dynamics of the infection depends. It was found that there exists a positive endemic equilibrium which represents the bacteriophage invasion of the bacteria whenever $b > b^*$(threshold value of burst size). The threshold value of burst size was found to be 16.0. Whenever $b < b^*$, the system approaches the infection-free stable equilibrium. Bremermann (1983) proposed a similar model, which is described below:

$$\begin{aligned} \frac{dS}{dt} &= \alpha S(1-\frac{S}{C}) - KSP \\ \frac{dI}{dt} &= KSP - \lambda I \\ \frac{dP}{dt} &= -\mu P + b\lambda I \end{aligned} \tag{11}$$

Where,

S – Density of susceptible bacteria

I – Density of infected bacteria

P – Density of phage

C - Carrying capacity of bacteria

α - Growth rate

b - Burst size

λ - Lysis rate.

In the model proposed by Bremermann (1983), the contribution of infected cells to the carrying capacity was not considered, and free phage loss due to adsorption was neglected.

2.4.3 Studies on Phage Therapy

Phage therapy is the use of bacterial viruses (bacteriophage) to treat bacterial infections. It has been practiced sporadically on humans and domestic animals for nearly 85 years since d'Herelle envisaged the possibility of using phage as therapeutic agents around 1917. Nevertheless phage therapy has remained outside the mainstream of modern medicine, presumably because of doubts about its efficacy, and possibly because it was eclipsed by antibiotics and other therapeutic agents (Levin and Bull, 1996). Studies of the bacteriophage as therapeutic agents have had mixed and unpredictable results. However Barrow and Soothill (1997) pointed clearly to the potential of phage therapy for use against antibiotic resistant bacteria under certain circumstances. Payne *et al.* (2000) predicted therapeutic response on basis of a simple mathematical model involving biologically meaningful parameters, which have to be measured experimentally before applying them to model to particular study systems. The model was described as follows:

$$\begin{aligned} \frac{dx}{dt} &= ax - bvx - H(t)x \\ \frac{dy}{dt} &= ay + bvx - ky - H(t)y \\ \frac{dv}{dt} &= kLy - bvx - mv - h(t)v \end{aligned} \qquad (12)$$

Where,

x - Density of susceptible bacteria

y - Density of infected bacteria

v - Density of phage

a - Growth rate of infected as well as uninfected bacteria

K - Adsorption constant

L - Burst size

k - Lysis rate

m - Decay rate for free phage

H(t) – Host (host for bacteria) responses to bacteria

h(t) – Host responses to phage

Some studies of bacteriophage therapy (Anderson and May, 1992) have assumed that even infected cells can also divide if the lysis time is greater than the doubling time or the time for cell division and thus they described the concept of the basic reproductive number for phage. This assumption may not be correct because as soon as cell becomes infected, phage-encoded proteins are expressed, and cell metabolism is used for phage production. Payne *et al. (*2000) solved these equations using computer simulations to give variety of predictions based on initial conditions and given parameters. However they did not validate the model by doing studies in the laboratory and used parameter estimates from other studies (Levin and Bull, 1996). In this study variability was explained by various density dependent threshold effects. A simple mathematical model was developed to capture the critical replication and density dependent qualities of the bacteriophage-host interaction for the lytic phage. It was reported that there was a threshold density of bacteria that must be present in order for the virus numbers to increase, which has been referred as the proliferation threshold (X_p). Density dependent threshold effects were also found in other studies (Wiggins *et al.*, 1985). Wiggins *et al*. (1985) found that there exists a delay in phage emergence when the host cell densities are

lower than threshold value, which will be typically equal to 1 x 10^4 cfu/ml. It was suggested that it may be due to the increased time required by the phage to adsorb to a host or find a host. Two different thresholds for the phage inocula are also derived as V_i, the phage inundation threshold and V_c, the clearance threshold and these are defined below. For V (initial dose of phage injected in mouse) > V_i phage will be able to reduce bacterial numbers to some extent, while for V > V_c phage will be able to eradicate the bacteria. Depending on the above thresholds and the time of inoculation of phage results of the experiments are categorized.

Levin and Bull (1996) developed a mathematical model to describe the relative effects of antibiotics and phage as a control measure against *E. coli* infection in mouse. The model developed was the extension of the model developed by Antia *et al.* (1994), and that of Levin *et al.* (1977) for a study of the population dynamics of phage-host interaction. They considered mouse as a homogeneous and essentially dimensionless habitat in which there are up to five interacting populations or substances; density of phage sensitive bacteria, density of phage resistant bacteria, intensity of mouse's immune response against the bacterium, density of phage, and concentration of antibiotics. They described the whole process by a set of five ordinary differential equations. The model did not include a delay term or a term for phage mortality. They assumed spontaneous lysis of infected cells. The model developed by Levin and Bull (1996), indicated that the combined immune defenses and phage should clear the infection rapidly. However this was not true and they postulated that the inconsistency between the model and the experiment can be attributed to assumptions they made, i.e., phage infection parameters are independent of the physiological state of the bacteria. They suggested that if they

modified parameters associated with phage i.e., adsorption and burst size and allow them to decline with time, they would better mimic experimental results.

It has been observed in several studies that when the host cell density is low, on the order of 10^4 cfu/ml or cells/ml, there is a much longer delay before phage numbers increase over the number of the input phage. This period has been explained as the time needed for the host cells to reach the replication threshold density, which was defined as the minimum concentration of bacteria which is required for phage production to occur (Payne *et al.* 2001, Payne *et al.* 2000, Wiggins *et al.* 1985). Kasman *et al.* (2002) found that there is no necessary threshold density required for the phage replication to occur. The delay in increase of phage numbers in case of low host cell densities was due to the decrease in adsorption. Kasman *et al.* (2002) considered the possibility that the apparent threshold density they observed was due to quorum sensing via molecules that were secreted by bacteria into the environment. These are soluble signaling molecules that alter the expression of dozen of genes and thereby regulate the metabolic state of the host cell when the sensing bacteria are exposed to them at a sufficient concentration. Quorum factors could therefore explain the dependence of the phage replication on cell density if, for example molecules that serve as phage receptors are expressed in response to quorum sensing, however no detectable quorum effect on the ability of phage to replicate was found. Kasman *et al.* (2002) studied phage infection mechanisms of M13 and P1 in *E. coli.* Kasman *et al.* (2002) explained the apparent threshold density due to the decrease in adsorption which depends on chance encounters between phage and host and thus in turn depend on the host cell and phage numbers. Phage rely entirely on chance encounters with their hosts, and so in liquid culture their ability to infect and reproduce

can be entirely predicted by the equations that describe the movements and coagulation (irreversible binding) of inert colloidal particles under the influence of Brownian motion (Schlesinger *et al.,* 1932). This is the same mechanism as described by the law of mass action used in several studies of phage adsorption.

$$P = P_0 e^{-kCt} \tag{13}$$

Where,

P - Density of free phage

P_0 - Total phage density

C - Density of the host cell

K - Adsorption constant

t - Adsorption time.

Kasman *et al.* (2002) considered in the equation that t (the time for adsorption) is sufficiently smaller than the culture doubling time so that the bacterial concentration remains fairly constant. Variations between the phage host systems due to the number of phage binding sites per cell, the diffusion rate constant of the virus and the efficiency with which the collisions between cells and phage result in infection are accounted for the empirical determination of the adsorption constant k for each system. Kasman *et al*. (2002) introduced the concept of MOI_{input} and MOI_{actual}. Traditionally, MOI is defined as the average number of virus particle infecting each cell, and is calculated as the ratio of the number of virus particles to the number of host cells. MOI_{actual} is the actual number of phage particles expected to bind per cell under the experimental conditions. Traditional MOI in a particular experiment i.e. MOI_{input} is the maximum possible MOI that can be

experienced by a cell, as it is not necessary that at the end of the adsorption period all the phage will bind to available cells. MOI input is average number phage per bacterium, if all phage bind at the end of adsorption period, then only MOI input will be equal to MOI actual.

$$MOI_{actual} = \left(1 - e^{kCt}\right) MOI_{input} \tag{14}$$

$$P_{bound} = \left(1 - e^{-kCt}\right) P_0 \tag{15}$$

C is the minimum concentration of bacteria at which MOI_{actual} will equal MOI_{input}

$$C = \frac{9.2}{kt} \tag{16}$$

When infection of all the cells is desired, an MOI of 10 is often used. It can be calculated from the basic probability that an MOI of 10 gives each cell a better than 99.99% chance of being infected by at least one viral particle. Kasman *et al*. (2002) study showed that for every host cell concentration there exists some number of phage that will be sufficient to cause the infection and replication. They found that below a minimum concentration of host cell density, number of phage to infect host cells will almost be same irrespective of how low is the cell density below the given level. MOI_{actual} was determined by Kasman *et al*. (2002) assuming that adsorption rate constant is independent of the cell density.

One of the reasons for the failure of phage therapy may be explained by the fact

that bacteria rapidly evolve resistance to phage attack (Bohannan *et al*., 2000). Antagonistic co-evolution is believed to play a pivotal role in structuring phage and host population dynamics. Co-evolution is largely driven by directional selection, with hosts becoming more resistant to a wider range of phage and phage becoming infective to wider range of hosts (Buckling and Rainey, 2002).

2.4.4 Models Specific to *E. coli*

Most of the previous studies have concentrated on the phage-host kinetics for the *E. coli* cells and their corresponding bacteriophage i.e. λ, T4 etc. All time and kinetic parameters differ with interacting species. Hadas *et al.* (1997) found that bacteriophage development depends on the physiology of host cells. They found that the parameters of phage development and cell lysis were growth rate dependent. The rate of phage release and burst size increased as the eclipse period and latent periods decreased and growth rate was increased. Hadas *et al.* (1995, 1997) investigated the intracellular growth of phage T4 inside *E. coli* cells. They determined kinetic parameters of intracellular T4 growth under different physiological states of cell. To distinguish between the possible effects of medium composition and those of cell size, low penicillin concentrations were used, which specifically block cell division (Hadas *et al.*, 1995). A competitive inhibitor of glucose uptake, methyl α-D-glucoside, was exploited to reduce cell growth rate. Hadas *et al.* (1995, 1997) focused on the intracellular growth of phage, and understanding of intracellular kinetics is required to model phage-host dynamics. Since kinetic parameters

depend on the physiological state of cells, model results should be applied to similar conditions as were used for the model development.

Rabinovitch *et al.* (1998), determined mathematical relationships for the number of mature T4 phage both inside cells and after lysis of an *E. coli* cells as a function of time after infection by a single phage with following parameters:

a) Delay time until first bacteriophage is completed inside the cell (also known as eclipse period)
b) Standard deviation of eclipse period.
c) Rate at which number of bacteriophage increase inside the cell.
d) Latent period or time when cell lysis takes place.
e) Standard deviation of latent period.

They used a destruction parameter to show the adverse effect of chloroform on phage. There are different burst times of individual cells (Ellis and Delbruck, 1939). These times are normally distributed, so plaques formed per bacterium should increase as *erfc* [(q-t)/Δ], where q is latent period or burst time and 2Δ is the width of distribution.

Currently, efforts are being made to completely understand the mechanism of bacteriophage infection of *E. coli* cells. "Three independent parameters eclipse period, latent period, and rate of phage maturation inside cell are essential and sufficient to describe bacteriophage development in its bacterial host (Rabinovitch *et al*., 2002)". Rabinovitch *et al.* (2002) hypothesized that these parameters depend solely on the culture doubling time (τ) before infection. The experimental values determined for eclipse and latent periods were plotted against the culture doubling time. Phage assembly rate was

plotted with its reciprocal (i.e., bacterial growth rate), and a good correlation was obtained with the predicted values.

They concluded that burst size depends on the synthesis of the phage components, and lysis time. The rate of synthesis and assembly of phage components depend on the contents of PSS (protein synthesizing system) and lysis time on the cell dimensions:

$$B = \alpha[\mu - \nu] = [0.254\tau - 0.00166\tau^2]e^{(92.1/\tau)} \qquad (17)$$

Where,

α - Rate of maturation of phage inside the cell

μ - Latent period

ν - Eclipse period

B - Burst size

τ - Doubling time for the culture

They found in their experiments that when the doubling time for culture approaches 160 min, the eclipse period becomes equal to the latent period and no production of phage will be anticipated, however they did not test this hypothesis. Also for very short doubling times difference between latent period and eclipse period tended to zero, and therefore should prevent phage production, but they found that in this case phage maturation rates can give higher burst sizes than expected. The recent finding that burst size of the temperate bacteriophage of *E. coli*, λ is larger in faster growing cells (Gabig *et al.,* 1998) extended the validity of the conclusions of this paper.

2.5 Additional Observations about Phage-Host Kinetics

Abedon *et al.* (2001) defined the term 'Latent period optima'; phage latent period is the time period between adsorption and phage induced host cell lysis, which is typically under control of a phage protein complex known as a holin. Holins control the activity of cell wall digesting endolysins, and mutations in holin genes can significantly modify the timing of the host cell lysis. Phage generation time and burst size are the functions of latent period, where larger burst size associated with longer latent periods and a shorter generation time associated with shorter latent periods. Therefore the latent period optimum is the latent period which maximizes the population growth of a specific phage. Abedon *et al.* (2001) compared two studies regarding the phage-host interaction performed by Abedon *et al.* (1989) and the one by Wang *et al.* (1996). There have been two schools of thought to describe the adsorption of phage to cells. One school of thought uses the exponential free phage decay approach (Levin *et al.,* 1977; Abedon *et al.* 1989), while Wang *et al.* (1996), described adsorption by using mean free time approach (MFT):

$$t_a = (kN)^{-1} \tag{18}$$

Where,

t_a - Mean free time

k - Adsorption rate constant

N - Cell density

It was found that exponential free phage decay approach better describes the adsorption

of a free phage cohort than does MFT, and MFT may not be adequate in describing phase adsorption, particularly at low host densities (Abedon *et al*., 2001). Phage generation time was defined as the sum of mean free time (adsorption time) and latent period. Phage growth rates are the function of both the length of the phage generations and phage burst size. Phage generation time is a function of the latent period and the rate of phage adsorption. Consequently the preponderance of phage population growth is made by those phage that, by chance adsorb their hosts sooner. Using MFT as an adsorption algorithm ignores these earlier adsorptions treating all adsorption as delayed to some average value (Abedon *et al*., 2001). The author employed the phage growth model by Levin *et al.* (1977) excluding the resource terms, and simulated it using arrays to describe the delay present. Latent period optima were calculated by simulations and explicitly considering phage growth by following equation:

$$P_t = P_0 B^{\left(\frac{t}{t_g}\right)} \qquad (19)$$

Where,

P_t - Phage density at time t

P_0 - Initial phage density

B - Burst size,

t_g - Generation time which is equal to sum of adsorption time or MFT (t_a) and latent period

Abedon *et al*. (1997) considered different growth media to determine the latent period

optima as a function of host quality. They found that lower host densities select for longer latent periods and higher host densities selects for shorter latent periods. It was found that for a given host density a decline in host quality leads to longer latent period optima.

Rabinovitch *et al*. (2002) found that kinetic parameters of phage-host interaction are dependent on the growth rates of host cells. Rabinovitch (2002) empirically determined latent period and burst size as a function of cell growth rate only, Hadas *et al*. (1997) studied variation in all kinetic parameters with growth rate by changing nutrient conditions and medium of growth of the cells.

Few studies have focused on the modeling of more intricate details of phage-host interaction, including, what happens inside infected cell, the result of which is lysis, or modeling why there is variability in cell lysis times for individual cells. However some of the studies have focused on these intricacies example of which is study conducted by Rabinovitch *et al.* (1999). In this study, the authors considered the importance of cell size, envelope thickness and lysozyme eclipse time on the final probability distribution of lysis.

Studies dealing with some other virus-host interactions may also be of some help to better understand the phage-host intracellular dynamics. An example of such a study is the study by Petricevich *et al*. (2001). In this study an insect cell-baculovirus expression system was modeled. The design of optimum infection conditions should be based on a thorough understanding of the mechanisms ruling cell-phage interactions. The rate of infection was found to be limited by baculoviruses adsorption to the cells, and mean time for this step was of one order higher magnitude than the other genome

penetration steps in the model. The model was developed to investigate the parameters governing virus binding to develop strategies aimed at reducing adsorption time and thus increasing infection rate.

Phage lysis of specific bacterial populations has been suggested to be an important factor for structuring marine bacterioplankton communities (Middleboe *et al*., 2001). The population dynamics of bacteriophage and four marine bacteria were studied, both by the batch culture and continuous culture experiments. Experiments were performed in two systems, one with added phage and other control without phage by Middleboe *et al.* (2001). State variables were bacteria, phage, protozoa, and dissolved organic carbon. The processes that were modeled were predation, uptake, respiration, infection, basic metabolism, nutrient addition, and dilution. The model was written as a function of state variables and parameters, which are theoretical estimates based on the ranges of empirically determined values obtained from the literature. It was found that viral lysis had only temporary effect on the dynamics and diversity of the individual bacterial host species. Following the inital lysis of sensitive host cells, growth of the phase resistant clones of the bacteria dominated and resulted in a distribution of bacterial strains in the phage enriched culture that was similar to the control culture after 50-60 hours incubation. After about 15 hours of the infection all the strains that were tested were phage resistant and it was consistent from the batch culture experiments. The re-growth of host cells in artificial seawater cultures with single-phage host systems indicated that mutations are more common in marine environment.

2.6 Conclusion

Biological systems are generally very complex. It is a challenging task to mathematically model any biological process. If the details of the process are clearly understood, a mechanistic model is desirable, with parameters having biologically significant meaning. The mechanism of phage-host interaction has been studied extensively and parameters are well defined. However, variation in parameters has been observed depending on the physiological condition of the host. It has also been found that parameters defining phage-host interaction also depend on the growth rates of hosts and host densities. Latent period and burst size are found to be interdependent. In the development of a model for phage-host interaction these factors may be considered.

Chapter 3

Materials and Methods

3.1 Bacterial Strains and Culture Media

Leuconostoc mesenteroides 1-A4 and *Leuconostoc pseudomesenteroides* 3-B11 strains were previously isolated from a commercial sauerkraut fermentation (Lu *et al*., 2003), and obtained from the USDA-ARS Food Fermentation Laboratory Culture Collection (Raleigh, NC). All bacterial stocks were kept at –84 ºC in MRS (Media for lactobacilli growth) broth containing 16% (v/v) glycerol. When required, frozen cultures were streaked on the fresh MRS agar (Difco), and overnight cultures (17 h) were prepared from the isolated colonies.

3.2 Phage

Phage 1-A4 and 3-B11 were enriched and isolated from commercial sauerkraut fermentation (Lu *et al*., 2003). These phage isolates were concentrated and purified (Lu *et al*., 2003).

3.2.1 Spot Test

The spot test method (Chopin *et al*., 1976) was used as an initial test for the presence of the phage by measuring the lytic activity. Three ml of soft agar was seeded with 0.1 ml of actively growing culture (2 x 10^8 cfu/ml), mixed gently, and poured on the MRS agar plate. After solidification, 10 µl of phage lysate was spotted on the lawn of bacteria. The plate was allowed to stand for 30 minutes before overnight incubation at 30 °C. A clear zone in the plate, resulting from the lysis of the host cells, indicated the presence of phage.

3.2.2 Lysate Preparation

A single plaque was picked and transferred into a tube containing 5 ml of MRS broth, 5 mM of $CaCl_2$, and an early log phase host culture at an optical density between 0.1-0.4. The tube was then incubated at 30 °C for 10 h. The phage lysate was centrifuged at 4000 x g for 10 min at 4 °C (Sorvall RC-5B centrifuge, Wilmington, DE). The supernatant was filtered using a 0.45 µm pore size syringe filter. The phage stock was then stored at 4 °C.

3.2.3 Determining Titer of Phage

Phage titer was determined as plaque forming units (Pfu)/ml using the double-layer agar plate method similar to that of Adams (1959). After appropriate dilution with saline, 0.1 ml of phage containing sample and 0.1 ml of actively growing culture (10^8 cfu/ml) were added to 3 ml of soft agar, (maintained at 48.5 °C in water bath) and 30 µl of 1 M $CaCl_2$ was also added, mixed gently and poured immediately on the MRS agar plates. Plates were kept for 30 minutes on the bench, to allow the agar to solidify, and the plates were incubated overnight at 30 °C. Bacteria forms a lawn on agar plate and phage lyse cells to form a zone of clearing. These zones of clearing are called plaques, these plaques are counted and titer is expressed in plaque forming units per milliliter (pfu/ml). This method for determination of titer is also known as double-layer agar plate method.

3.2.4 Optimizing Multiplicity of Infection (MOI)

Multiplicity of infection is defined as the ratio of number of virus particles to that of bacteria. *L. mesenteroides* 1-A4 was grown in MRS broth at 30 ºC to an optical density (O.D.) of 0.1 at 630 nm, measured using a spectrophotometer (Novaspec II Pharmacia LKB, Piscataway, NJ). This corresponds to an initial cell density of 5 x 10^7 cfu/ml. The early log phage cells were infected with MOI (multiplicity of infection) of (0.01, 0.1 and 1.0 pfu/cfu). After incubation at 30 ºC for 9 h, the phage lysates were centrifuged at 9000 x g for 5 min. The supernatant was filtered using a 0.45 µm syringe filter and assayed to determine the phage titer by using the method mentioned above.

The MOI resulting in highest phage titer after 9 h was considered as the optimal MOI and was used in subsequent large scale production of phage.

3.2.5 Preparation of New Lysate from Existing CsCl - Purified Phage

CsCl - purified phage were used for lysate preparation. An actively growing bacterial culture was infected at an MOI between 0.01-0.05 in MRS medium supplemented with 5 mM $CaCl_2$. The cell and phage mixture was then incubated at 30°C for 7 h. After 7 h, the cell-phage suspension was filter sterilized using a 0.45 μm syringe filter and the supernatant was stored at 4 °C. To determine the MOI, cell counts were correlated with the measured optical density using standard curves. Standard curves were made by measuring optical densities of MRS medium with growing cells and determining cell counts at regular interval of time. R^2 values for the standard curves of *L. mesenteroides* 1-A4 (Fig. 1) and *L. pseudomesenteroides* 3B-11 (Fig. 2) were 0.98 and 0.99 respectively. Viable cell counts were determined by using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Bethesda, MD) for plating samples on MRS agar, and a colony counter (Protos Plus: Bioscience International, Rockville, MD) for colony counting. Optical densities were measured using spectrophotometer (Novaspec II, Pharemacia LKB, Piscataway, NJ). Standard curve was then used to approximate the cell counts corresponding to a specific O.D. value. Titer of the lysates tends to decrease with time, so new lysates were produced monthly.

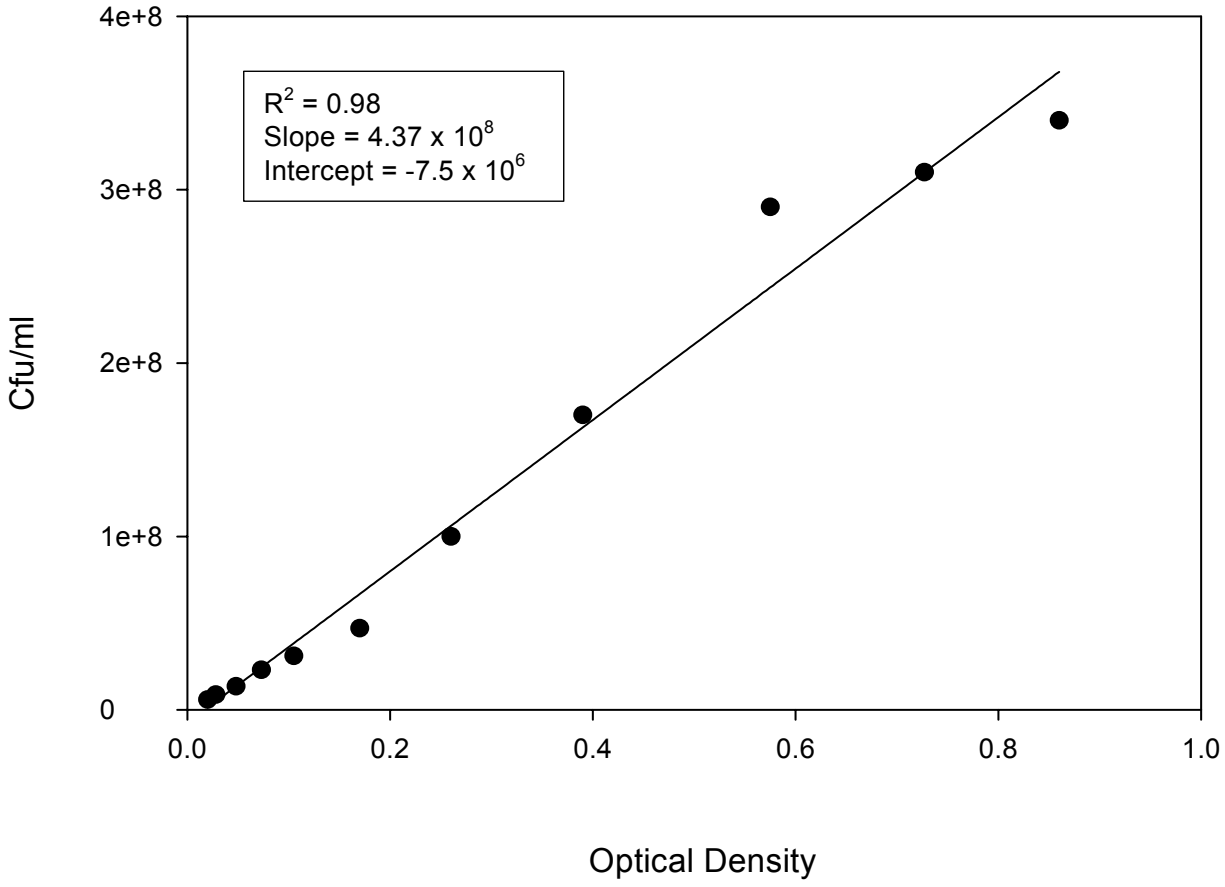

Figure 1 Standard curve for *L. mesenteroides* 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$ at 30 ºC

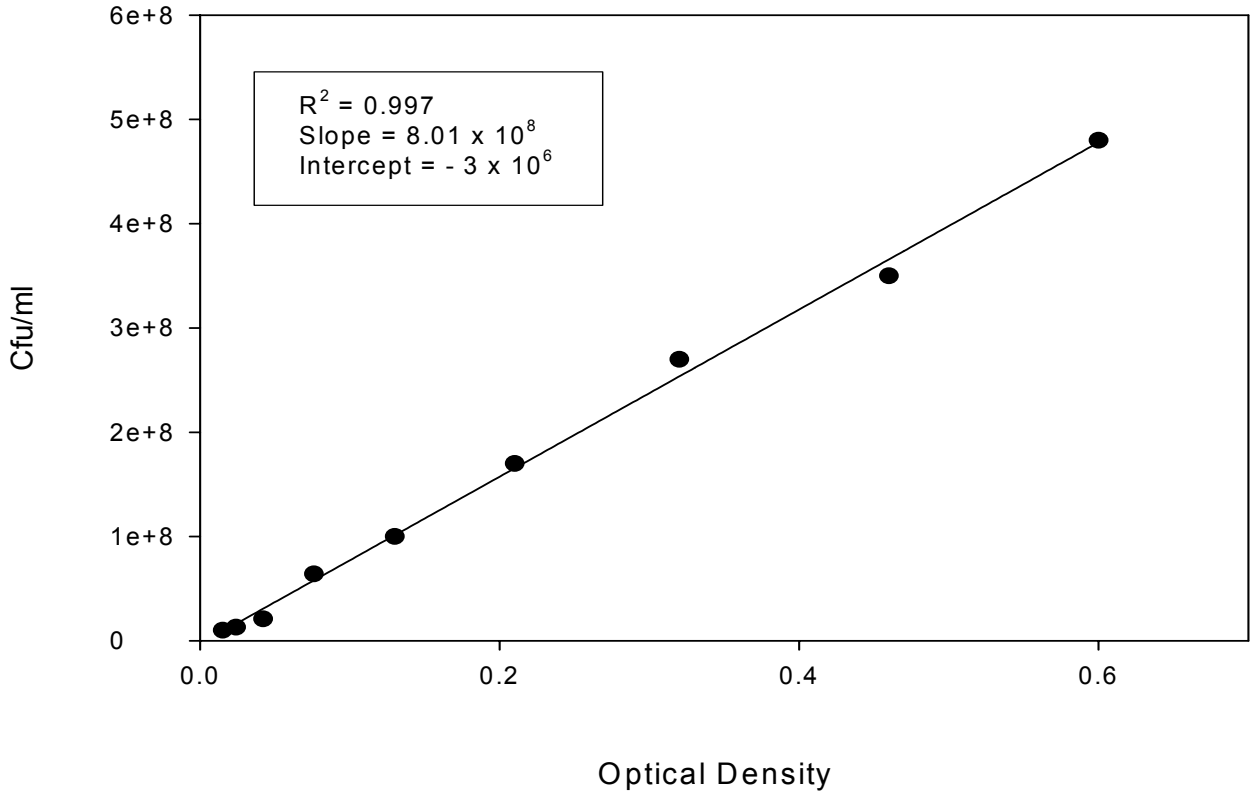

Figure 2 Standard curve for *L. pseudomesenteroides* 3-B11 in MRS medium supplemented with 5 mM $CaCl_2$ at 30 ºC

3.3 Determination of Parameters for Phage-Host Interaction

Parameters defining the phage-host interaction were bacterial growth rates, carrying capacity of bacteria, burst size, latent period and adsorption rate constant. These parameters were determined for phage-host systems studied, *Leuconostoc mesenteroides* 1-A4, *Leuconostoc pseudomesenteroides* 3-B11 and their corresponding phage 1-A4 and 3-B11 respectively. In the following sections, the methods for determination of these parameters are described.

3.3.1 Bacterial Growth Rates

Bacterial growth rates were calculated from the optical density measurements using, micro-titer plate reader, and also from viable cell counts obtained by spiral plating.

Twenty µl of the overnight culture (17 h) was diluted with 180 µl of MRS media and overlaid with 75 µl of mineral oil in micro titer plate wells. These fermentations were placed inside the micro titer plate reader (ELX 808 IU, Ultra Microplate Reader, Biotek Instruments Inc., Winooski, VT) and internal temperature was maintained at 30 °C, optical density was monitored every one hour up to 24 hours. To check for the variation in temperature, a data logger was used to monitor temperature and determine the variability. Optical densities of these 200 µl fermentations were measured by using micro titer plate reader. Eight replicates were performed for both bacteria *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11.

In addition to OD measurements, viable bacterial counts were determined using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Bethesda, MD). On MRS agar plates, colony counts were obtained by an automatic colony counter (Protos Plus: Bioscience International, Rockville, MD). Growth curves were made by measuring cell counts with time. From the growth data, data points in the exponential part of the curve were considered, linear regression was done and growth rate constants (μ) were calculated.

3.3.2 Effect of Calcium Chloride on the Bacterial Growth Rates

While monitoring the growth of bacteria L. mesenteroides 1-A4 in MRS media supplemented with 30 mM $CaCl_2$ it was observed that bacteria did not grow to the usual carrying capacity indicating an inhibitive effect of $CaCl_2$ on the growth rate of bacteria. The effect of $CaCl_2$ on the growth rates of *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11 was investigated. The growth rates for *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11 were determined in MRS medium supplemented with 0, 1, 2, 5, 8, 10, 12, and 15 mM $CaCl_2$. Above 15 mM, turbidity from $CaCl_2$ prevented OD measurements. Growth rates were evaluated using optical density data from Micro titer plate reader (ELX 808 IU, Ultra Microplate Reader, Biotek Instruments Inc., Winooski, VT), as described in 3.4.1. Eight replicates were performed.

3.3.3 Latent Period and Burst Size

One step growth experiments were performed as defined by Ellis and Delbruck (1939) to determine latent period and burst size. Host cells were infected with phage in

the early exponential phase [O.D. = 0.3] at an MOI of 0.04 approximately for *L. pseudomesenteroides* 3-B11 and at an MOI of 0.25 approximately for *L. mesenteroides* 1-A4. Five mM calcium chloride was added to aid adsorption. After allowing adsorption for 10 minutes, infected cells were pelleted by centrifugation at 8000 x g for 5 minutes at 4 ºC (Sorvall RC-5B centrifuge, Wilmington, DE). Pelleted infected cells were then resuspended in fresh MRS broth supplemented with 5 mM $CaCl_2$. Samples were taken every 5-10 minutes up to 2 h and were immediately titered by the double-layer agar plate method. Three independent replicates of one step growth experiments were performed for both phage 1-A4 and phage 3-B11 to observe the variation in parameters, latent period and burst size. Non-linear regression was done on the data using four parameter sigmoidal function using Sigmaplot v8.0. Latent period has been defined as the time between adsorption and the beginning of the first burst as indicated by the initial increase in the titer. The burst size was calculated as the ratio of final phage count at the end of latent period to the initial phage count.

3.3.4 Adsorption Rate Constant

Adsorption experiments were performed with the modifications to the method described by Ellis and Delbruck (1939). Instead of lag phase cultures, actively growing cultures were used. Bacterial cells were infected with phage at an early exponential phase and 5 mM $CaCl_2$ was added. Cell phage suspension was then filtered using 0.45 µm syringe filter to obtain free phage over time. Filtered samples containing free phage were then titered immediately using double-layer agar plate method at every 5 minutes up to time less than the first latent period. Free phage concentration obtained by titering

supernatant was then plotted with time. Independent adsorption experiments were performed by varying the optical density of cells at the time of infection and also the multiplicity of infection. For *L. mesenteroides* 1-A4 adsorption experiments were done at optical density of 0.20, 0.15, and 0.22 at MOI of 0.13, 0.09, and 0.1 respectively. For *L. pseudomesenteroides* 3-B11 adsorption experiments were done at optical density of 0.2, and 0.3 at MOI of 0.03, and 0.02 respectively. Data obtained was fitted with an exponential decay model using Sigmaplot v8.0, and adsorption rate constant was determined at the time zero using initial cell and phage densities.

3.4 Development of the Model

Leuconostoc mesenteroides 1-A4 and its corresponding phage 1-A4 were used for the development of model. The model was then validated using two phage-host systems: *L. mesenteroides* 1-A4, phage 1-A4 and *L. pseudomesenteroides* 3-B11 and phage 3-B11 respectively. Validation of the model was done in MRS media supplemented with 5 mM $CaCl_2$, and all the kinetic studies were performed at 30 ºC.

The initial model was similar to that described by (Beretta and Kuang, 1998). In this model the phage mortality term (other than lysis), was neglected. The authors used an additional phage mortality term, due to protozoan grazing, which was particularly applicable for their case, as they developed the model for marine bacteriophage infection. The model was described by a set of three ordinary differential equations (ODEs) and is described below:

$$
\begin{aligned}
N(t) &= S(t) + I(t) \\
\frac{dS}{dt} &= \alpha S(1 - \frac{S}{C}) - KSP \\
\frac{dI}{dt} &= KSP - \lambda I \\
\frac{dP}{dt} &= -KSP + B\lambda I \\
\lambda &= \frac{1}{L}
\end{aligned}
\tag{20}
$$

Three variables were: the density of susceptible cells (S), the density of infected cells (I), and the density of phage (P). K was the adsorption rate constant, N was the sum of the susceptible cells and the infected cells (total bacterial density at any time), α was the growth rate of bacteria, B was the burst size, L was the latent period and λ was the lysis rate. This model was similar in some aspects to other models developed for phage-host interaction assuming continuous lysis of cells (Bremermann, 1983; Payne *et al*., 2000; and Beretta, 1998). The rate of lysis was defined to be the reciprocal of the latent period. The assumptions made were as follows:

1) All of the cells were assumed to be sensitive to phage attack, 2) The medium was homogeneous and mixed, so that there was a random encounter probability between bacteria and phage, 3) Infected cells do not divide, 4) Only one phage infects a cell, and the rest all remain in solution, 5) In the absence of phage, the bacterial population can only grow to the carrying capacity, which is the maximum population which can be reached, 6) The adsorption rate constant does not depend on the physiological state of the cell, and cell density, 7) Lysis of cells takes place continuously, 8) All phage are capable

of binding to the cells. The kinetic parameters for *Leuconostoc mesenteroides* 1-A4 and corresponding phage 1-A4 were determined for this model including growth rate, burst size, latent period, carrying capacity, and adsorption rate constant. The model was integrated by using ODE23 solver in MATLAB v6.5.1 using experimentally determined parameter values. Predicted phage counts and infected cell counts were compared to plaque assay counts at different times. The sum of square error term (SSE) for the observed and predicted values was used to check the predictability of the model. In continuous time model predictions, there was no lag observed, however there existed a clearly defined lag in the experimental data. Lag was observed, soon after infection up to two latent periods. Phage numbers did not increase for the first latent period as was found with validation studies, whereas the model predicted a continuous increase in the phage concentration. To avoid this problem, a step function was used, so that there was no lysis up to the first latent period and thereafter lysis was assumed to be a continuous process. However in this case lysis rate was doubled and was twice the reciprocal of the latent period, and this reduced SSE values. The modified model is described below:

$$\begin{aligned}
&\frac{dS}{dt} = \alpha S(1 - \frac{S}{C}) - KSP \\
&\frac{dI}{dt} = KSP - a\lambda I \\
&\frac{dP}{dt} = -KSP + aB\lambda I \\
&\text{for } t \leq \quad a = 0, \\
&\text{else} \quad a = 1 \\
&\lambda = \frac{2}{L}
\end{aligned} \tag{21}$$

During the validation of the model, it was also found that the model predicted complete lysis of the cells and predominance of phage in the medium, but in validation experiments it was found that bacterial population decreased with time, and phage population increased. After a certain period of time, the bacterial population remained constant at low levels and then started to increase again, while the phage population remained constant. This indicated the presence of a resistant population of bacteria that was not susceptible to phage attack and grew continuously. This was verified in the laboratory by picking up surviving colonies from an agar plate containing actively growing cells, infected with very high phage concentration. These colonies were then grown and checked for phage resistance. Therefore a differential equation was added to describe the change in a resistant cell population. The additional variable improved the fit, and it was found that the value of adsorption rate constant was lower than what was observed in laboratory, and it decreased with time. As there was a delay observed up to two latent periods, and continuous lysis was not a mechanistic assumption. The model was further modified and instead of ODEs, delay differential equations (DDEs) were used.

Finally the model was described by a set of delay differential equations, which described the phage-host interaction mechanistically (Fig. 3). In the model bacteria was divided in to two classes: Susceptible and resistant cells. Susceptible cells are infected by phage, and after latent period are lysed to release an average of B phage per cell. Resistant cells grow unaffected by the presence of phage logistically. Assumptions were same as for our starting model with the following changes, 1) There is a population of resistant cells, that are not susceptible to phage attack 2) This population grows

logistically unaffected by phage 3) There is a delay present in the emergence of progeny phage from lysis of cells 4) Adsorption rate constant varies with time depending on the physiological state of the cells and cell densities. Definition and units of parameters and variables used in the model are listed in Table 1. The model developed is described below:

$$\begin{aligned}
\frac{dS}{dt} &= \alpha \cdot S\left(1 - \frac{S + I + M}{C}\right) - KSP \\
\frac{dI}{dt} &= KSP - a.KS(t - L)P(t - L) \\
\frac{dP}{dt} &= -KSP + a.B \cdot K.S(t - L)P(t - L) \\
\frac{dM}{dt} &= \beta.M(1 - \frac{S + I + M}{C})
\end{aligned} \tag{22}$$

S: The density of susceptible cells

I: The density of infected cells

P: The density of phage

M: The density of resistant cells.

Alpha (α): The growth rate of susceptible cells

β: The growth rate of resistant cells.

K: The adsorption rate constant, and K takes values depending on time based on step functions as follows:

For $0 \leq t < 2.5$; $K = K_0$

$2.5 \leq t < 3$; $K = K_1$

$3 \leq t < 4$; $K = K_2$

$4 \leq t$; $K = K_3$

C: The carrying capacity

B: The burst size

L: The latent period.

Model

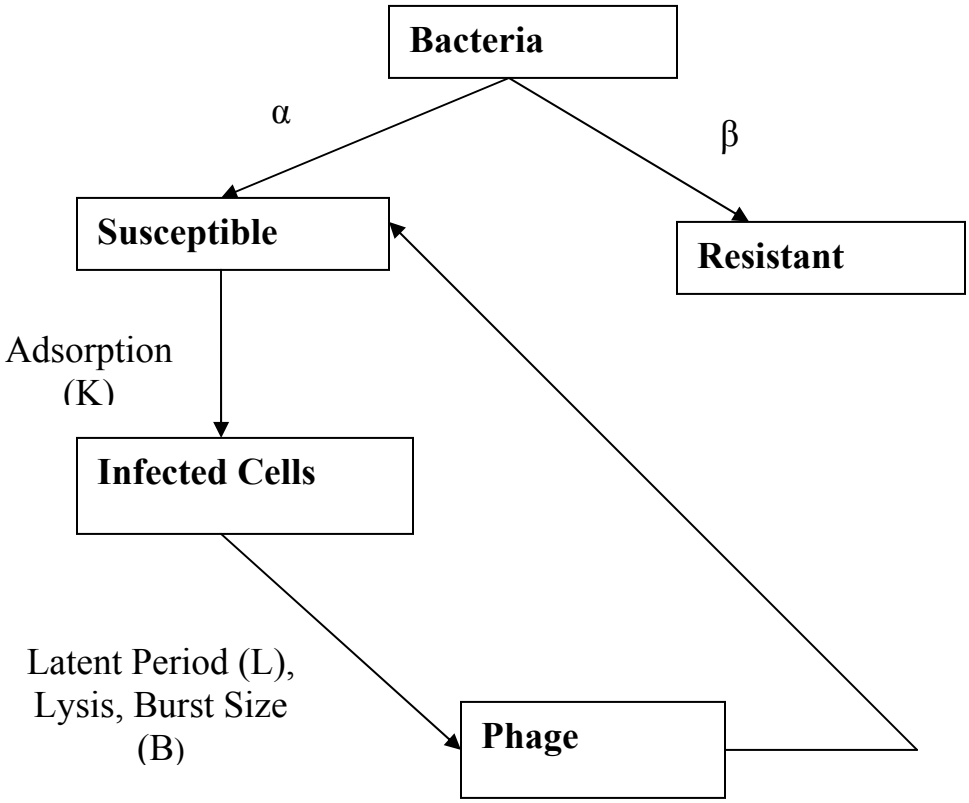

Figure 3 Flow diagram for the phage-host model

Table 1 Definition and units for parameters and variables in the model

Variable	**Definition**	**Units**
Phage Density (P)	Phage concentration in the medium	pfu/ml
Susceptible cell density (S)	Bacterial cell concentration which are susceptible to phage attack	cfu/ml
Resistant Cell density (M)	Bacterial cell concentration resistant to phage attack	cfu/ml
Infected Cells (I)	Cells which are infected by phage (these cells form plaque)	pfu/ml
Growth Rate of Susceptible bacteria (α)	Growth rate of susceptible bacteria	(h^{-1})
Growth rate of resistant bacteria (β)	Growth rate of bacteria which are resistant to phage infection	(h^{-1})
Latent Period (L)	Average time period between adsorption of phage to cells and their lysis	h
Burst Size (B)	Average yield of phage per lysis of a bacterium	Dimensionless
Carrying Capacity (C)	Maximum density of cells which is possible in the medium	cfu/ml
Adsorption Rate Constant (K)	Rate of encounter between phage and bacteria multiplied by the probability of successful infection	ml/h

Using a constant adsorption rate constant, the model predicted that phage quickly killed all the cells, and phage concentration then became constant. The data showed it took longer than the model predictions for the phage to kill all the susceptible cells (*L. mesenteroides* 1-A4 and phage 1-A4). The model was tested for changes in the parameter values. A constant value for the adsorption rate could not improve the fit, even if other parameters were changed. It was possible that the adsorption rate varied with time and decreased as cell numbers increased, which may be due to the decrease in number of the successful encounters between phage and cells due to their increase in number, or may be due to production of metabolites by the bacteria, affecting the ability of phage to adsorb. For this reason adsorption rate was varied with time using step functions which were then considered to be separate parameters. The model consisted 9 parameters with 6 specific parameters [α, β, C, K_0, L, B] and three other parameters were included which were [K1, K2, K3], these three parameters were the estimated values of the adsorption rate constant at different times (Page 57). From the results of parameter optimization it was clearly shown that for low initial cell concentration, the value of the adsorption rate was higher. The initial density of infected cells was always taken to be zero, and the initial concentration of resistant cells was taken as a percentage of total bacterial density. For *Leuconostoc mesenteroides* 1-A4, this ratio was $1/10^6$ of total cells, and for *Leuconostoc pseudomesenteroides* 3-B11, it was $1/10^5$. There was no significant difference between the growth rates of susceptible cells (α) and resistant cells (β), so these growth rates were considered equal, and number of parameters was reduced to eight. These modifications made the adsorption rate constant dependent on the initial

conditions and made the model predictions more accurate over wider range of initial phage and host concentrations.

3.5 Validation

Cells at a known initial concentration were infected with the known concentration of phage and 5 mM $CaCl_2$ was added to the cell phage suspension. Initial concentration of cells and phage were varied for different validation experiments for both phage–host systems (*L. mesenteroides* 1-A4 and phage 1-A4; *L. pseudomesenteroides* 3-B11 and phage 3-B11). The model was used to estimate the time after which the phage population became constant, based on initial conditions. Samples were taken every 20-30 minutes. Total cell counts were obtained by spiral plating the samples, and samples were titered at the same time to obtain total phage concentration. Cell and phage counts were taken up to several (6-12) hours depending on the initial cell and phage counts and the model predictions. Four different validation experiments were done for *L. mesenteroides* 1-A4 and phage 1-A4 and two validation experiments for *L. pseudomesenteroides* 3-B11 and phage 3-B11. Initial cell and phage concentrations for these validations experiments are listed in table 2.

Table 2 Different sets of initial conditions for phage host systems 1-A4 and 3-B11

	***Leuconostoc mesenteroides* 1-A4 and Phage 1-A4**			
Set #	Initial Susceptible Cell Density (S_0) (cfu/ml)	Initial Infected Cell Density (I_0) (cfu/ml)	Initial Phage Density (P_0) (pfu/ml)	Initial Resistant Cell Density (M_0) (cfu/ml)
1	8×10^7	0	8×10^7	8×10^1
2	7.5×10^7	0	2×10^6	7.5×10^1
3	2.66×10^7	0	2×10^7	2.66×10^1
4	7.5×10^5	0	1.4×10^3	0.75×10^0
Set #	***Leuconostoc pseudomesenteroides* 3-B11 and Phage 3-B11**			
1	4.8×10^7	0	1.31×10^5	4.8×10^2
2	2.92×10^6	0	2.92×10^2	2.92×10^1

3.6 Parameter Optimization

Parameters defining phage-host interaction were experimentally determined in the laboratory. In any biological system there is variability, even when the conditions are kept identical. The values determined for the parameters varied even under identical conditions, and a range for each parameter was obtained. Experimentally determined values of the parameters were taken as an initial set, and then parameters were varied using a random walk algorithm to minimize total sum of square errors between observed and predicted logs of cell and phage counts. Since cell and phage counts varied greatly from 10^0 to 10^{12}. When there is large variation between numbers, as it was in this study, a relatively small difference in observed and predicted value but higher in magnitude may result in very large SSE, even though the overall fit may be good between other data points. Therefore, all the statistics were applied to the log values of total phage and cell concentrations. The observed values from the validation studies were the total phage counts by plaque assays, and the total bacterial counts by spiral plating method. Total phage counts were compared with the sum of predicted phage density. Total bacterial counts obtained from spiral plating were compared with the sum of the predicted susceptible cells, and resistant cells as predicted from the model. The sum of squared error term (SSE) was calculated for the observed cell counts and sum of predicted susceptible and resistant cell counts and this statistic was denoted by SSE (cells). Similarly SSE (phage) was calculated for the observed total phage count and sum of predicted free phage and infected cell counts. The sum of squared error total [SSE (T)] was obtained by adding SSE (cells) and SSE (phage). SSE (T) was the statistic that was minimized while doing the parameter optimization. In the first approach of parameter

optimization, a set of parameters was selected randomly from an experimentally determined range of parameters. For every iteration, a random set of parameters was used to integrate the solutions of the delay differential equations using DDE23 solver in MATLAB v6.5.1 and then solutions were calculated at the time points, for which observed values were taken. This was repeated for all the experimental sets with different initial conditions. Then all the observed values, and corresponding predicted values were stored in an array. The array was then passed to a function which returned SSE (total) for all the values. Among all the iterations performed, parameter set which resulted in the least SSE (total) was stored. This routine was followed for different number of iterations and best results were noted. In the other approach, a random walk algorithm was used. In this approach, a set of parameter values was used as starting point, and steps were taken changing the value of each parameter randomly by a fixed step size specified manually by the user (Step size was different for each parameter depending on the magnitude to avoid scale differences). When the SSE (total) was reduced, the parameter vector (set of parameters) was updated as a new starting point. There is a common problem in this approach, if the parameter vector reaches a point, such that any new parameter vector resulting by taking steps from that position does not improve the solution, then the final answer will be the same. However it is possible that, there may be a different parameter vector which could further reduce the SSE (T). This problem can be referred to as being trapped in a local minima. To remove this problem, different starting points were used obtained from results of the first approach (as described above) and final solutions were compared. The parameter set obtained after parameter optimization was used to integrate differential equations for individual experimental

conditions. It was found that the SSE (T) remained high around 300 for the 4 sets of the phage-host system 1-A4 compared to SSE (T) values around 2 for an individual set, when a single parameter set was used to integrate the solutions for different experimental conditions. Parameters optimization was done for individual sets given an initial parameter vector and carried out for a specified number of iterations. Parameter sets resulting in reasonably low SSE (T) values (≈ 1-5), were recorded. A coefficient of variation was determined for each parameter for the same dataset. The average value of parameters were taken and used to integrate solution for each dataset and SSE (T) was calculated. A coefficient of variation was determined among the parameters for different datasets (experiments), by considering average values of parameters for a particular set.

Some of the parameters numerically determined from optimization were different from the experimentally determined values, which is possible due to two reasons. First, model may be incapable of simulating phage-host interaction exactly, and thus may need further modifications. Secondly, model is correct but experimentally determined values may not be accurate. Variation between parameters was observed for different validation experiments. It was found that variation of the initial number of phage and cells altered the adsorption rate constant. This rate was found to be higher with decreasing initial cell numbers, and the adsorption rate constant decreased with time. After running simulations it was found that the growth rate of the resistant cells and susceptible cells was not predicted to be significantly different. The model predictions were very good giving high R^2 (≈ 0.9-0.99) values for the fit between predicted and observed counts for the individual sets. R^2 values were calculated as the ratio of sum of squares regression to sum of squares total calculated between the predicted and observed data. R^2 was not adjusted for

degrees of freedom as almost same number of data points were used for calculation in each case.

It was found that after adjusting for adsorption rate constant based on the initial cell and phage density, it was possible to achieve good prediction ($R^2 > 0.85$) for all the conditions.

As seen in previous studies, kinetic parameters defining phage-host interaction vary with the physiological condition of host and growth rate (Hadas *et al*., 1997; Delbruck, 1940; and Rabinovitch *et al*., 2002). Thus, it is also possible that other parameters will change with time. However, in present study only adsorption rate constant was allowed to vary and other parameters were assumed to be constant. This resulted in an improved fit between predicted and observed data.

Chapter 4

Results

4.1 Experimental Determination of the Parameters

Parameters defining the phage-host interaction are bacterial growth rates, latent period, burst size, carrying capacity of bacteria, and adsorption rate constant. These parameters were experimentally determined, and the results are described in subsequent sections. These results provided realistic estimates of parameters defining phage-host interaction and were also used to determine starting values of parameters used for parameter optimization.

4.1.1 Bacterial Growth Rates

The bacterial growth rates were calculated by using the Gompertz function on the optical density data obtained by the micro titer plate reader. The specific growth rate of *Leuconostoc mesenteroides* 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$ was 0.30 h^{-1} and the growth rate of *Leuconostoc pseudomesenteroides* 3-B11 was 0.31 h^{-1} at 30 ºC. The standard deviation between eight replicates was found to be very small (< 0.001). Independent growth curves were also made by measuring the actual viable counts with time in addition to the optical density data to determine the variation in calculation of the growth rates.

For viable cell count data regression was done on the data points in the exponential part of the growth curve and the growth rate constants (μ) were determined.

Growth rate constants calculated from these growth curves were found to be 0.53 h^{-1} and 0.51 h^{-1} for *Leuconostoc mesenteroides* 1-A4, and *Leuconostoc pseudomesenteroides* 3-B11 respectively in MRS medium supplemented with 5 mM $CaCl_2$. The growth rates of resistant cells, calculated from the optical density data were found to be 0.30 h^{-1} and 0.27h^{-1} for *Leuconostoc mesenteroides* 1-A4 and *Leuconostoc pseudomesenteroides* 3-B11 respectively. From these results a range of values of growth rates was determined for both *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11 (Table 3). Carrying capacity was determined as the maximum cell concentration reached in MRS broth supplemented with 5 mM $CaCl_2$ for both bacteria *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11 respectively. These results are summarized in Table 3.

	***L. mesenteroides* 1-A4**	***L. pseudomesenteroides* 3-B11**
Susceptible Cells	0.3 – 0.53 h^{-1}	0.31 – 0.51 h^{-1}
Resistant Cells	0.3 h^{-1} (O.D. Data)	0.27 h^{-1} (O.D. Data)
Carrying Capacity	9 x 10^8 cfu/ml	8 x 10^8 cfu/ml

Table 3 Bacterial growth rates and carrying capacity in MRS media supplemented with 5 mM $CaCl_2$

4.1.2 Effect of $CaCl_2$ on the Growth Rates of *L. mesenteroides* 1-A4 and *L. pseudomesenteroides* 3-B11

For *Leuconostoc mesenteroides* 1-A4, growth rate decreased from 0.33 h^{-1} (S.D. = 0.0003) at 0 mM $CaCl_2$ to 0.26 h^{-1} (S.D. = 0.0007) at 15 mM $CaCl_2$. It indicated an inhibitive effect of calcium chloride on the growth rate of bacteria. It was observed, that the bacterial growth rate remained nearly constant (≈ 0.300) in a range of concentration

from 2mM to 8mM for *L. mesenteroides* 1-A4 (Fig. 4). This effect was also observed for *Leuconostoc pseudomesenteroides* 3-B11, where growth rate remained constant in range 4 mM to10 mM (Fig. 5). For *Leuconostoc pseudomesenteroides* 3-B11, growth rate decreased from 0.35 h^{-1} (S.D. = 0.0004) at 0 mM to 0.28 h^{-1} (S.D. =0.0004) at 15 mM $CaCl_2$ concentration in MRS media. Five mM Calcium chloride concentration was selected to help the adsorption of phage to cells in order to facilitate phage-host kinetic studies.

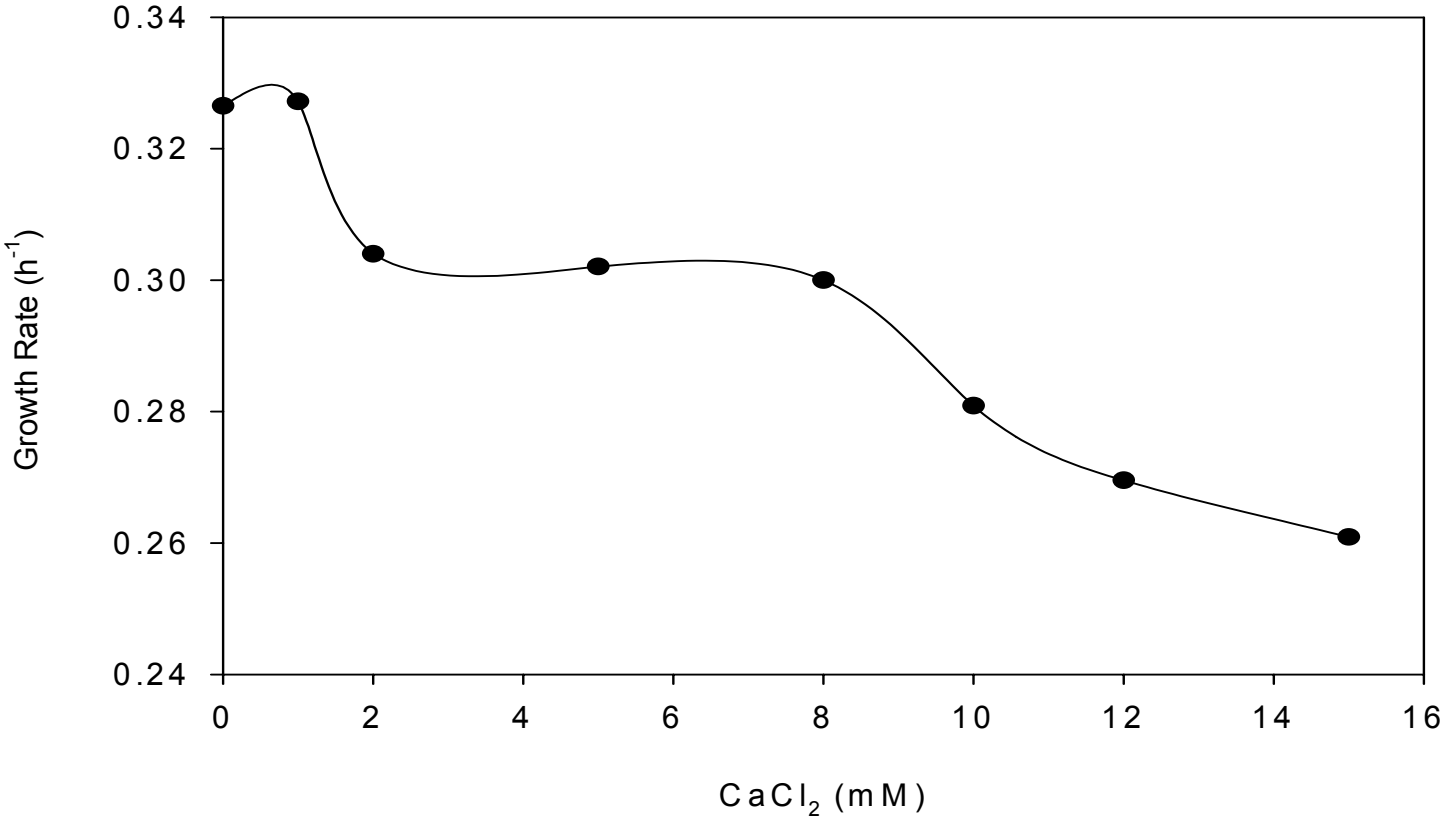

Figure 4 Effect of $CaCl_2$ on the growth rate of *L. mesenteroides* 1-A4 in MRS medium at 30 ºC

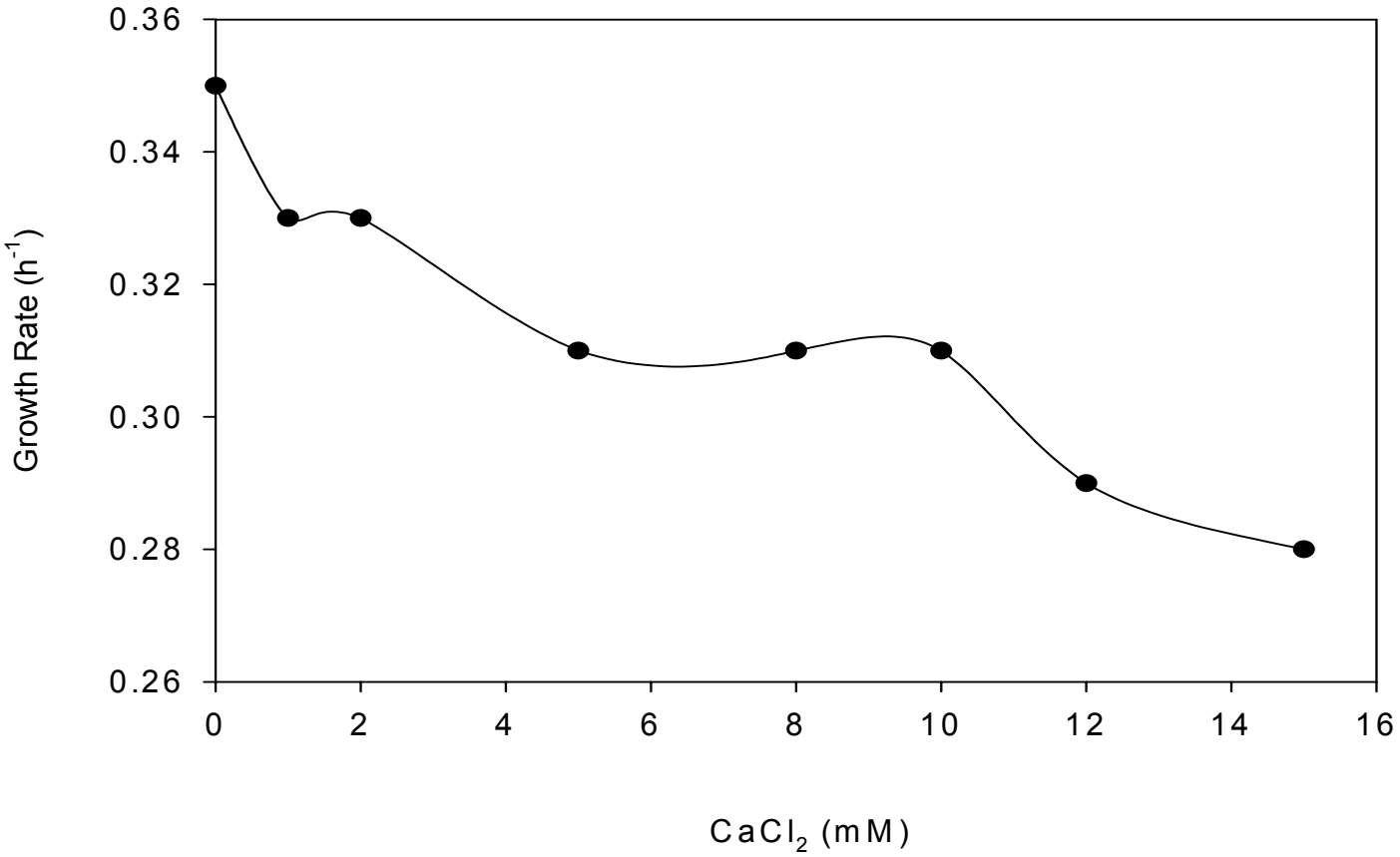

Figure 5 Effect of $CaCl_2$ on the growth rate of *L. pseudomesenteroides* 3-B11 in MRS medium at 30 ºC

4.1.3 Latent Period and Burst Size

One step growth experiment is used to determine the parameters latent period and burst size. It has been reported in the literature (Ellis and Delbruck, 1939) that the latent period and burst size vary from experiment to experiment, even under identical conditions. To observe this variation, independent replicates of one step growth curves were performed. Experimental conditions including O.D. of cells at the time of infection, MOI, temperature and calcium chloride concentration in MRS medium were kept constant in replicate experiments. Variation was observed in the values of parameters, latent period and burst size calculated from the results of replicate one step growth experiments. Typical one step growth experiments are shown for phage 1-A4 and phage 3-B11 in Figures 6 and 7 respectively. In a typical one step growth experiment, the phage concentration remains constant for a period of time (also known as latent period), and then increases until all cells infected during latent period are lysed. From the results of replicate one step growth curves, it was found that latent period for phage 1-A4 ranged from 16 min to 24 min with mean value of 20 min (S.D. = 4 min). In the determination of latent period, the time allowed for adsorption of phage to cells, and the time for centrifugation of cells prior to the start of one step growth curves, were neglected. Burst size for phage 1-A4 ranged from 11-37 with mean value of 24 (S.D. = 13). For phage 3-B11 latent period varied from 39 min to 51 min with a mean value of 44 min (S.D. = 6.25 min), and burst size was found to be varying from 37 to 62 with a mean value of 47 (S. D. = 13). As expected, a range for latent period and burst size was obtained and the results are summarized in the Table 4.

Phage 1-A4			**Phage 3-B11**		
Cells infected at O.D.= 0.3 with MOI ≈ 0.25			Cells Infected at O.D. = 0.3 with MOI ≈ 0.04		
Replicates	**Burst Size**	**Latent Period (min)**	Replicates	**Burst Size**	**Latent Period (min)**
1	37	20	1	42	39
2	24	16	2	37	42
3	11	24	3	62	51
Average	24	20	**Average**	47	44

Table 4 Results of one step growth experiments for phage 1-A4 and 3-B11 in MRS with 5 mM $CaCl_2$

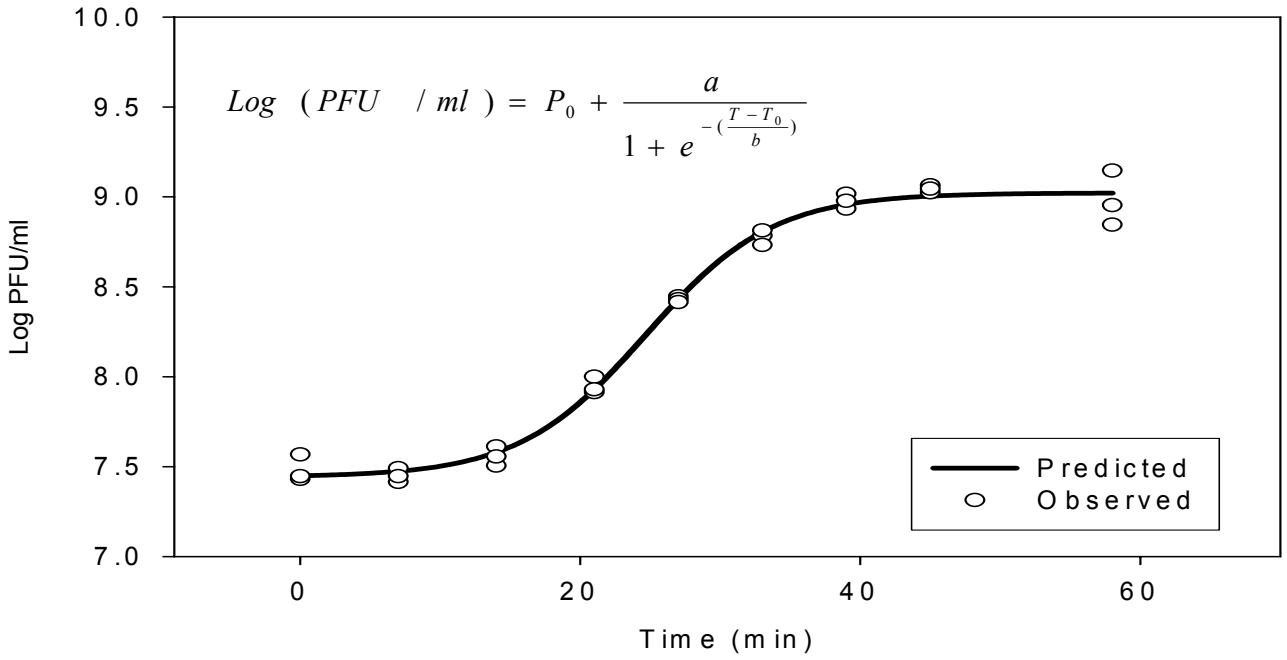

Figure 6 One step growth curve of *L. mesenteroides* 1-A4 phage 1-A4 in MRS medium with 5 mM $CaCl_2$ at 30 ºC

Burst size (Difference between lower asymptote and upper asymptote) = 37
T_0 is the time when slope of the exponential part is the maximum.
Latent Period = T_0-b (Buchanan *et al*. 1989, Buchanan and Klawitter, 1991) = 24.67 - 4.532 = 20 min.

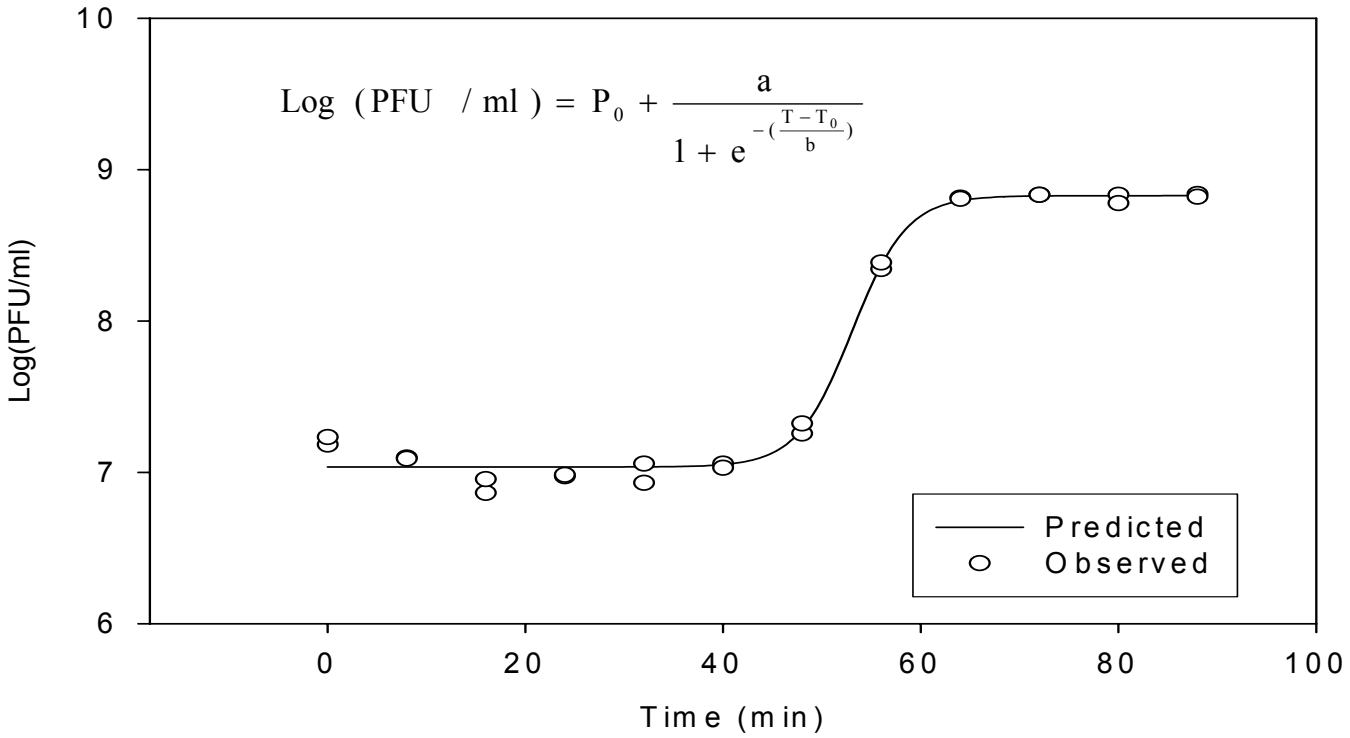

Figure 7 One step growth curve *of L. pseudomesenteroides* 3-B11 phage 3-B11 in MRS medium with 5 mM $CaCl_2$ at 30 ºC

Burst Size = 61.65
Latent Period = 53 – 2.77 = 51.23 min.

4.1.4 Adsorption Rate Constant

In adsorption experiments cells were infected with phage in MRS medium supplemented with 5 mM $CaCl_2$. The time of mixing phage and cells was considered as time zero or starting point, and free phage density in MRS medium was measured with time. Adsorption experiments were repeated by varying initial density of cells, and MOI was also varied to some extent to observe the variation in determined values of adsorption rate constant. Adsorption experiments for phage-host 1-A4 were done at O.D. of 0.2, 0.15, 0.22 at MOI of 0.13, 0.09, and 0.1 respectively. Free phage concentration decreases as adsorption increases until first latent period is over. Reduction in free phage density for 1-A4 at O.D. of 0.2, 0.15, and 0.22 are shown in Figures 8-10. As can be observed from the figures, free phage concentration in MRS followed exponential decay model for all the cases. Slight variation was observed in the calculated values of the adsorption rate constant. However it should be noted that O.D. and MOI were varied to a small extent, with O.D. $\approx$ 0.2 and MOI $\approx$ 0.1. The confidence for the value of adsorption rate constant was obtained from the results of different adsorption experiments. Similarly two adsorption experiments were performed for phage-host 3-B11 at O.D. of 0.2 and 0.3 at MOI of 0.02 and 0.03 respectively. Adsorption experiments for phage-host 3-B11 at O.D. of 0.2 and 0.3 are shown in Figures 11-12 respectively. Free phage reduction for phage-host 3-B11 was also fitted with exponential decay model.

Mean value of the adsorption rate constant for varying initial cell and phage numbers was found to be 4.97×10^{-8} ml/h (S. D. = 3×10^{-9}) for phage 1-A4 and 4.97×10^{-8} ml/h (S. D. = 1.2×10^{-8}) for phage 3-B11. The variation between adsorption rate constant values determined from different experiments was relatively small for both

phage 1-A4 and phage 3-B11. The following equation was used to fit the free phage measurement data.

$$P = ae^{-bT} \tag{23}$$

Where,

P: Free phage density at time T

a, b: Parameters obtained from the exponential decay model

Since free phage decrease is described by following equation in our model, following equations describe calculation of adsorption rate constant at time zero.

$$\frac{dP_f}{dt} = -KSP_f \tag{24}$$

Where,

P_f: Free phage density at time t

K: Adsorption rate constant

S: Susceptible cell density

At t = 0,

$$\left(\frac{dP_f}{dt}\right)_{t=0} = -KS_0P_{fo} \tag{25}$$

Where,

S_0: Susceptible cell density at time zero

P_{f0}: Phage density at time zero

K: Adsorption rate constant at time zero

From exponential decay model,

$$\frac{dP_f}{dt} = -abe^{-bt} \quad (26)$$

So substituting t=0 in above equation:

$$(\frac{dP_f}{dt})_{t=0} = -KS_0P_0 = -ab \quad (27)$$

$$K = \frac{ab}{S_0P_0}$$

As, $a \approx P_0$

$$K = \frac{b}{S_0}$$

Results from adsorption experiments are summarized in following table:

Phage 1-A4				**Phage 3-B11**		
S. No.	S_0 cfu/ml	MOI	K (ml/h)	S_0 cfu/ml	MOI	K (ml/h)
1	7.98×10^7	0.125	4.7×10^{-8}	1.57×10^8	0.02	5.82×10^{-8}
2	5.58×10^7	0.089	5.3×10^{-8}	2.37×10^8	0.03	4.1×10^{-8}
3	8.85×10^7	0.096	4.9×10^{-8}			
Average			4.97×10^{-8}	**Average**		4.97×10^{-8}

Table 5 Adsorption rate constant values for phage 1-A4 and 3-B11 in MRS medium with 5 mM $CaCl_2$

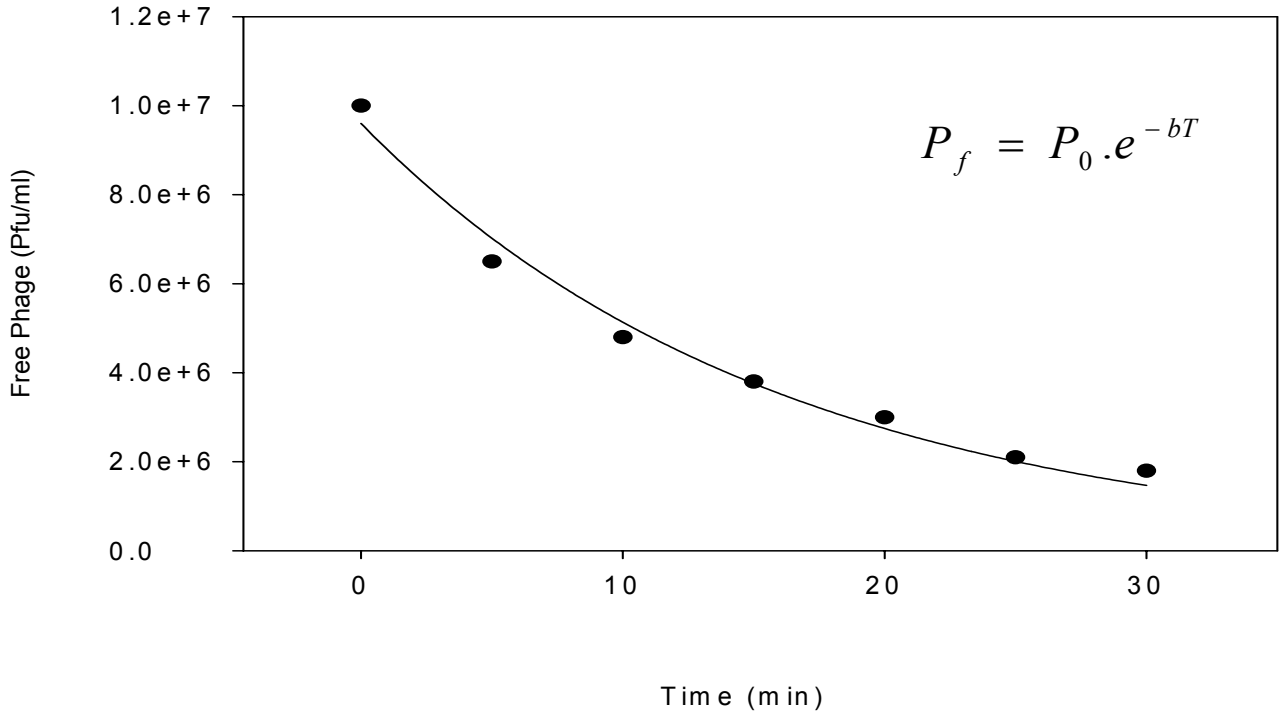

Figure 8 Free phage decay curves for adsorption of phage 1-A4 to *L. mesenteroides* 1-A4 in MRS media supplemented with 5 mM $CaCl_2$ at 30 ºC

$S_0 = 7.98 \times 10^7$ cfu/ml from standard curve corresponding to O.D. of 0.20, MOI = 0.13
$K = 0.0626/7.98 \times 10^7 = 7.84 \times 10^{-10}$ ml/min = 4.7×10^{-8} ml/h

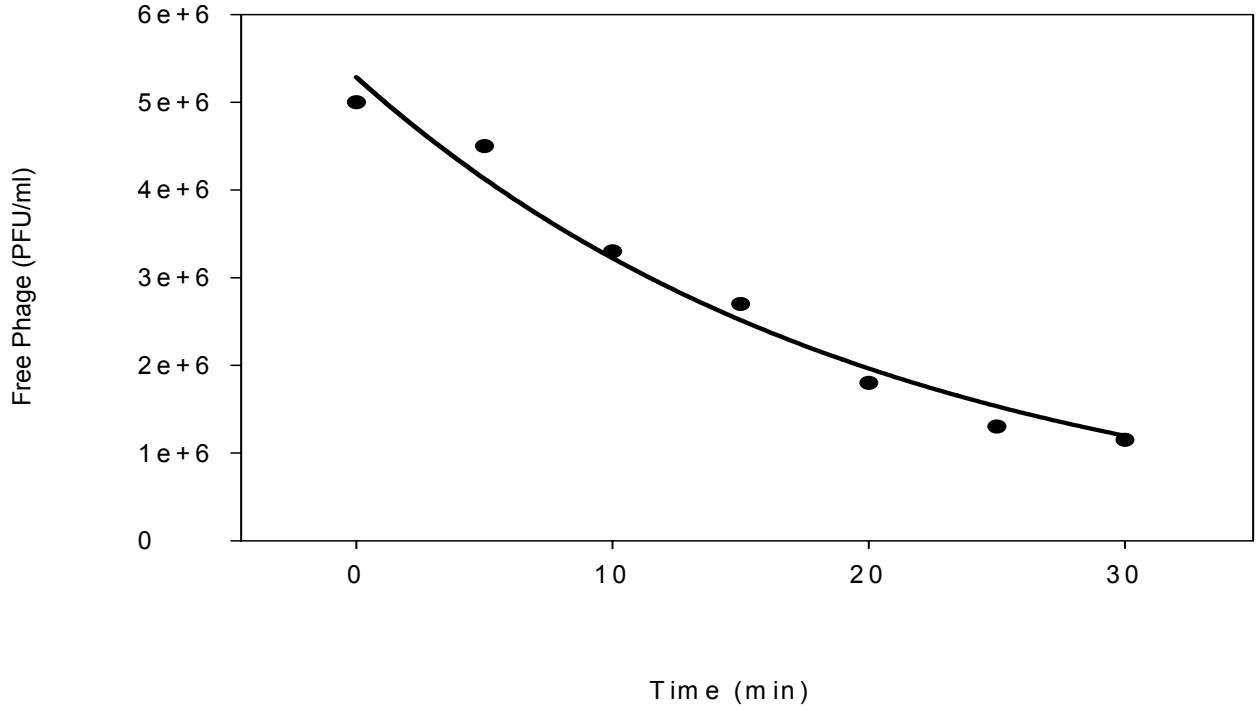

Figure 9 Free phage decay curves for adsorption of phage 1-A4 to *L. mesenteroides* 1-A4 in MRS media supplemented with 5 mM $CaCl_2$ at 30 ºC

$S_0 = 5.579 \times 10^7$ cfu/ml corresponding to O.D. = 0.15, MOI = 0.09
$K = 0.0495 / 5.58 \times 10^6 = 8.87 \times 10^{-10}$ ml/min = 5.3×10^{-8} ml/h

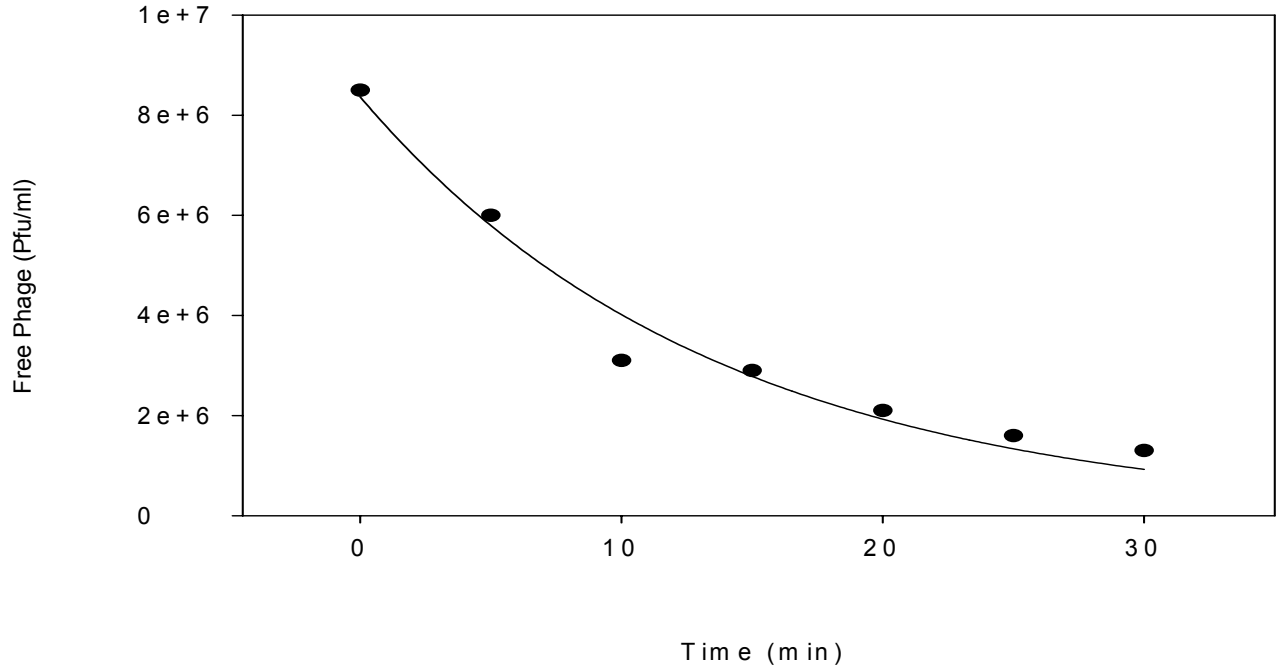

Figure 10 Free phage decay curves for adsorption of phage 1-A4 to *L. mesenteroides* 1-A4 in MRS media supplemented with 5 mM $CaCl_2$ at 30 ºC

$S_0 = 8.85 \times 10^7$ cfu/ml corresponding to O.D. = 0.22, MOI = 0.1
$K = 0.0734/8.85 \times 10^7 = 8.29 \times 10^{-10}$ ml/min = 4.97×10^{-8} ml/h

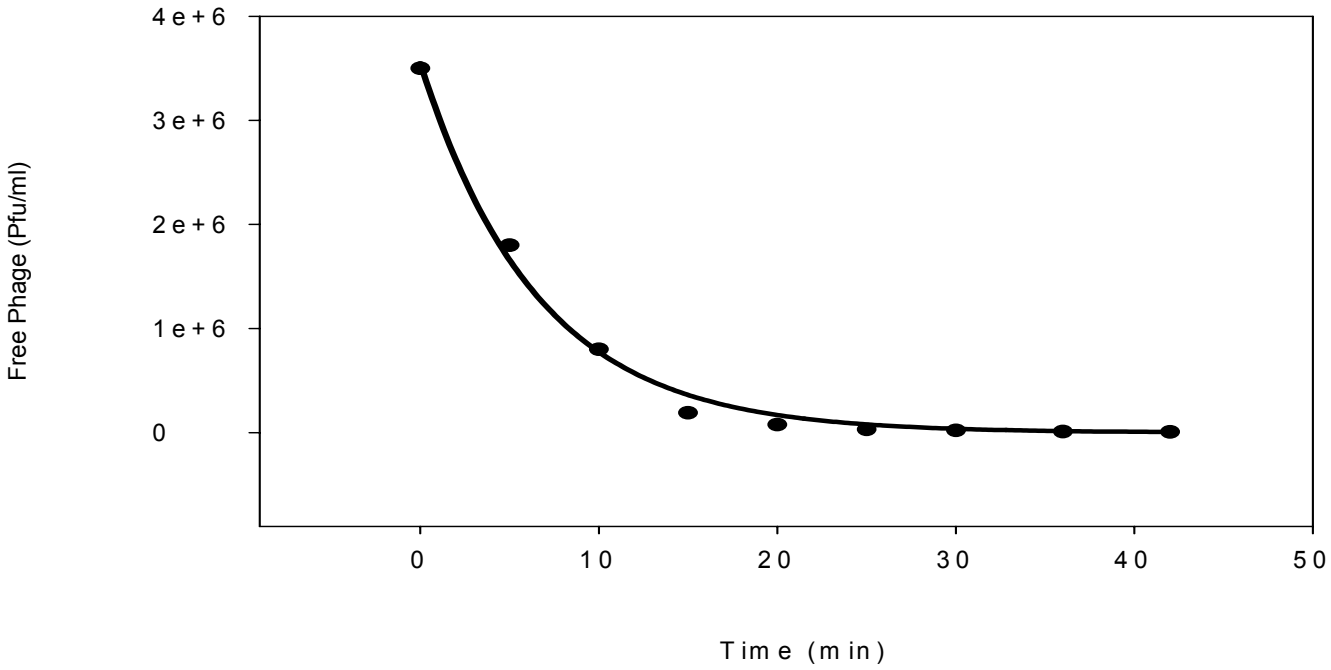

Figure 11 Free phage decay curves for adsorption of phage 3-B11 to *L. pseudo mesenteroides* 3-B11 in MRS media supplemented with 5 mM $CaCl_2$ at 30 ºC

$S_0 = 1.57 \times 10^8$ cfu/ml corresponding to O.D. of 0.2 from Standard Curve, MOI = 0.02
$K = 0.1521/1.57 \times 10^8 = 9.7 \times 10^{-10}$ ml/min = 5.82×10^{-8} ml/h

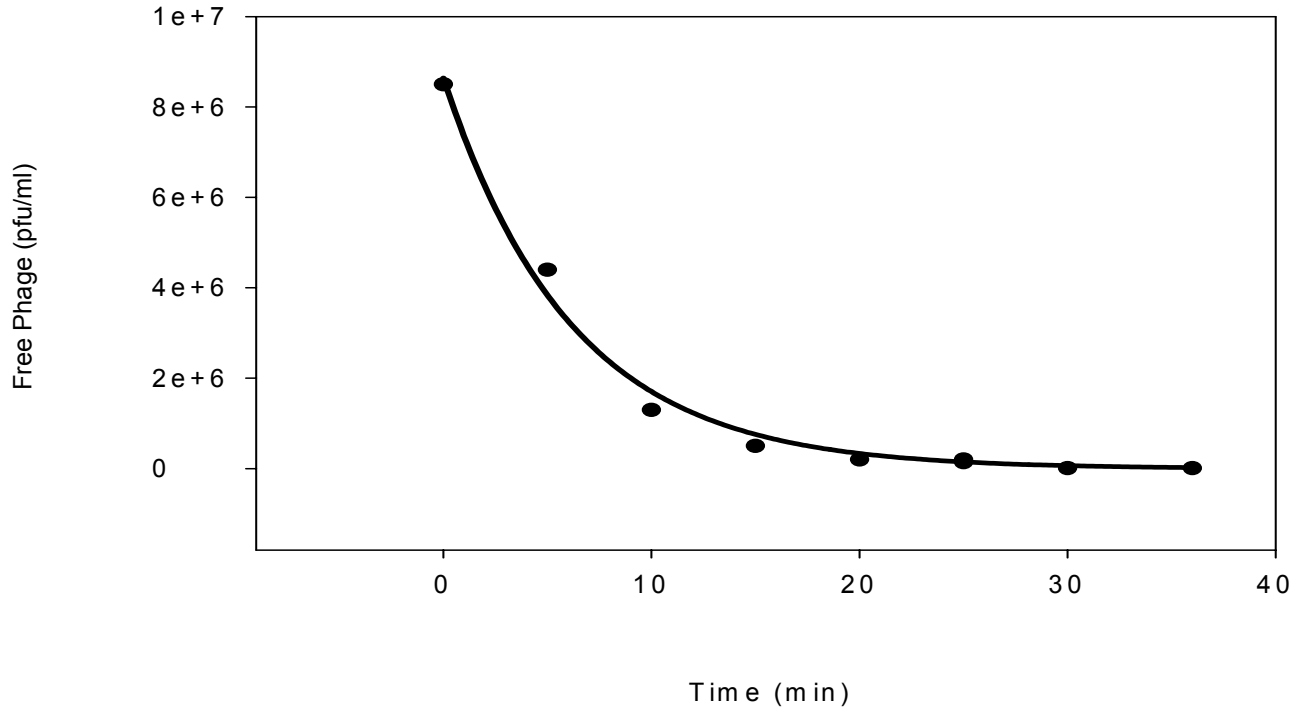

Figure 12 Free phage decay curves for adsorption of phage 3-B11 to *L. pseudo mesenteroides* 3-B11 in MRS media supplemented with 5 mM $CaCl_2$ at 30 ºC

$S_0 = 2.37 \times 10^8$ cfu/ml corresponding to O.D. of 0.3 from Standard Curve, MOI = 0.03
$K = 0.1622/2.37 \times 10^8 = 6.84 \times 10^{-10}$ ml/min = 4.1×10^{-8} ml/h

4.2 Validation of the Model

The model predictions were obtained by evaluating solutions of delay differential equations at desired time points for a specific initial condition and a parameter vector. Initial condition refers to total initial phage and cell densities. Parameter vector refers to a set of values for all the parameters used in the model.

Initially parameters were optimized for individual sets, which resulted in different parameter vectors for each set, these parameter values were used to calculate the model predictions. The model was then tested for different initial conditions using a same parameter vector to predict for two or more different initial conditions simultaneously. Details on validation of the model are described in section 3.5.

4.2.1 Parameter Optimization and Validation for Individual Sets

The model predictions were found to be in close agreement with the observed phage and cell numbers. The model predictions fitted the observed phage and cell density data, with high R-square values (≈ 0.95) using parameter values obtained after optimization of individual sets. The results of parameter optimization for individual sets are summarized in tables 6-9 for phage-host system 1-A4 and 10-11 for phage-host system 3-B11 respectively. The symbols used to denote various parameters are described in section 3.4 (page 58 and 60). These data show average values of parameters with their respective standard deviations and percentage variances within a particular experimental condition (particular initial condition).

The coefficient of variance was about 20 % or less for most of the parameters obtained after parameter optimization for a particular initial condition case. The average values of parameters (Tables 6-11) were used for the model predictions, which resulted in similar predictions corresponding to least possible SSE (T) values. Validation results comparing model predictions with observed total cell and phage counts are shown in Figures 13-16, corresponding to sets 1-4 for phage-host system 1-A4 and in 17-18 for sets 1 and 2 of phage-host system 3-B11 respectively.

Parameter optimization for individual sets resulted in different parameter vectors for different sets, however the SSE (T) values calculated using these parameter vectors for model predictions were similar in magnitude. Mean values of SSE (T) and standard deviations were 1.85 (0.33), 1.14(0.11), 4.83(0.40), and 1.7 (0.17) for sets 1, 2, 3, and 4 of phage-host system 1-A4 respectively. These values were 3.5 (0.41), 2.28(0.7) for sets 1 and 2 of phage-host system 3-B11 respectively. Separate R-square values were calculated for the observed and predicted phage data and cell density data. R-square values were not normalized for the number of data points, as all conditions had roughly same number of points. A general trend was seen in both observed and predicted data in all cases. Cell numbers first increased and then started decreasing and became roughly constant and started increasing again. Phage number continuously increased and became constant after few hours (Figures 13-18). Initial increase in cell numbers suggests that cell growth is faster than phage growth, and as a result cell numbers increase. Thereafter phage numbers increase to certain level and kills susceptible cells, the cell lysis rate is more than the cell production rate resulting in continuous decrease in cell densities. At

the end of kinetics all susceptible cells are replaced by resistant cells and phage population becomes constant.

Set -1 $S_0 = 8 \times 10^7$ cfu/ml, $P_0 = 8.2 \times 10^6$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 8 \times 10^1$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.59	1.02E+09	1.36E-09	0.38	45.98	8.71E-10	3.22E-10	1.05E-10	1.93
Standard Deviation	0.11	1.74E+08	2.33E-10	0.08	11.19	1.28E-10	1.16E-10	2.12E-11	
C.V. (%)	18.32	16.94	17.07	19.80	24.33	14.69	36.04	20.15	

Table 6 Results of parameter optimization for validation experiment # 1 of *L. mesenteroides* 1-A4 and phage 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$

Set -2 $S_0 = 7.5 \times 10^7$ cfu/ml, $P_0 = 2 \times 10^6$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 7.5 \times 10^1$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.66	9.76E+08	2.07E-09	0.46	51.01	5.9E-10	2.96E-10	2.14E-10	1.28
Standard Deviation	0.11	1.41E+08	6.36E-10	0.11	7.97	1.16E-10	4.95E-11	2.02E-11	
C.V. (%)	16.24	14.47	30.77	24.74	15.62	19.66	16.75	9.43	

Table 7 Results of parameter optimization for validation experiment # 2 of *L. mesenteroides* 1-A4 and phage 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$

Set -3 $S_0 = 2.66 \times 10^7$ cfu/ml, $P_0 = 2 \times 10^7$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 2.66 \times 10^1$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.62	1.24E+09	4.58E-09	0.41	25.23	2.76E-09	9.54E-10	5.74E-10	2.08
Standard Deviation	0.15	2.37E+08	1.36E-09	0.08	7.28	8.72E-11	2.37E-10	2.34E-11	
C.V. (%)	24.12	19.16	29.66	20.84	28.86	3.16	24.90	4.08	

Table 8 Results of parameter optimization for validation experiment # 3 of *L. mesenteroides* 1-A4 and phage 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$

Set -4 $S_0 = 7.5 \times 10^5$ cfu/ml, $P_0 = 1.4 \times 10^3$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 0.75 \times 10^0$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.82	9.83E+08	7.03E-08	0.43	74.01	7E-08	5.5E-08	1.55E-09	4.82
Standard Deviation	0.04	4.95E+07	3.89E-09	0.01	7.96	2.83E-10	6.36E-10	2.9E-10	
C.V. (%)	4.28	5.04	5.53	2.44	10.75	0.40	1.16	18.76	

Table 9 Results of parameter optimization for validation experiment # 4 of *L. mesenteroides* 1-A and phage 1-A4 in MRS medium supplemented with 5 mM $CaCl_2$

Set -1 $S_0 = 4.8 \times 10^7$ cfu/ml, $P_0 = 1.31 \times 10^5$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 4.8 \times 10^2$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.47	1.04E+09	1.65E-08	0.70	45.85	1.33E-08	1.57E-09	1.27E-09	5.4
Standard Deviation	0.07	4.04E+07	4E-09	0.06	2.31	4.62E-09	1.39E-10	3.3E-11	
C.V. (%)	14.32	3.88	24.27	9.01	5.03	34.76	8.85	2.59	

Table 10 Results of parameter optimization for validation experiment # 1 of *L. pseudomesenteroides* 3-B11 and phage 3-B11 in MRS medium supplemented with 5 mM $CaCl_2$

Set -2 $S_0 = 2.92 \times 10^6$ cfu/ml, $P_0 = 2.92 \times 10^2$ pfu/ml, $I_0 = 0$ cfu/ml, $M_0 = 2.92 \times 10^1$ cfu/ml									
Parameters	$\alpha=\beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Average	0.54	1E+09	3.74E-08	0.63	46.52	3.78E-08	3.31E-08	2.16E-08	1.39
Standard Deviation	0.05	3.95E+07	6.66E-09	0.01	1.63	7.63E-09	1.11E-09	3.48E-09	
C.V. (%)	9.35	3.95	17.81	1.25	3.51	20.19	3.34	16.10	

Table 11 Results of parameter optimization for validation experiment # 2 of *L. pseudomesenteroides* 3-B11 and phage 3-B11 in MRS medium supplemented with 5 mM $CaCl_2$

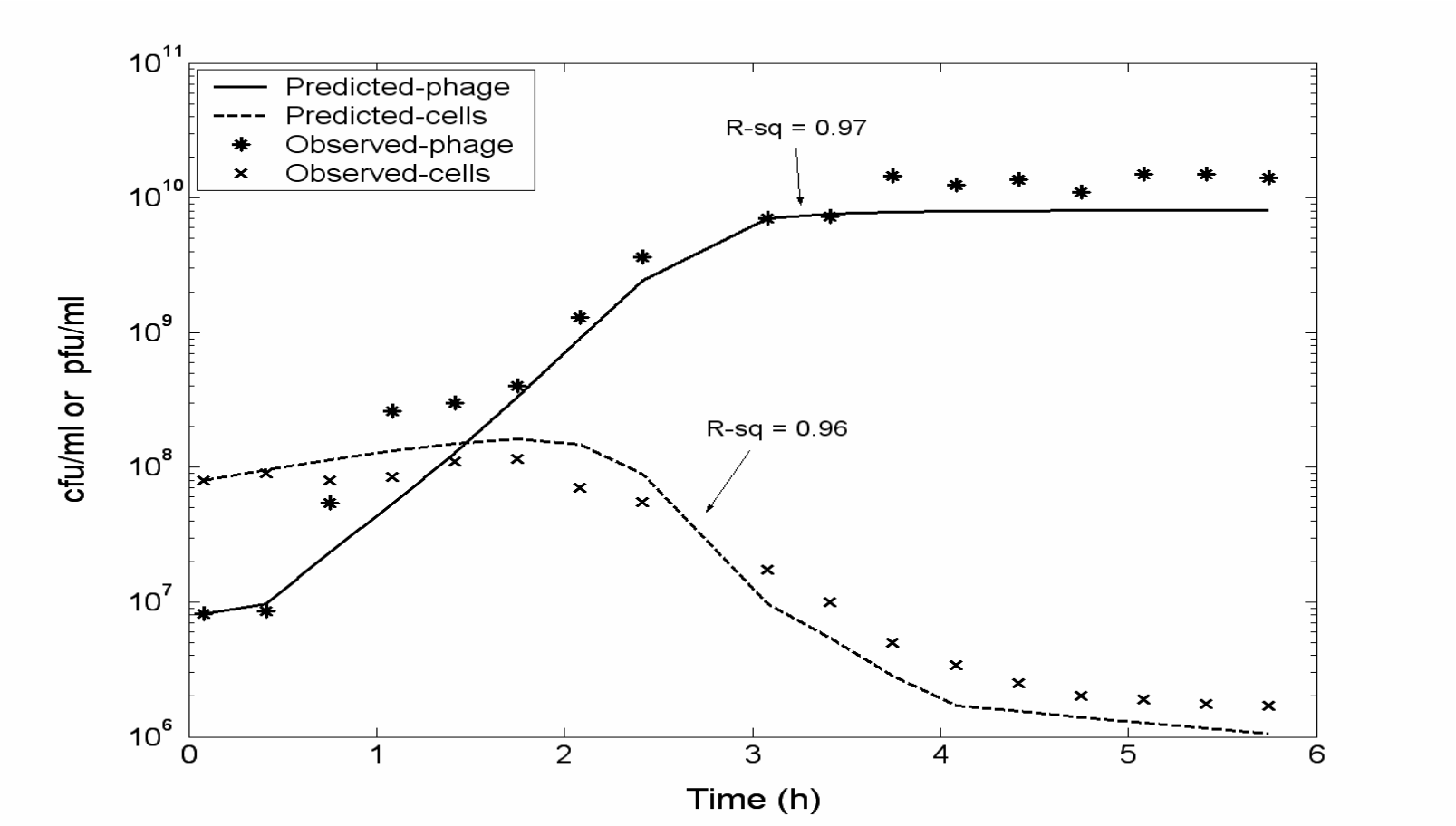

Figure 13 Validation results for experiment # 1 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used are average values of parameters obtained after parameter optimization for set # 1.

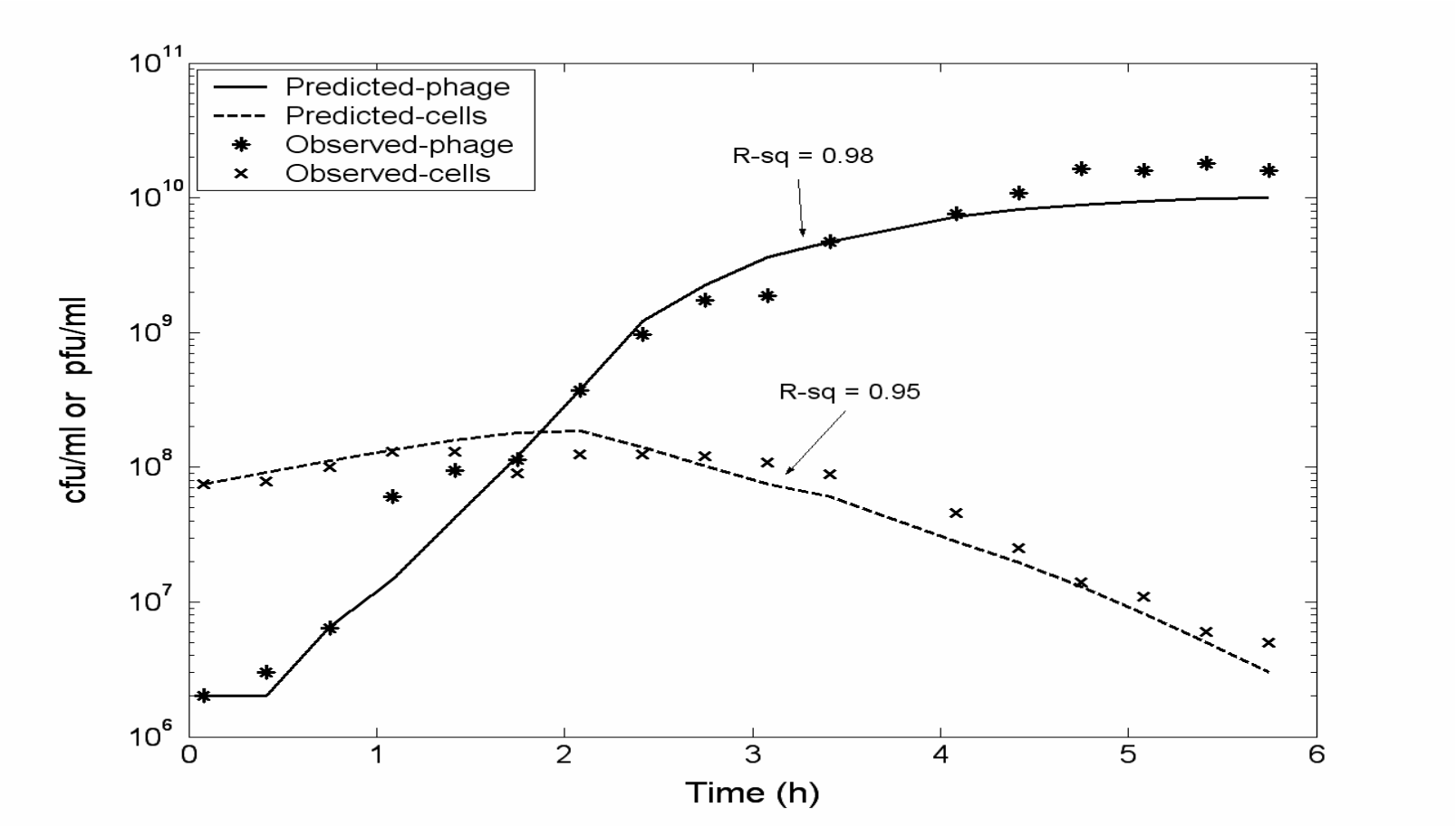

Figure 14 Validation results for experiment # 2 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used are average values of parameters obtained after parameter optimization for set # 2.

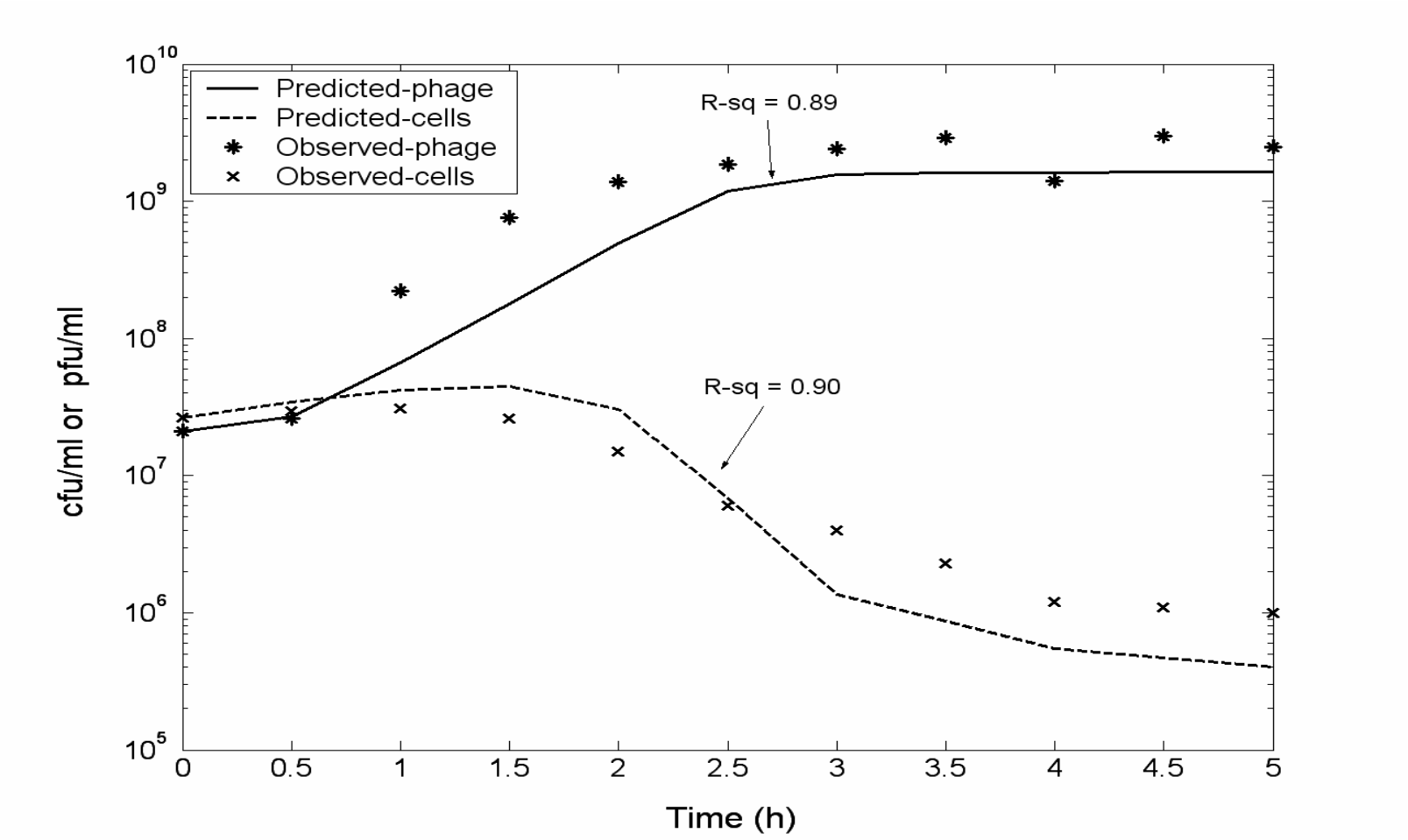

Figure 15 Validation results for experiment # 3 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used are average values of parameters obtained after parameter optimization for set # 3.

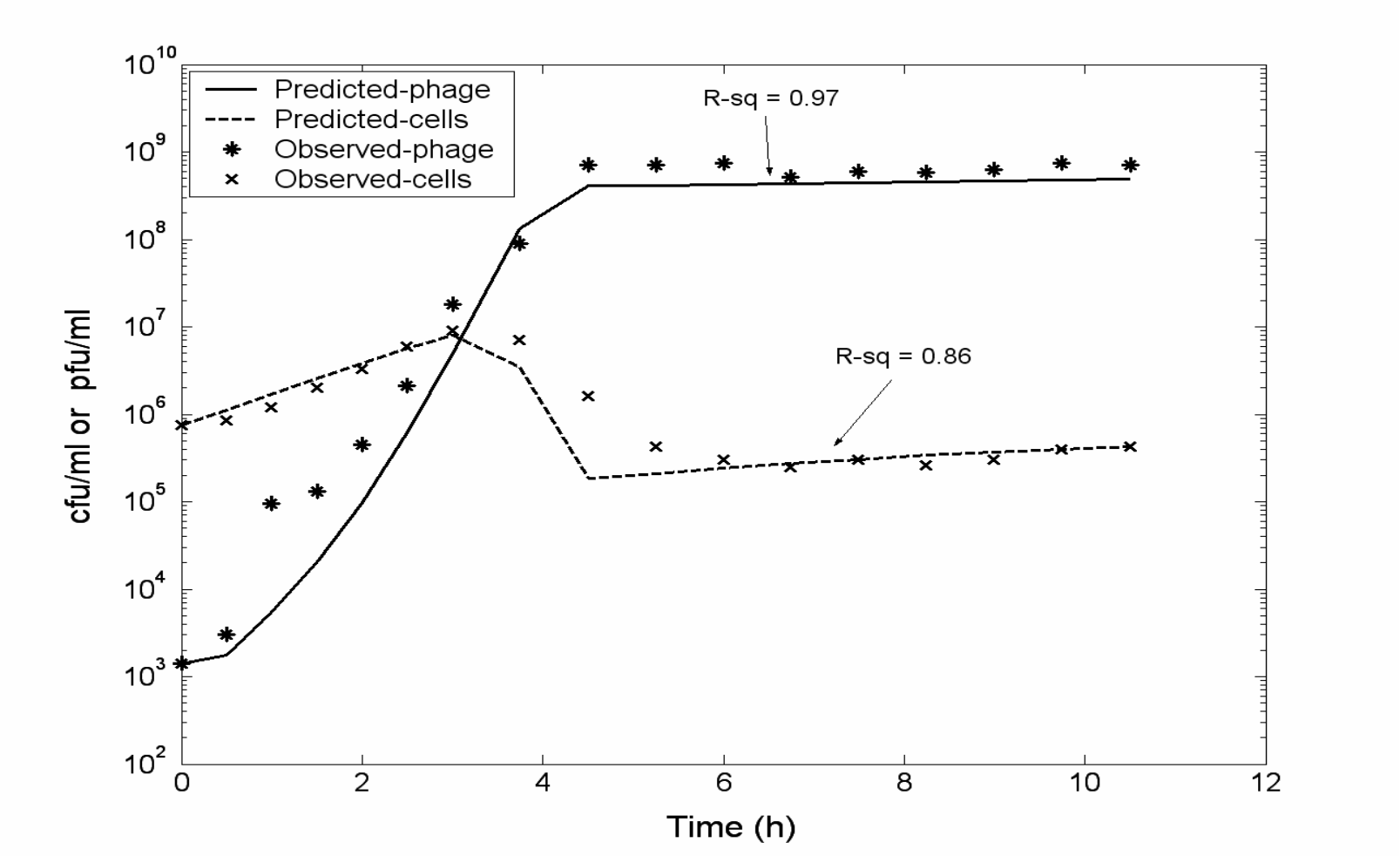

Figure 16 Validation results for experiment # 4 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used are average values of parameters obtained after parameter optimization for set # 4.

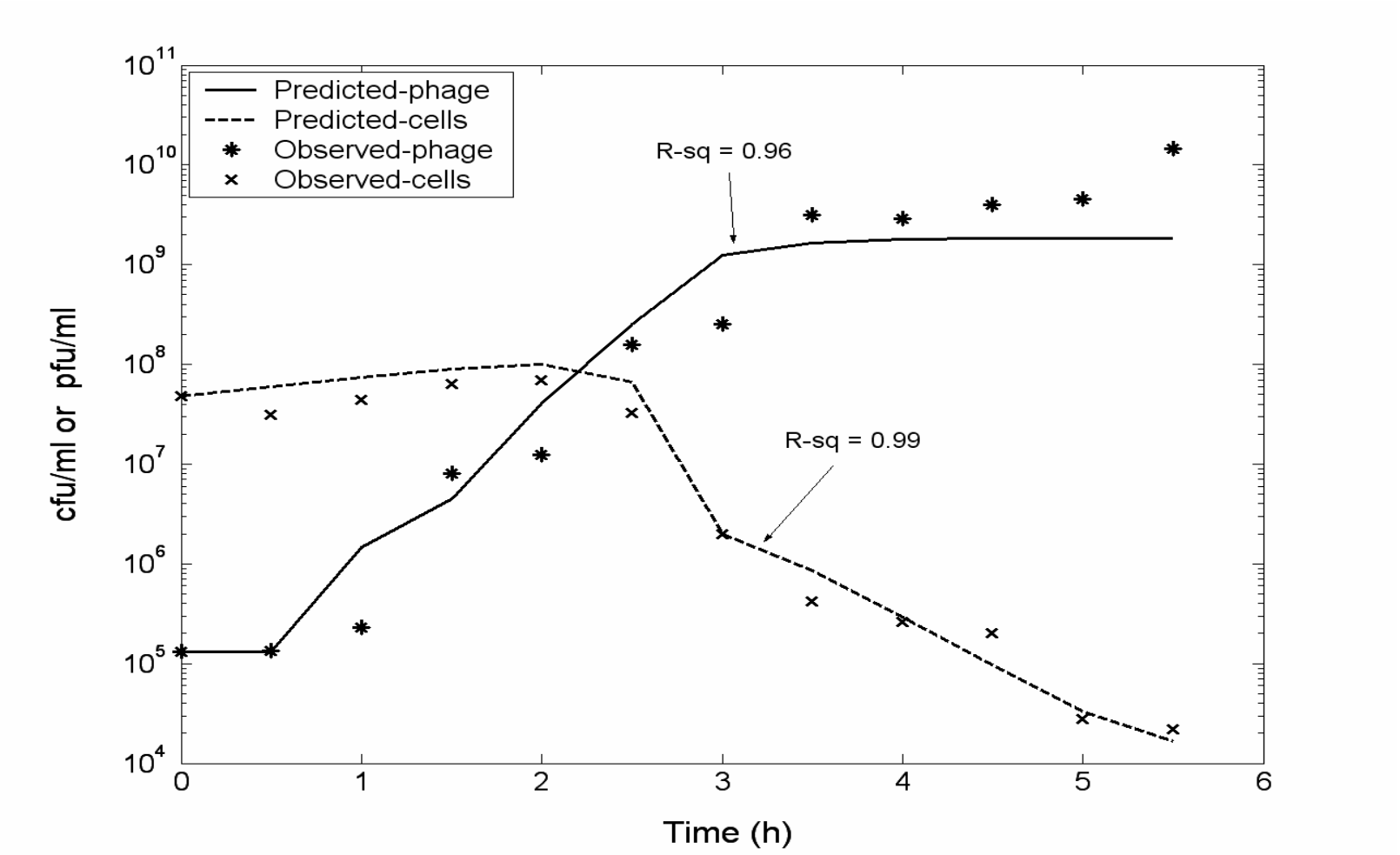

Figure 17 Validation results for experiment # 1 for *L. pseudomesenteroides* 3-B11 and phage 3-B11. Parameters used are average values of parameters obtained after parameter optimization for set # 1.

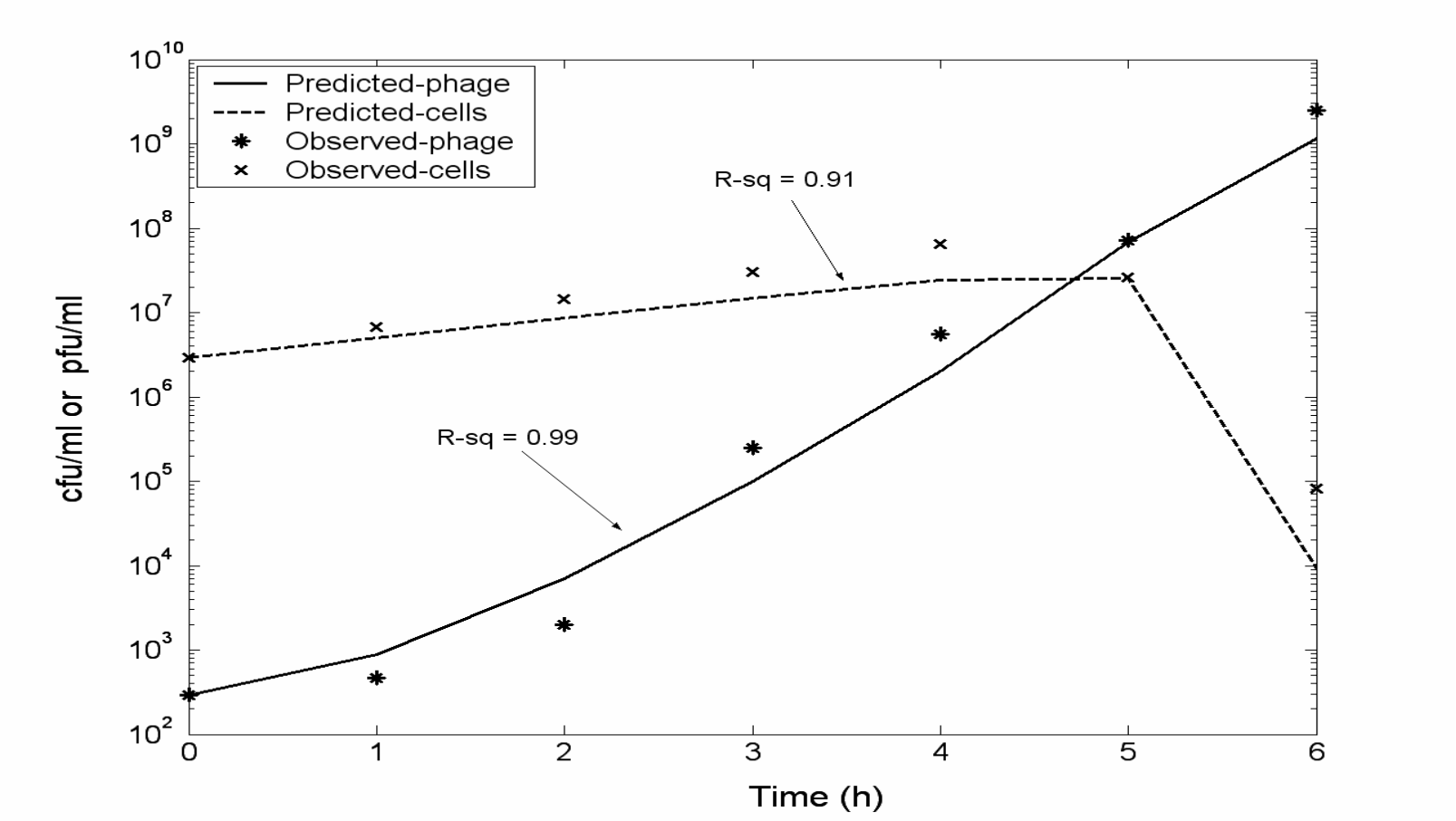

Figure 18 Validation results for experiment # 2 for *L. pseudomesenteroides* 3-B11 and phage 3-B11. Parameters used are average values of parameters obtained after parameter optimization for set # 2.

4.2.2 Parameter Optimization and Validation for Multiple Sets

To attain the objective of finding a common parameter vector, which can be used to predict for all cases with different initial conditions, parameter optimization was done for more than one sets. When same parameter vectors were used for predictions of more than one set, fit was decreased. It was found that a particular parameter set could not be used to find equally good predictions for all the cases simultaneously. As can be observed from Table 12, SSE (T) increased to very high value (382) when same parameter set was used to predict for all different initial density cases. However it was possible to find a parameter set, which predicted with high R-square values ($R^2 \approx 0.97$) and low SSE (T) (≈ 1.5) values for sets with similar initial cell and phage densities (Set 1 and Set 2, $S_0 \approx 10^8$ cfu/ml, $P_0 \approx$ 2-8 x 10^6 pfu/ml). Validation results for set 1-2 of phage-host system 1-A4 using parameter vector obtained after parameter optimization for sets 1 and 2 simultaneously are shown in figures 19-20.

When parameter optimization was done for sets 1, 2 and 3 for phage-host system 1-A4 simultaneously, and same parameters were used to predict for all sets fit was decreased for all the cases resulting in total SSE (T) value of 19. Validation results using same parameter set for datasets 1, 2, and 3 of phage-host system 1-A4 are shown in figures 21-23. As can be observed from these figures the fit was better for set 1 and set 2 (similar initial conditions) than for set 3. Low R^2 values were observed for set 3, especially a very low R^2 value of 0.2 for cell density data. Parameter optimization was based on minimizing the SSE (T), so the results of parameter optimization gave a parameter vector which resulted in lower SSE (T) values for two similar initial density cases (Sets 1 and 2) than compared to set 3. It is apparent from the results that it was not

possible to find a common parameter vector, which could be used to find equally good predictions for all different initial density cases. The possible reason for this may be that some parameters may vary with cell density or phage density. To understand the variation in parameter values depending on initial densities of cell and phage, the coefficient of variance was determined among different initial density cases and was compared to variance values for these parameters within individual cases. These results are discussed in detail in the section 4.2.3.

Parameters	$\alpha = \beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)	SSE(T)
Sets 1 and 2	0.60	1.03E+09	2.09E-09	0.40	35.24	7.30E-10	5.23E-10	1.08E-10	3.15
Sets 1, 2, and 3	0.62	1.02E+09	1.58E-09	0.44	48.60	6.11E-10	3.59E-10	1.66E-10	19.00
Sets 1,2,3, and 4	0.76	1.15E+09	1.95E-09	0.45	55.62	5.16E-08	2.58E-08	4.92E-10	382.0

Table 12 Results of parameter optimization for two or more datasets simultaneously for *L. mesenteroides* 1-A4 and phage 1-A4.

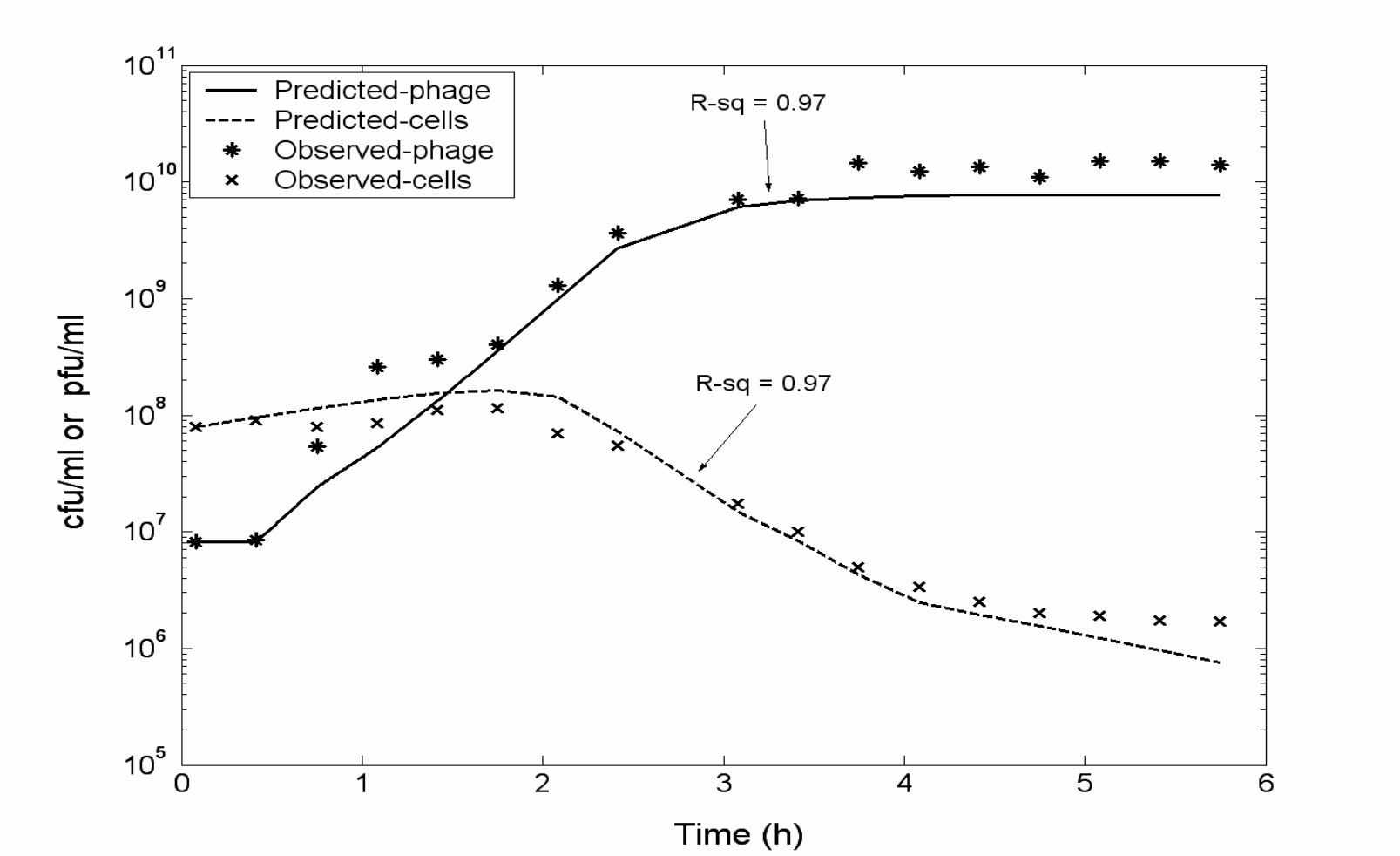

Figure 19 Validation results for experiment # 1 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used were obtained after parameter optimization for set 1 and 2 simultaneously.

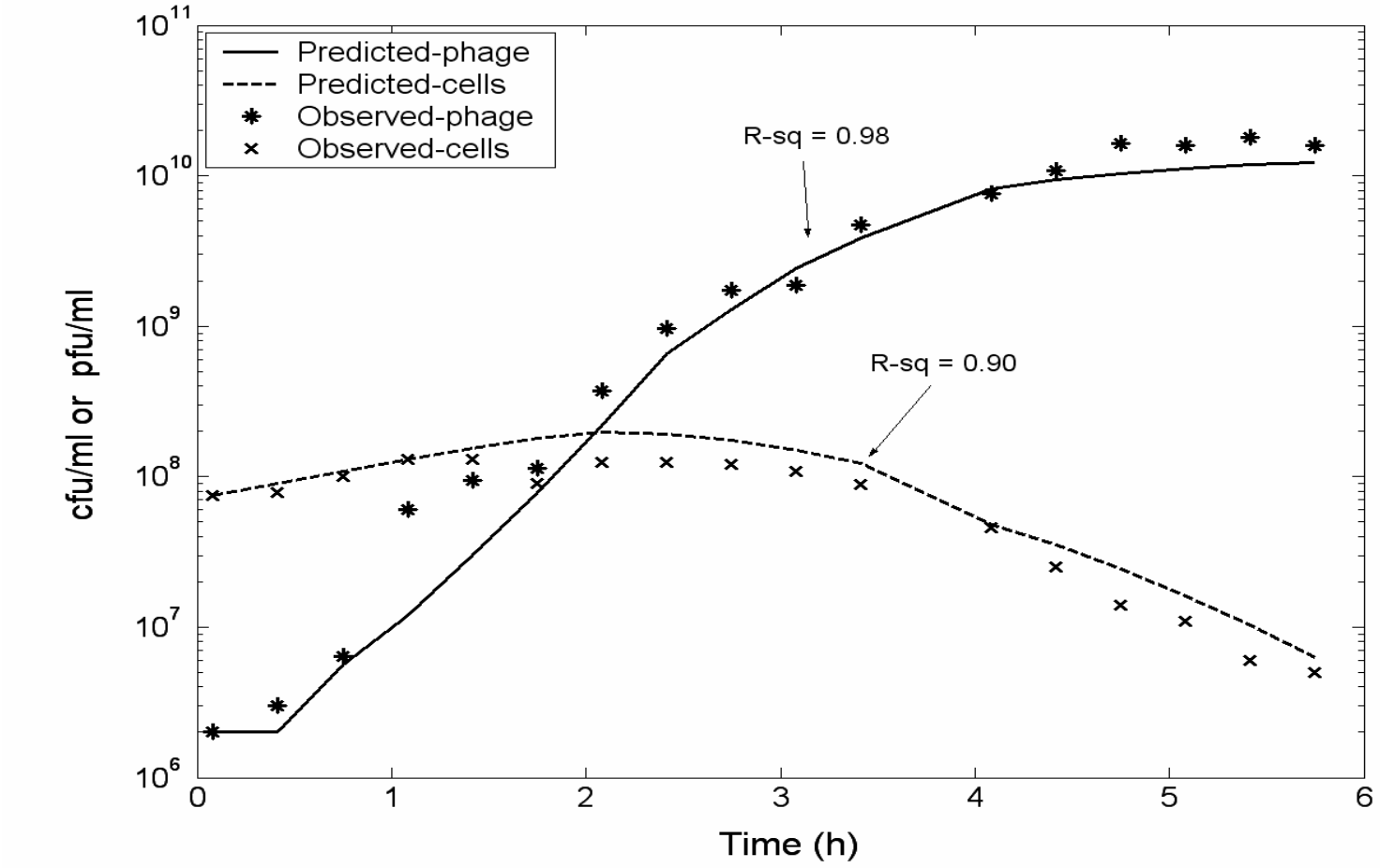

Figure 20 Validation results for experiment # 2 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used were obtained after parameter optimization for set 1 and 2 simultaneously.

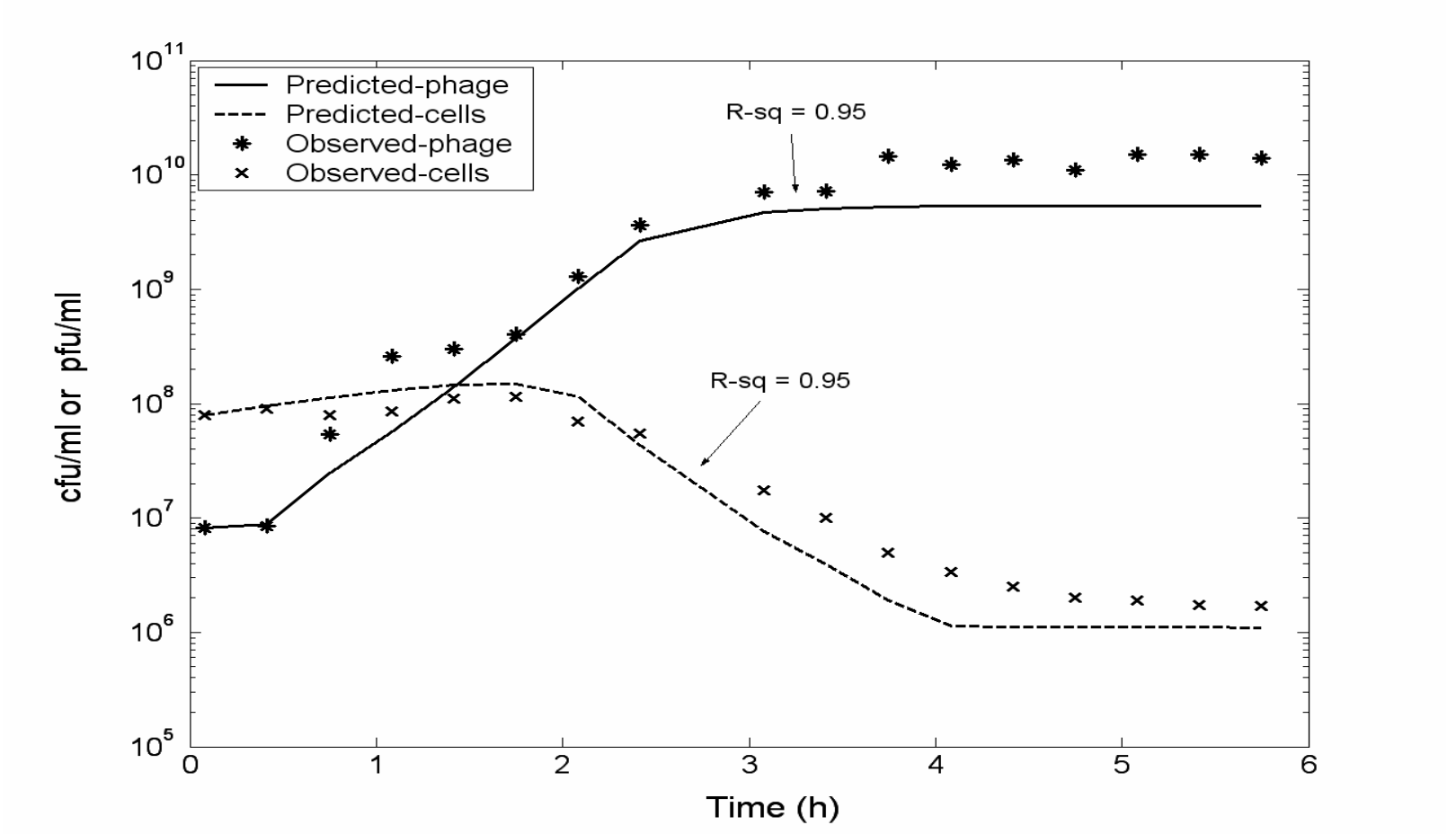

Figure 21 Validation results for experiment # 1 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used were obtained after parameter optimization for set 1, 2, and 3 simultaneously.

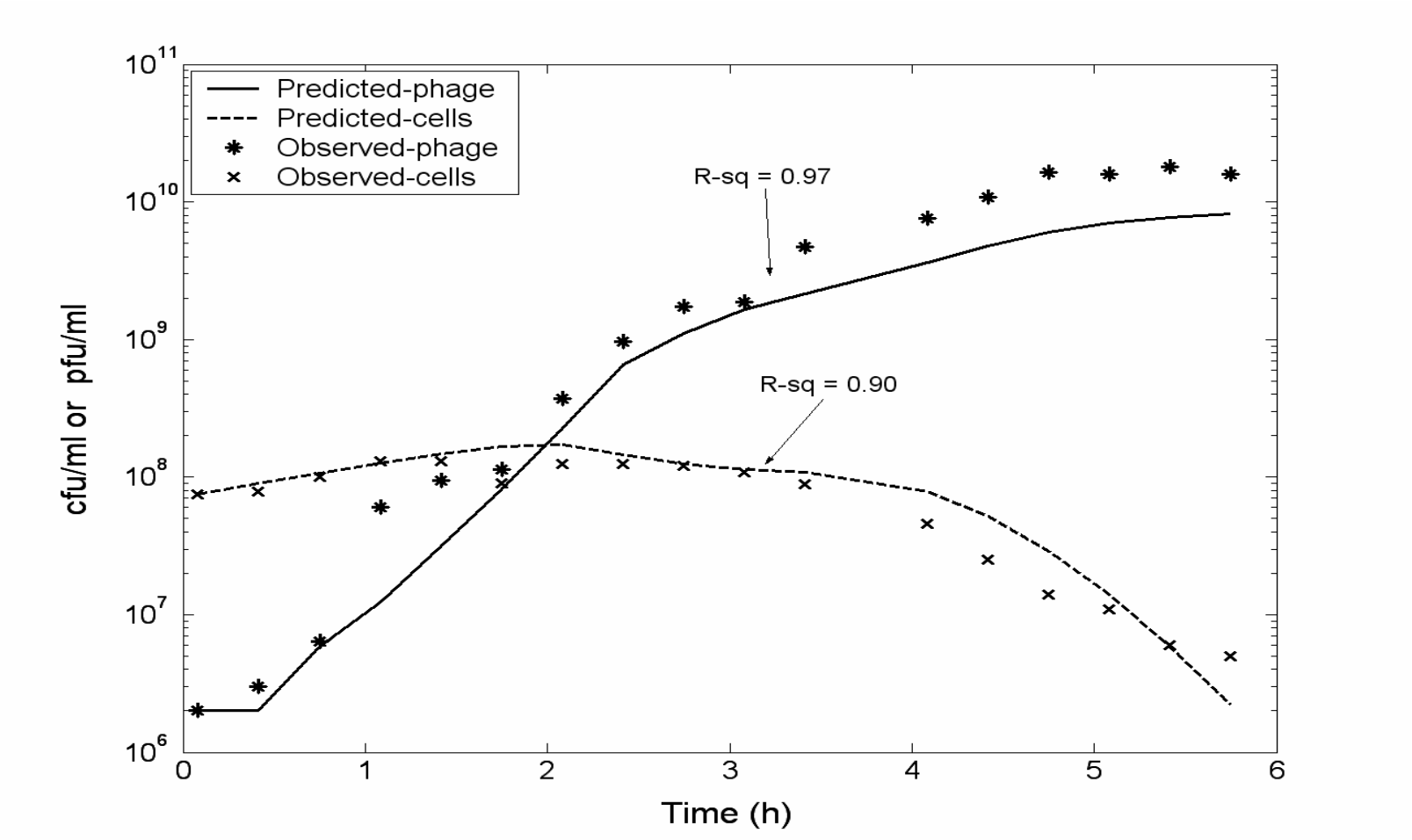

Figure 22 Validation results for experiment # 2 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used were obtained after parameter optimization for set 1, 2, and 3 simultaneously.

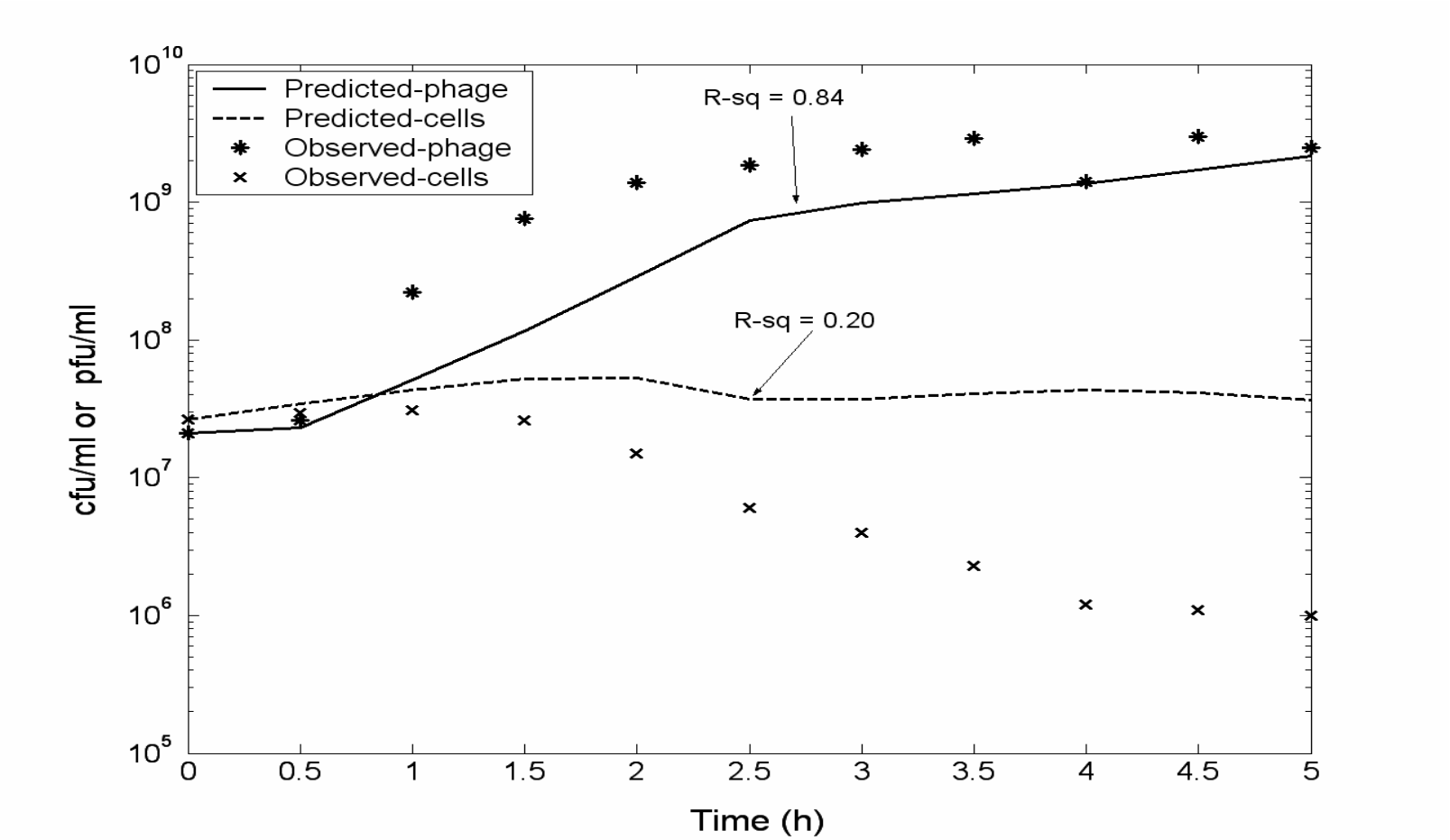

Figure 23 Validation results for experiment # 3 for *L. mesenteroides* 1-A4 and phage 1-A4. Parameters used were obtained after parameter optimization for set 1, 2, and 3 simultaneously.

4.2.3 Variation in Parameters with Different Initial Conditions

Coefficient of variance was determined for the all the parameters among different initial density cases. Results are shown in table 13 for phage-host system 1-A4 and table 14 for phage-host system 3-B11. It was observed that the coefficient of variance among different validation experiments was significantly higher for parameters, burst size and adsorption parameters at different times, than what was observed within a particular validation experiment. This variation was slightly higher for burst size in the case of phage-host system 1-A4 (C.V. = 41 %), and was almost the same (C.V. = 1 %) for the phage-host system 3-B11. However, the coefficient of variance was very high (> 100 %) for adsorption rate constant values (at different times) as compared to what was observed within a particular validation dataset (C.V. ≈ 20 %). This suggested that the parameter value for the adsorption rate constant varied greatly with different initial densities. For other parameters, the percentage coefficient of variance with different initial conditions was similar in magnitude (< 20 %) to that found within a specific initial density case.

After adjusting for adsorption rate constant based on the initial cell and phage density, it was possible to achieve reasonably good ($R^2 \approx 0.90$) predictions for all the validation experiments using the same values for other parameters specific to a particular phage-host system. From the results of parameter optimization (Tables 15 and 16) it was found that adsorption rate constant decreases with time, and value of adsorption rate constants were found to be higher with lower initial cell densities. In table 15 the adsorption rate constant values are listed for different times and different initial cell densities for phage-host system 1-A4. Similar data is listed for phage-host system 3-B11 in Table 16.

Parameters	$\alpha = \beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)
Set – 1	0.59	1.02E+09	1.36E-09	0.38	45.98	8.71E-10	3.22E-10	1.05E-10
Set – 2	0.66	9.76E+08	2.07E-09	0.46	51.01	5.90E-10	2.96E-10	2.14E-10
Set - 3	0.82	9.83E+08	7.03E-08	0.43	74.01	7.00E-08	5.50E-08	1.55E-09
Set – 4	0.62	1.24E+09	4.58E-09	0.41	25.23	2.76E-09	9.54E-10	5.74E-10
Average	0.67	1.06E+09	1.96E-08	0.42	49.06	1.86E-08	1.41E-08	6.1E-10
Standard Deviation	0.10	1.25E+08	3.38E-08	0.04	20.03	3.43E-08	2.72E-08	6.55E-10
C.V. (%)	15.20	11.80	172.85	8.38	40.83	184.92	192.60	107.43

Table 13 Coefficient of variation for parameters among different experimental conditions for *L. mesenteroides* 1-A4 and phage 1-A4

Parameters	$\alpha = \beta$ (h^{-1})	C cfu/ml	K_0 ml/h	L (h)	B	K_1 (ml/h)	K_2 (ml/h)	K_3 (ml/h)
Set – 1	0.47	1.04E+09	1.65E-08	0.70	45.85	1.33E-08	1.57E-09	4.06E-09
Set – 2	0.54	1E+09	3.74E-08	0.63	46.52	3.78E-08	3.31E-08	2.16E-08
Average	0.50	1.02E+09	2.69E-08	0.66	46.19	2.55E-08	1.74E-08	1.29E-08
Standard Deviation	0.05	3.05E+07	1.48E-08	0.05	0.47	1.73E-08	2.23E-08	1.24E-08
C.V. (%)	10.70	2.98	54.88	7.79	1.02	67.87	128.61	96.74

Table 14 Coefficient of variation for parameters among different experimental conditions for *L. pseudomesenteroides* 3-B11 and phage 3-B11

Time (h)	K_t	$S_0 = 8 \times 10^7$ (cfu/ml)	$S_0 = 7.5 \times 10^7$ (cfu/ml)	$S_0 = 2.66 \times 10^7$ (cfu/ml)	$S_0 = 7.5 \times 10^5$ (cfu/ml)
0-2.5	$\mathbf{K_0}$	1.36E-09	2.07E-09	4.58E-09	7.03E-08
2.5-3	$\mathbf{K_1}$	8.71E-10	5.9E-10	2.76E-09	7.00E-08
3-4	$\mathbf{K_2}$	3.22E-10	2.96E-10	9.54E-10	5.50E-08
> 4	$\mathbf{K_3}$	1.05E-10	2.14E-10	5.74E-10	1.55E-09

Table 15 Adsorption rate constant values at different times for different initial concentration of *L. mesenteroides* 1-A4 cells

Time (h)	K_t	$S_0 = 4.8 \times 10^7$ (cfu/ml)	$S_0 = 2.92 \times 10^6$ (cfu/ml)
0-2.5	$\mathbf{K_0}$	1.65E-08	3.74E-08
2.5-3	$\mathbf{K_1}$	1.33E-08	3.78E-08
3-5	$\mathbf{K_2}$	1.57E-09	3.3E-08
> 5	$\mathbf{K_3}$	1.27E-09	2.16E-08

Table 16 Coefficient of variation for parameters among different experimental conditions for *L. pseudomesenteroides* 3-B11 and phage 3-B11

4.2.4 Conclusions

It was observed that phage kills susceptible bacterial cells quickly (2-6 hours), even when phage are present at low numbers ($\approx 10^3$ pfu/ml). Susceptible bacteria are replaced by resistant bacteria after few hours (5-10 h). The resistant cells, those are selected in the presence of phage, apparently are similar to the starter culture or the susceptible cells in their fermentation capabilities. It was found that parameters vary with cell densities, especially adsorption rate constant. Adsorption rate constant was also found to be decreasing with time of phage-host kinetics. Variation in burst size and adsorption rate constant was found to be higher than other parameters. With eight specific parameters, model was able to predict phage and host cell densities with reasonably good accuracy ($R^2 > 0.90$). Further possible modification in the model, and suggested future work is discussed in detail in Chapter 5.

Chapter 5

Discussion

5.1 Bacterial Growth Rates

It was found that the growth rates of bacteria measured from direct plate counts were higher (≈ 0.50 h^{-1}) than that to measured from optical density measurements (≈ 0.30 h^{-1}). Correlation between optical density readings and actual cell counts is linear over a limited, usually over a tenfold range (McMeekin *et al*., 1993). The advantages of optical density measurements are their speed, simplicity and non-invasiveness which allow many measurements to be taken on a single culture. There are however limitations. The system usually provides self consistent data for a particular set of conditions, but is limited to measuring growth in clear liquid media. The lower limit of detection is typically 10^6 cfu/ml. Because the present study had bacterial numbers that were greater than 10^6 cfu/ml and exponential phase cultures (≈ 10^8 cfu/ml) were used, optical density measurements were suitable. The growth rate of bacteria is an important parameter in modeling phage-host interaction. It has been found that other parameters of phage-host interaction depend on the physiological condition of the host (Delbruck, 1940; Hadas *et al*., 1997; and Rabinovitch *et al*., 2002). Rabinovitch *et al*. (2002) found that the latent period, burst size and the rate of phage maturation can be expressed as a function of the culture doubling time. The growth rates of bacteria which were measured in the laboratory were found to be between 0.31-0.53 h^{-1} for *L. mesenteroides* 1-A4 and 0.31 - 0.51 h^{-1} for *L. pseudomesenteroides* 3-B11. The growth rates of bacteria calculated from

parameter optimizations were found to vary between 0.45 - 0.80 h^{-1} for *L. mesenteroides* 1-A4 and 0.39-0.58 h^{-1} for *L. pseudomesenteroides* 3-B11. The growth rates calculated from parameter optimizations were in similar range to those experimentally determined values for *L. pseudomesenteroides* 3-B11, and were found to be higher for *L. mesenteroides* 1-A4. This may be due to two reasons, first because of variability in the experimental measurement of growth rates; second, the model may need further modifications. Growth rates of resistant bacteria were not significantly different from that of susceptible bacteria for both experimentally determined and numerically determined values of growth rates. This was expected, as both are similar strains of bacteria.

Phage require divalent cations in the process of adsorption (Cheery and Watson, 1949, Oki and Ozaki, 1967). Calcium chloride is used to provide Ca^{2+} ions for this purpose. However for certain phage Ca^{2+} ions are also needed in other stages of phage growth such as for multiplication of phage inside cells (Rountree, 1955), for penetration of phage genome inside cells (Watanabe *et al*., 1972). In our study, with addition of Calcium chloride, plaque formation increased significantly, indicating that Ca^{2+} was required during some stage of phage growth. Initially 30 mM $CaCl_2$ was added to MRS for phage-host kinetics, however, it was found that 30 mM concentration had inhibitory effect on the growth rate of bacteria. The effect of $CaCl_2$ was determined on the growth rates of bacteria. It was found that growth rates decreased with increasing concentration of $CaCl_2$. Interestingly growth rate decreased when $CaCl_2$ was increased from 0-2 mM, then remained constant for 2-8 mM $CaCl_2$, and then decreased again for further addition of $CaCl_2$ in MRS medium. 5 mM $CaCl_2$ concentration was selected for phage-host kinetics, so that the growth rate was not significantly affected.

5.2 Latent Period and Burst Size

A four parameter sigmoidal function was used to fit the one step growth curves and data, and the latent periods were calculated (McMeekin *et al*., 1993). Latent period was calculated excluding the time allowed for adsorption (10 min) and time for centrifugation of cells. As some phage adsorb earlier the actual latent period may be longer than the calculated values. Different latent periods were obtained from independent replicates of the one step growth curve. The range of latent period was 16-24 min for phage 1-A4 and 39-51 min for phage 3-B11. Latent periods calculated from parameter optimization were similar to these values for both phage (22-28 min for phage 1-A4 and 38-42 min for phage 3-B11).

It has been observed that burst size varied from a few phage to several hundreds (Ellis and Delbruck, 1939). This explains the variation in average burst size obtained from one step growth curves. Experimentally determined values of burst size varied from 11-37 for phage 1-A4 and 37-62 for phage 3-B11. A large variation was observed in burst size for phage 1-A4 obtained by parameter optimization, with a coefficient of variance (CV) of 40 % and the average value, 49, was higher than experimentally determined values. For phage 3-B11, the average burst size calculated from parameter optimization was 46, and had very low CV (1 %).

In one step growth experiments the titer of phage lysate, when multiplied with the amount of phage lysate and added to the cells, should give initial maximum possible phage count. However it was observed that initial count measured after tittering infected cells was always 5-10 times higher. Ellis and Delbruck (1939) in their growth

experiments found that phage titer increased 1.6 times after the starting of one step growth curve experiments and before latent period. They attributed this rise to an increase in the efficiency of plating, as phage are already adsorbed. Delbruck (1940) also found that adsorbed phage will be assayed higher than the free phage, because adsorption of phage is the first step in the process of lysis. In adsorption experiments, free phage was titered, so the initial value of free phage was the same as expected by multiplying the titer of phage lysate to the amount of lysate added to infect cells.

5.3 Adsorption

Values for the adsorption rate constant at time zero, obtained by parameter optimization, were found to vary depending on the initial host cell density, with higher values of adsorption rates at lower initial cell densities [$\approx 7 \times 10^{-9}$ ml/hr at S_0 (susceptible cell density) $\approx 10^6$ cfu/ml, and $\approx 2 \times 10^{-9}$ ml/hr at $S_0 \approx 1 \times 10^8$ cfu/ml] for phage 1-A4. The experimentally determined mean value for the adsorption rate constant was found to be 4.97×10^{-8} ml/h (S.D. = 3×10^{-9}) for phage 1-A4. Adsorption experiments were performed at initial cell concentration $\approx 10^8$ cfu/ml. Values obtained after parameter optimization were lower than expected. For phage 3-B11, the adsorption rate constants were also found to be higher at lower cell densities (1.65×10^{-8} ml/hr at $S_0 = 4.8 \times 10^7$ cfu/ml and 3.65×10^{-8} ml/hr at $S_0 = 2.9 \times 10^6$ cfu/ml). Average value of adsorption rate constant at time zero was 4.97×10^{-8} ml/h (S.D. =1.2×10^{-8}), when calculated at initial cell densities near 2×10^8 cfu/ml. For phage 3-B11, the difference in the experimentally determined and numerically determined values of adsorption rate constants were less compared to phage 1-A4 (numerical value of 1.65×10^{-8} ml/h for 3-B11 as compared to 2

x 10^{-9} ml/h for cell densities ≈ 5 x10^{7} cfu/ml). Experimentally determined values for adsorption rate constants are similar to those reported in the literature (Kruegger, 1931; Schlesinger, 1932; Ellis and Delbruck, 1940; and Levin *et al*. 1977).

It has been reported in literature that the adsorption rate constant remains the same for varying bacterial, and phage concentrations (Schlesinger, 1932). Adsorption rate constants have been calculated in literature using high bacterial (> 10^{6} cfu/ml), and phage numbers. The limitations in time set for adsorption studies require high bacterial concentrations (>10^{6} cfu/ml) so that a decrease in free phage is measurable (Delbruck, 1940). However, there is no strong evidence, that the adsorption rate constants are independent of the cell and phage concentrations. From the results of our validation kinetic studies performed with two *Leuconostoc* species and their specific phage, it has been found that, the adsorption rate constant decreases with time, and is dependent on the initial cell concentration. The results of parameter optimization studies strongly suggest that the adsorption rate constant may vary during phage-host interaction within wide limits. It is of course not a surprising observation, as the same kind of observation was found by Delbruck in 1940. The adsorption rate constant was found to be 60 times higher depending on the physiological condition of the host (Delbruck, 1940). From the parameter optimization studies there is a strong correlation that the adsorption rate constant is higher when the initial cell concentration were lower, but surprisingly it decreased during the course of the phage-host kinetics experiments, when the cell numbers were decreasing. This may be due to the production of metabolites by bacteria, resulting in altered or reduced capability of phage leading to the successful infection. Another possible explanation is that, the adsorption rate constant varies with MOI, and as

phage-host kinetics proceed, the MOI increases continuously. Therefore adsorption rate constant may decrease with an increase in MOI. The coefficient of variance was determined for all the parameters among different validation experiments. It was observed that the coefficient of variance among different validation experiments was higher (CV ≈ 40 % for burst size, CV ≈ 100-200 % for adsorption rate constants) for burst size and adsorption rate, than what was observed within a particular validation experiment. This variation was slightly higher for burst size in the case of phage-host system 1-A4 (CV ≈ 40%), and was almost the same for the phage-host system 3-B11 (CV ≈1%). However, coefficient of variation was higher for adsorption rate constants (at different times) when compared to what was observed within a particular validation dataset (100-200 % for phage 1-A4 and 50-100 % for phage 3-B11).

In the present study, only the adsorption rate was varied with time (using step functions), however it is also possible that other parameters may also change with time depending on the physiological condition of the host. After adjusting for the adsorption rate constants based on the initial cell and phage density, it was possible to achieve reasonably good predictions ($R^2 > 0.9$) for all the validation experiments keeping other parameters constant specific to a particular phage-host system. Levin and Bull (1996) also suggested that if they modified parameters associated with phage i.e., adsorption and burst size and allow them to decline with time; their model would better approximate the results.

It has been found that latent period varied depending on the host density (Wang *et al*., 1997). Wang *et al*. (1997) suggested that phage will evolve a shorter latent period when the host density is high or host quality is good. Longer latent periods are associated

with larger burst sizes. The rate of phage adsorption decreases with the decrease in host quality. Latent period decreases and adsorption rate decreases with higher growth rates (Abedon *et al*., 2001). All these studies suggested that kinetic parameters defining phage-host interaction also depends on the host cell density.

5.4 Further Modifications of the Model

In the present study the model was developed with several assumptions. It was assumed that kinetic parameters defining phage-host interactions were independent of each other and assumed to be constant. It has been reported in the literature that these parameters depend on the physiological state of the host. Latent period, burst size and adsorption constant depend on the growth rate of the host (Hadas *et al*., 1997, Rabinovitch *et al*., 2002). There is an apparent interdependence between parameters latent period and burst size, longer the latent period, larger the burst size (Abedon *et al*., 2001). These parameters may also change with time of phage-host interaction. In our model only adsorption rate was allowed to vary with time. In further modifications of the model additional factors may be considered, such as dependence of parameters on the growth rate of the cell. In parameter optimization, other parameters may be expressed as function of growth rate and then results can be compared to the present results. The functional dependence of latent period, burst size, and adsorption rate constant on the growth rate of host is found in very few studies (Rabinovitch *et al*., 2002), and it may vary for different phage-host systems. Results of the model may be compared by varying the parameters whose interdependence has to be determined while holding other

parameters constant. This approach may give information about the functional dependence of parameters on each other. Parameters have also been found to vary with the host density (Wang *et al*., 1997), which was observed for adsorption rate constant in the present study. In our study the dependence of parameters on host density was determined by measuring the coefficient of variation between parameters. Maximum variation was found in adsorption rate followed by the burst size. Adsorption rate constant can be described as a function of host density, and incorporating this in the model will make model more robust. The model can then be used for a wider range of host concentrations using the same parameter set specific to a particular phage-host system. To understand the variation in adsorption rate constant with time, adsorption experiments can be conducted with varying initial cell and phage densities. The values of adsorption rate constants thus obtained can be correlated with cell density, phage density and MOI to better understand the variation of adsorption rate constant.

5.5 Future Work

The model has been validated using two *Leuconostoc* species and their corresponding phage isolated from commercial sauerkraut fermentation. The model validation has been done in broth media (MRS), and environmental variables such as temperature, pH, and acid concentration were kept constant in validation kinetics. The effect of temperature, pH and acid concentration on kinetic parameters should be considered as a future work. The results of the model showed that susceptible bacterial population is quickly killed by phage (4-6 h) and presence of phage selects for phage

resistant population, which increases continuously with time. It may be required to understand the fermentation capabilities of the resistant cells. Provided, resistant cells are similar in fermentation capabilities to that of susceptible cells (starter culture), the presence of phage may not be a significant concern to the quality of sauerkraut produced at the end of the fermentation.

Starter cultures are introduced in sauerkraut fermentation tanks at the level of 10^6 cfu/ml, and phage may be present in fermentation tanks at very low levels of the order 10^1-10^3 pfu/ml. The model was developed in the broth (MRS), which is more homogeneous than the commercial fermentation environment. In commercial fermentation environment, solids are also present such as salt, cabbage tissues. It is possible that the parameters such as adsorption rate may differ to experimentally observed values in commercial sauerkraut fermentation. Adsorption kinetics of phage depends on diffusion, and random encounter probability between phage and host. Due to spatial heterogeneity in commercial fermentation, this probability may decrease and lower adsorption rates are expected than what are measured in laboratory. Phage may also adsorb to resistant cells, which will lead to lower rates of successful infection, and an advantage to susceptible cells over phage, due to decrease in adsorption rate. Commercial sauerkraut fermentation is carried at temperatures around 18 ºC. Kinetics in the present study was performed at 30 ºC. It is possible that at lower temperature, growth rate of cells may be lower and parameters such as burst size and latent period may vary. It may be desirable to measure growth rates at 18 ºC. Decrease in growth rate will be expected to be accompanied with small burst size. Present study may serve as a foundation for the application of mathematical models in vegetable fermentations. The

model should be tested in commercial sauerkraut fermentation using estimated values of parameters for a specific phage-host system, and then adjusting parameters to check the predictability of model. The model has been developed for one to one interaction between phage and host, therefore the model should first be tested in sauerkraut fermentation using a starter culture and a specific phage. Interactions between many phage and host may be present in sauerkraut fermentation and the model will have to be modified to accurately predict such cases. Validation of the model in commercial fermentation will be required for measurements of phage and host density with time.

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