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AN INVESTIGATION OF THE POSSIBILITIES OF SCALING-UP AND MASS-PRODUCING ENTOMOPATHOGENIC NEMATODES (EPNs)

Fasemore Olufemi Alexander

A thesis submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2011

Declaration

I declare that this research is my unaided work. It is submitted for the Degree of Doctor of Philosophy (Chemical) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for examination in any other university

Signature Date

Abstract

The aim of this study was to design a bioreactor system for the cultivation of entomopathogenic nematodes (EPNs). These are worm-like living invertebrate organisms that can be used as bio-pesticides. The *Heterorhabditis indica* nematode from Ireland and the South African strain *Heterorhabditis Bacteriaphora* were used in the reseach. Both of these species feed on the *Photorhabdus luminescens* bacteria, and the experiments were carried out using *Photorhabdus luminescens* and these EPNs. We cultured *Photorhabdus luminescens* bacteria in both shake flasks and bioreactors in order to determine the optimal conditions for bacterial growth, and found that both the volume of the initial bacteria inoculum and the length of the period during which the bacteria were cultured in nutrient broth were important factors. The doubling time of *Photorhabdus luminescens* bacteria was estimated to be about 3–4 minutes in the batch reactors and about 20 minutes in the shake flasks.

In the scale-up experiments we conducted, the slower growth of bacteria in the shake flask was thought to offer a better match for the growth rate of the EPNs, in that it provided the EPNs with sufficient bacteria to feed off. In the experiments using reactor systems (a fermenter and an external recycle reactor), it proved difficult to match the growth rate of *Photorhabdus luminescens* bacteria and the EPNs. Because the reactor systems are supplied with adequate air, the bacterial growth rate increased: the bacteria multiplied quickly, reached their peak population density and then declined in number as the nutrients in the growth medium depleted. Since the growth rate of the bacteria and that of the EPNs did not match, the EPNs in the reactor did not have sufficient bacteria to feed on, and therefore died.

For this reason we proposed that the bacteria-rich medium (fed batch) should be replenished in the reactor order to ensure that the growth of

bacteria is continuous, and that the life cycle of the EPNs progresses at the faster rate that the reactor offers. To ensure that the growth rate of the bacteria and EPNs match, we recommend that in future a double reactor setup is used and that the bacteria-rich medium is transferred at a specific feed rate into the reactor in which the EPNs are being cultivated.

Dedication

I dedicate this thesis to God Almighty, my Wife (Mamorapelo), my Son (Olulana) and my Siblings, Tunde, Jumoke, Seye and Kayode. (All Things are possible through Jesus for them that believe)

Finally I do appreciate the prayers of my Mother (Juliana) and the fervent wishes of my Father (Akin) that I should succeed in life.

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I do express my gratitude to Lubanza Ngoma and Mirabelle Nyamboli, who were my laboratory mates.

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Abbreviations

CFU	Colony Forming Units
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic acid
E.Coli	*Eschericia coli*
EPN	Entomopathogenic Nematodes
IJ	Infective Juvenile
LCM	Liquid Culture Medium
LPB	Lipid Broth Medium
OD	Optical Density
PCR	Polymerase Chain Reaction
TKN	Total Kjeldahl Nitrogen

1 Chapter One

An Overview

This thesis describes the experimental investigation carried out by the researcher in order to study and understand the growth patterns of the entomopathogenic nematode (EPN) species *Heterorhabditis indica* (Ireland) and *Heterorhabditis bacteriaphora* (South Africa). The growth of the *Photorhabdus luminescens* bacteria, which are symbionts of the nematodes, was also studied in order to understand the nature of their relationship with the EPNs.

To determine the factors that influence the growth of *Photorhabdus luminescens* bacteria and EPNs, we conducted a number of experiments in which *Photorhabdus luminescens* bacteria and the chosen EPNs were cultivated submerged in a liquid medium. This was done to assess the possibility of cultivating EPNs in submerged culture. The experiments were carried out at different scales, starting from shake flask experiments of different volume capacity and extending to varying laboratory-scale reactor types and volumes.

The supply of *Photorhabdus luminescens* bacteria was found to be critical to the growth of EPNs. Chapter 2 describes the investigation carried out to study the factors that influence the survival of the *Photorhabdus luminescens* bacteria. The impact on the growth of the *Photorhabdus luminescens* bacteria of running the experimental set-up as batch and continuous processes was evaluated, as were the effects of nutrients such as nitrogen, phosphorous and chemical oxygen demand compounds (COD), which are essential to the growth of micro organisms.

Chapter 3 outlines the investigation we undertook into the possibility of growing EPNs submerged in different types of liquid culture media. Egg yolk, lipid broth and nutrient broth media were used at shake flask level, and the results compared and evaluated. Another set of experiments concerned the availability of *Photorhabdus luminescens* bacteria as feed for the EPNs. A brief comparison was made between the *Heterorhabditis bacteriaphora* (South Africa) and *Heterorhabditis indica* (Ireland). Among the key conclusions drawn from the experimental results were the importance of factors such as reproducibility and the impact of adding fresh feed as pointers to ensuring optimum growth.

Chapter 4 discusses the scaling-up of the experimental process for producing EPNs in submerged culture. We tested the cultures at the shake flask to the reactor scale to identify the factors affecting the production of the EPNs in the different set-ups. These factors included the impact of air and of air pressure, the addition of fresh *Photorhabdus luminescens* bacteria feed, and media volumes in the reactor. The results reflected the different effects of the container volumes and types, which are discussed and analysed.

Chapter 5 is the section containing the conclusion and recommendations for further research. In it we highlight the core factors affecting the production of EPNs, as observed in the experimental process, and also propose steps to be taken to improve the processes used for the production of EPNs in submerged culture in the future.It also includes the discussions of th modelling of the EPNs lifecycle.

Each chapter in this thesis was written as a paper for publication, and as such has its own introduction, literature survey, conclusion and so on. The thesis is presented in this format. The reader can read the chapters in whichever sequence he/she prefers.

2 Chapter Two

Factors affecting the Growth Rate and Nutrient Utilization Dynamics of *Photorhabdus luminescens* under Batch and Continuous Culture

ABSTRACT

The bacteria *Photorhabdus luminescens* is a symbiont of the entomopathogenic nematode (EPN) *Heterorhabditis indica*. Experiments in culturing *Photorhabdus luminescens* bacteria in a shake flask and bioreactors were carried out to determine which factors affect bacterial growth, and in particular which properties of that growth could have significance for the design and operation of a system for the production of EPNs.

The doubling time of *Photorhabdus luminescens* bacterial growth was evaluated at about 3–4 minutes in both types of batch reactor used under a range of experimental conditions, while in the shake flasks it was about 20 minutes. After the stationary phase of bacterial growth we adopted a system of adding a continuous feed of fresh medium, which resulted in a short exponential phase. This showed that growth was dependent on the availability of nutrients.

The pH was lowest at the peak exponential phase of the *Photorhabdus luminescens* experiment. This could be attributed to the high CO_2 content of the medium. Subsequently, we observed a continuous pH increase during the stationary and death phases of the experiment. Colour was found to be an important indicator of culture contamination.

The dynamics of bacterial growth were monitored by plate counts, while the depletion of nutrients in the bacterial cultures was charted by measuring the amounts of nitrogen, chemical oxygen demand and phosphorous remaining in the medium. These were separately evaluated to investigate to what degree each supported the growth of *Photorhabdus luminescens* bacteria. Nitrogen was found to be the most important substrate for bacterial growth.

This experimental work on the dynamics of nutrient depletion helped to elucidate different but regular patterns in nutrient demand.

2.1 INTRODUCTION

Before detailing the procedures involved in the experiments summarized above, the author considers it appropriate to provide some background on scientific work that has already been published on EPNs.

Entomopathogenic nematodes are potentially of high economic importance when used as agents for insect pest bio-control, and have therefore generated a great deal of scientific interest. Various views on the commercial viability of the mass production of EPNs have been published. (Ehlers, 2001; Gaugler, 2001) The most widely-used mass culture procedures at present include three methods: *in vivo* cultivation of *Galleria mellonella* larvae; and *in vitro* solid and *in vitro* submerged liquid media culture. Bedding (1984) provides an excellent example of the *in vitro* culture of EPNs on a large scale using solid media, while Ehlers (2000) has successfully cultivated these nematodes, also *in vitro*, but using a liquid medium.

The production of EPNs has been carried out mainly in Europe and the Americas, and has involved co-culturing the different species with their associated bacterial symbionts. Species that have been successfully

cultivated include *Heterorhabditis indica* and its endosymbiont *P. luminescens*, which has a facultative relationship with the former. At present there are a number of ongoing investigations to see how other nutrients affect the growth of EPNs. *Heterorhabditis indica* was first isolated in the sugar cane fields of Tamil Nadu, in southern India. Ehlers et al. (2000) described it as high-yielding in submerged culture, and reported a mean count of about 457 000 IJ/mℓ and a highest sample count of 648 000 IJ/mℓ in a monoxenic *P. luminescens* culture. The Australian scientist Bedding (1983) mass-produced *Neoaplectana bibionis*, with its associated symbiotic bacteria *Xenorhabdus*, in autoclavable bags. Gaugler et al. (2002) discussed automated technology for the *in vivo* mass production of the *Heterorhabditis bacteriaphora* EPNs. Surrey and Davies (1996) also reported the successful cultivation of *H. bacteriophora* in an airlift fermenter inoculated with the *Photorhabdus luminescens* bacteria.

The scientific investigation of EPNs led to the identification of a number of different species. *Caenorhabditis elegans* is a widely-reported non-entomopathogenic nematode which feeds on the bacteria *Escherichia coli,* a member of the Enterobacteriacae, as do both *Photorhabdus* and *Xenorhabdusi*. Suda et al. (2005) used *C. elegans* to study the uptake of oxygen by nematodes in submerged liquid culture. Other species worked on include *H. heliothidis* and *Steinernema glaseri,* both identified initially by Dr Brooks at the University of North Carolina. (Zervos et al., 1990)

The key to future technological development of economically viable EPN mass-production should be based on the *in vitro* submerged liquid fermentation process reported on by Strauch and Ehlers (1998), Gaugler (2001) and Torre (2003). Various scientists have postulated a number of solutions to the challenge of optimizing nematode yields. For example, Torre (2003) suggested that the impact of hydrodynamics and oxygen transfer-rate on mating in submerged media is extremely important. Friedman (1990), on the other hand, laid more emphasis on technological

developments, particularly advances in the direction of medium composition and the effect of protein nutrient consumption by bacteria and nematodes in both axenic and monoxenic cultures. Similar studies concerning the effect of different media on the production of high densities of healthy infective juveniles were carried out by Yang et al. (1997), who compared the quality of growth of the *S. carpocapsae* Beijing strain cultured in three different artificial media.

Bailey (1986) described the process of cell growth in a liquid medium as being affected by both biological and environmental factors. An example of a biological factor is that increasing the densities of nematodes or bacteria causes a rise in chemical toxicity, pH shifts and nutrient depletion in the culture medium. His research was carried out to evaluate the impact of nutrient depletion on cell growth. The study described in this thesis also focuses on nutrient depletion, using *P. luminescens* in an egg yolk medium to investigate the growth rate of these organisms in order to explain the effect of bacterial dynamics on the nematode partner *H. indica*. Bailey suggested that a reduced supply of certain nutrients in the culture medium might have a more significant limiting effect on the growth of a particular microorganism than the lack of other nutrients. In the experiments carried out by the researcher, it was found that among those nutrients that are vital to the proper functioning of the cell is nitrogen, which forms about 12–13% of the bacterium cell body.

Much research has been done on the effects of various nutrients on EPN growth and yield. Hatab and Gaugler (2001) worked on the influence of diet composition and lipid elements on the *in vitro* culture of *H. bacteriophora*. The experiments carried out by Gil et al. (2002) clarified the impact of surplus glucose addition on EPN growth, while Ferris et al. (1998) investigated the effect of nitrogen mineralization on the metabolic activities and the growth dynamics of EPNs in submerged culture. Their work provides an invaluable starting-point for a further study of the

pattern of nutrient depletion in the bacto-helminthic complex *P. luminescens* and *H. indica* nematodes reared in submerged egg-yolk medium. Parameters such as pH, dissolved oxygen content and optical density (600nm) were also monitored.

The principal nutrients required in most aeration-based systems are carbon compounds (COD), nitrogen, phosphorous and sulphur, all of which are used for metabolism in biological systems. (Bailey, 1986) These essential compounds are required for the growth and reproduction of the nematodes. Bailey also emphasized the importance of these nutrients and proteins for every aspect of cell development and activity, such as cell wall growth and chromosomal functions.

Different scientists have supplied further definitions of these principal compounds. Bailey describes COD compounds as the amount of oxygen that a 1-litre sample of medium will absorb from an acidic solution of dichromate. A typical expression of chemical oxygen breakdown in an aerobic system is shown in equations 1 and 2:

$$COD + O_2 \rightarrow 6\ CO_2 + 6\ H_2O \quad [1]$$

Assuming COD is a glucose compound

$$C_6H_{12}O_2 + O_2 \rightarrow 6\ CO_2 + 6\ H_2O \quad [2]$$

Metcalf and Eddy (2004) measured the nitrogen concentration as total Kjeldahl nitrogen (TKN), as shown in equation 3:

$$\text{Total Kjeldahl Nitrogen: Organic Nitrogen} + NH_3 + NH_4^+ \quad [3]$$

The materials and methods section that follows describes the measurement of COD and nitrogen compounds, and outlines the analysis

of nutrients carried out by the researcher. It also elucidates how these relate to the growth curve of the *Photorhabdus luminescens* bacteria isolated from the *Heterorhabditis species of* EPNs.

The long-term objective of the research was to try to grow EPNs successfully and reproducibly. However, because the EPNs feed on the bacteria we first need to determine the important characteristics of the growth of the latter, and in particular identify what limits the growth of the bacterial system. This makes it possible to understand what significance these data might have for the behaviour of the EPN system.

The investigation of the constraints and factors affecting the growth of the bacteria is outlined below. Its importance relates to how the results will inform the experiments performed on the EPN system, and how both sets of experiments will yield indicators that determine reactor design and operation.

2.2 MATERIALS AND METHODS

2.2.1 The isolation of *Photorhabdus luminescens* from EPNs in *in vivo* culture

The EPN used in the laboratory tests described below was *G. mellonella* experimentally infected with the nematode *H. indica*. The multiplication of the EPN *in vivo* was observed by means of a stereo microscope. The isolation of bacteria was carried out under sterile conditions using a laminar-flow hood. *P.luminescens* bacteria were extracted from the nematodes with a streaking rod that had been sterilized by plunging it into ethanol absolute (Saarchem) 99% and exposing it to a naked flame. It was then dipped into the infected cadavers of the larvae and streaked onto nutrient agar plates, which were placed upside down in an incubator at 25^0C. The plates were checked for the growth of bacterial colonies after 24

hours. A colony of *Photorhabdus* was then taken from the plate and used as an inoculum for an autoclaved nutrient broth (Merck) solution.

The egg yolk medium (Surrey & Davies, 1996) was made up of 12.5g spray dried egg; 23g yeast extract (Merck); 2.3g sodium chloride (NaCl) 99% purity (Saarchem); 40mℓ corn oil and 1 000mℓ distilled water. The liquid culture medium (LCM) consisted of 10g Tryptic soy broth (Biolab); 10g nutrient broth (Biolab); 5g yeast extract (Biolab); 0.35g potassium chloride (KCl) (Saarchem); 0.21g calcium chloride ($CaCl_2$); 2.3g sodium chloride (NaCl) 99% purity (Saarchem); 2.5g casein (Merck); 2.5g peptone (Merck); 30mL sunflower oil and 1 000mℓ distilled water. Both media were sterilized before use.

2.2.2 Inoculation of submerged liquid media

P. luminescens bacteria isolated from infected insect cadavers were introduced into the egg yolk medium after a growth period of 18–48 hours in nutrient broth. The amount of bacterial inoculum added to the medium was between 1–10% of its volume, depending on the environment in which the experiment was being carried out. Under laboratory conditions using a laminar-flow hood, the researcher proposed that the inoculum should be in a proportion of 8–10% of the volume of medium as an extra precaution to prevent contamination.

2.2.3 Bacterial nutrient depletion of *Photorhabdus luminescens*

The nutrients measured were the nitrogen, phosphorous and COD, which provided an indicator of carbon, phosphorous and nitrogen depletion in the medium. The optical density (OD) of the samples was measured at five-hour intervals, and the number of colony-forming units (CFUs) was estimated using serial dilutions. Ten mℓ of each remaining sample was

then centrifuged, and the supernatant and pellet separated and collected. The pellet was diluted with 0.5mℓ of the original sample, after which the determination of the amount of nitrogen and COD in the various samples was made according to the instructions that accompanied the C9800 Hanna reactor (Hanna instruction manual, 2002).

2.2.4 The counting of colony-forming units for bacterial growth

A CFU count was carried out using autoclaved sterile water for dilution. The bacteria in the medium were well mixed before a 1mℓ sample was collected, using a sterile eppendorf pipette. The bacterial sample was then transferred to a vial containing 9mL of autoclaved water, and mixed. A further 1mℓ sample was collected from that vial and added to another vial containing 9mℓ of water, and the process was repeated until about 10^7 to 10^9 bacteria concentration dilution was reached, depending on the experiment in hand. In the next stage samples were taken from each of the vials, introduced onto agar plates, left to grow, and the CFUs counted after 24 hours.

2.2.5 The measurement of nitrogen in submerged media

The total amount of nitrogen measured in the samples of bacteria in medium fell within the total high range specified by the Hanna instruction manual (2002). The experimental procedure required the preparation of a standard or blank solution made by adding potassium persulfate in a sachet to a total nitrogen HI solution digestion bottle, together with 0.5mℓ of distilled water, and mixed by inverting the bottle several times for about 30 seconds. It was then inserted into the C9800 Hanna reactor and heated at 105^0C for 30 minutes before being removed from the reactor and allowed to cool to room temperature. Sodium persulfate powder in a sachet was then added to the bottle and mixed for about 15 seconds, after

which the bottle was allowed to rest for about three minutes, without mixing, for digestion purposes. Total nitrogen reagent (HI 93767-0) in a sachet was added next, and mixed for 15 seconds. The bottle was then left undisturbed for about two minutes for completion of digestion (Hanna, 2002).

At this point 2mℓ of the bacteria sample was collected in a syringe and added into the nitrogen reagent bottle. Effective mixing was achieved by inverting the bottle 10 times before using the C241 Hanna Photometer to calibrate the contents of the bottle. Care was taken when mixing because of the increase in temperature of the nitrogen reagent bottle. The photometer programme was set for total high range nitrogen (Programme 7) before measurement, and a lint-free cloth was used to dry the outside of the bottle before calibration.

The measurements of total high range nitrogen in the samples collected were the same as those for the standard reagent heating and digestion procedures. However, because the concentration of nitrogen differed in the supernatant and pellets, they required varying degrees of dilution before using the C241 Hanna Photometer to calibrate the contents of the bottle. The need for dilution of a representative sample before the addition was greater in the case of the pellets, because their nitrogen content was above the permissible range of 150mg/ℓ measurable by the C241 photometer (Hanna, 2002).

2.2.6 The measurement of chemical oxygen demand in submerged media

The determination of COD was made using the supernatant of a centrifuged sample. The corresponding pellets were recovered and the original non-separated samples collected at different times during the experiment. The meter measured a maximum COD of 1 649mg/mℓ.

The samples were diluted in order to obtain readings within the correct range, and the dilution factor was taken into consideration when back-calculating. The samples were well homogenized in a vortex before the addition of reagents, to ensure proper mixing.

A 0.2mℓ sample was then added into the reagent vial, which was denoted as reagent set HI 93754B-25 (Hanna), and 0.2mℓ of deionised water was added into the blank vial. The researcher ensured that both vials were at an angle of 45^0 when samples were added, and afterwards sealed the caps of the vials tightly and mixed the contents by inverting them 15–20 times. Because the mixing led to an increase in temperature of the vials, care had to be taken to prevent burns when handling them. A lint-free cloth was used to wipe the vials if their outer surfaces were wet, after which they were inserted into the reactor at 150^0C and heated for two hours. Thereafter, the vials were allowed to cool for 20 minutes, which allowed their temperature to drop to 120 ^{0}C, mixed by inverting them about 35–40 times, and placed in a test tube rack to cool. The COD content of the vials was measured at room temperature by a C214 photometer that had been calibrated by using a blank to determine the appropriate setting. The contents of the vials were not mixed after the final cooling in order to ensure that the readings were not distorted. The vial used as a blank was expected to be stable for 1–2 weeks at room temperature (Hanna, 2002).

2.2.7 Measurement of phosphorous in submerged media

The phosphorous measured in the samples of bacteria in medium fell within the total phosphorous range specified by the Hanna instruction manual (2002). The experimental procedure required the addition of potassium persulfate for phosphorous in a sachet and about 5mℓ from the reagent vial to a digestion bottle, which was then shaken until its contents were completely dissolved. It was then inserted into the C9800 Hanna reactor and heated at 150^0C for 30 minutes before being removed from

the reactor and allowed to cool to room temperature. The next step was to remove the cap of the digestion bottle and add about 2mℓ of sodium hydroxide solution of about 1.54N, replace the cap, and mix the contents by inverting the bottle a couple of times before using it to zero the C241 Hanna Photometer by inserting the vial into the meter. (Hanna, 2002).

Effective mixing was achieved by inverting the bottle 10 times before using it to calibrate the C241 Hanna Photometer. The photometer programme was set for total phosphorous (Programme 13) before measurement, and a lint-free cloth was used to dry the outside of the bottle before calibration.

The measurements of total phosphorous in the samples collected were done by adding one packet of HI 93758-0 phosphorous reagent. This was done by removing the cap of the vial (digestion bottle), adding the reagent, closing the bottle tightly, and mixing gently for about 2 minutes until all the powder was completely dissolved. The vial was then inserted into the C241 Hanna Photometer for measurement. The phosphorous content of the vial can be read off the meter display.

2.2.8 The measurement of pH

A Eutech Instrument alpha- pH 800 was used to measure pH, together with a pH/ ORP controller meter and a pH probe (Cole Palmer) that was sterilized by autoclaving or washing with both chlorine and ethanol. Calibration was carried out using standard buffers of pH 7 and 10.

2.2.9 The measurement of dissolved oxygen

A B & C Electronics OD565.2 oxygen meter was used in conjunction with a Cole Palmer dissolved oxygen probe that had been autoclaved beforehand at 120 ^{0}C at a pressure of 1 bar. The probe was calibrated adding 5mℓ of sodium dithionite in 100–150mℓ distilled water. The lower calibrating point was established by inserting the probe into the solution, after which it was rinsed in the sodium diotinide solution and allowed to stand in ambient air, or inserted in distilled water, to reach the upper calibrating point. After that the probe could be used to determine the amount of dissolved oxygen in the media.

2.2.10 Measurement of optical density

The researcher used a WPA/ Biowave Labotec 521900 diode array/ UV light- based spectrophotometer at 600nm to measure the optical density of the contents of the vials. The spectrophotometer was calibrated using a blank sample of the egg yolk medium.

2.2.11 Centrifuging of samples

A Hettia Universal centrifuge was used at 70rpm for 20 minutes.

2.2.12 The fermenter reactor set-up

The **fermenters** were glass reactors, each fitted with a stainless steel lid with flexible tubing connected to it, to allow the feed to be pumped into the reactor. Air was pumped in from the top through a stainless steel pipe that ran from the lid to the bottom of the reactor, and then distributed by means of a sparger. This was done with an Air cadet pump made by Barnant Company (Birmingham, Illinois 60010), operated at a maximum of 50Kpa. (Once this pressure level is exceeded, there is a likelihood that medium content will be lost owing to excessive pressure, which causes foaming and a subsequent overflow of medium containing nematodes. This is discussed in greater detail in 4.3.3 below.) The pump head used was a Master Flex, model 77201-62. The air was sterilised using a Cole-Palmer syringe filter, sterile G.D PTFE membrane and PP housing. The rotameter fitted was capable of measuring an air pressure of up to approximately 100mm of air. The temperature in the reactors was maintained at 26^0C by means of a stainless steel pipe that ran through the reactor and opened through the lid, where flexible tubing connected to the opening was fitted to the Jutabo water bath.

The fermenter reactor was equipped with three monitoring devices (Eutech probes): for pH, temperature and dissolved oxygen.

2.3 RESULTS AND DISCUSSION

The results obtained from the bacterial growth count and the nutrient depletion experiment for batch and continuous operation are presented and discussed in this section.

The various factors influencing the growth of *Photorhabdus luminescens* bacteria were evaluated under a range of conditions in order to determine their reproducibility and the factors that influenced their growth. Only a few of the results are presented here, to show the kinds of results we obtained, the trends that we identified, and how we reached our conclusions.

Table 2.1 shows the different conditions under which the bacteria were tested.

Table 2-1: Examples of results; Factors influencing growth of *P.luminescens* bacteria

Experiment	Time spent by initial inoculum in nutrient broth	Type of medium in reactor or shake flask	Volume of initial inoculum	Volume of medium	Reactor Type
1	48 hours	LCM	40mℓ	1000mℓ	fermenter
2	48 hours	Egg yolk	40mℓ	1000mℓ	fermenter
3	< 18 hours	LCM	10mℓ	1000mℓ	fermenter
4	48 hours	LCM	0.5mℓ	50mℓ	shake flask

The growth curves for the four different experiments in Table 2.1 are given in Figure 2.1 below. These results are fairly typical of all the various experiments we performed.

It is important to note that the results in Figure 2.1 show some variability in respect of the time at which growth occurs in the different experiments. The first observation that was made was that the shake flask experiments were never able to achieve the high concentrations of bacteria that the fermenters operating in batch mode could. Not all the experiments in the fermenters achieved the higher concentrations, as can be shown by Experiment 3, the possible reasons for which will be discussed later.
Furthermore, the experiments carried out in the fermenters typically had shorter lag times before the exponential phase started.

In Experiment 3 a higher air pressure was introduced into the reactor, which caused foaming. We also used a shorter inoculation time and a smaller volume of inoculant. We were unable to ascertain which of these (whether individually or in combination) caused the poorer results we obtained. We therefore concluded that there is variability in the performance of the fermenters and that optimisation is necessary.

The difference in performance between the fermenters and shake flasks is attributable to the greater availability of air in the fermenters. Air is pumped into the bioreactor through a sparger at a maximum of about 15kpa, and the fine air bubbles ensure good mixing, resulting in higher concentrations of bacteria, as is evident in the results shown for experiments 1 and 2. In contrast, the air supply in the shake flask is limited because the outlet of the flask is covered with aluminium foil, which inhibits air flow. The small amount of air trapped in the shake flask in these experiments (such as Experiment 4) is distributed to assist the growth of the bacteria by the swirling action of the shaker. However, this is not sufficient to allow the shake flask experiments to reach the bacterial counts that we achieved in the fermenters.

An important finding arising from analysis of the growth curves in Figure 2.1 is the fast growth experienced in experiments 1 and 2, which was

evident in the first 30 hours of the experiment. This demonstrated that in a reactor with proper aeration and an adequate volume of inoculum, the luminescence bacteria can sustain continuous growth for 30 hours. At that point growth starts to decrease.

The possibility of creating conditions that sustain continuous growth of luminescence bacteria in liquid culture media for longer periods will be explored in a later section.

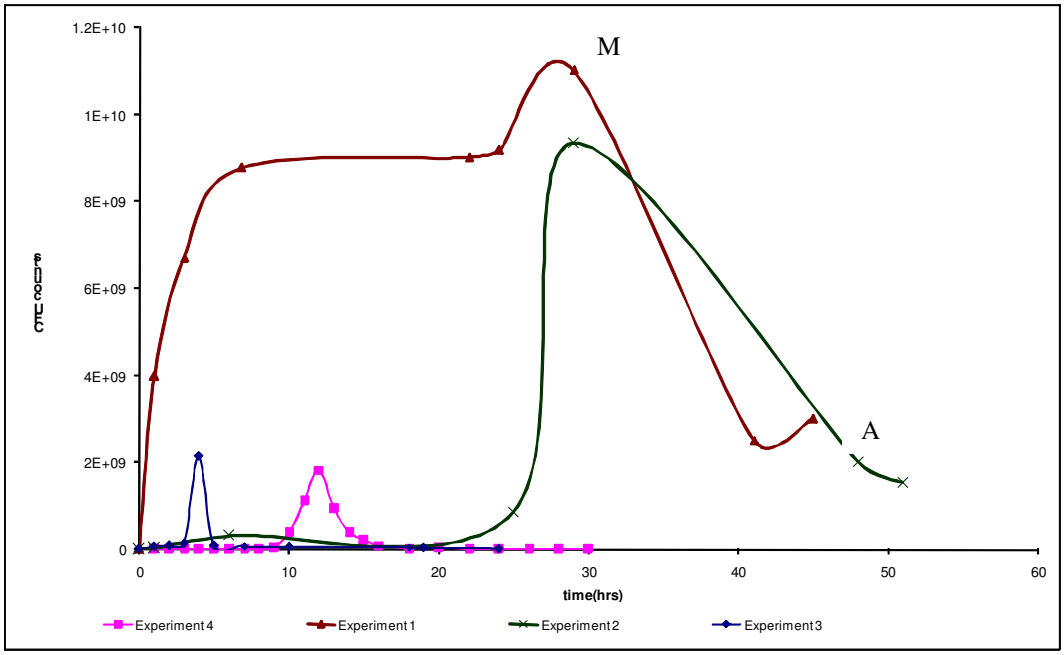

Figure 2.1: Comparison of the growth curves of *Photorhabdus luminescens* bacteria for different batch experiments, as described in Table 2.1

N.B

Experiment 1: LCM in fermenter (well aerated)

Experiment 2: Egg yolk medium in fermenter(well aerated)

Experiment 3: LCM in fermenter (well aerated)

Experiment 4: LCM in shake flask (covered with foil, no aeration)

A characteristic of the growth of *Photorhabdus luminescens* in medium is the gradual change in colour that can be observed in the different media. In the LCM samples the bacteria produce a transparent light yellow colour,

while those cultivated in egg yolk appear reddish-orange or reddish-yellow.

The experimental problems the researcher encountered in the experiments in the fermenters were: foaming; loss of reactor content due to high air pressure; gradual evaporation of the water content of the reactor, caused by the bubbling of air; and the clogging of probes. The best means of controlling loss of water, which may lead to an increase in the salt load of the reactor, is to monitor the volume of liquid in the reactor. If significant changes are observed, sterilized autoclaved water should be added. The only feasible solution to the clogging of probes, a problem that occurs frequently, is to have a supply of spare probes for replacement purposes.

The log exponential plots for the experimental results illustrated in Figure 2.1 are shown in Figure 2.2, which clearly defines the different growth phases in the fermenter and shake flask experiments. We see that a very high gradient occurs in the exponential phase for the fermenter when compared with that of the shake flask. The log plot peaks at maximum exponential phase, has a very short stationary phase and then enters the death phase, which is characterized by a drastic decrease in the number of luminescence bacteria. Phase variation of the bacteria may take place at this stage. Two major attributes of the *P. luminescens* at the primary and exponential phases are their anti-biotic activity, which enables them to fight off foreign contaminants, and their ability to auto-fluoresce.

Equation 4 was used to calculate the doubling time of the *Photorhabdus luminescens* bacteria.

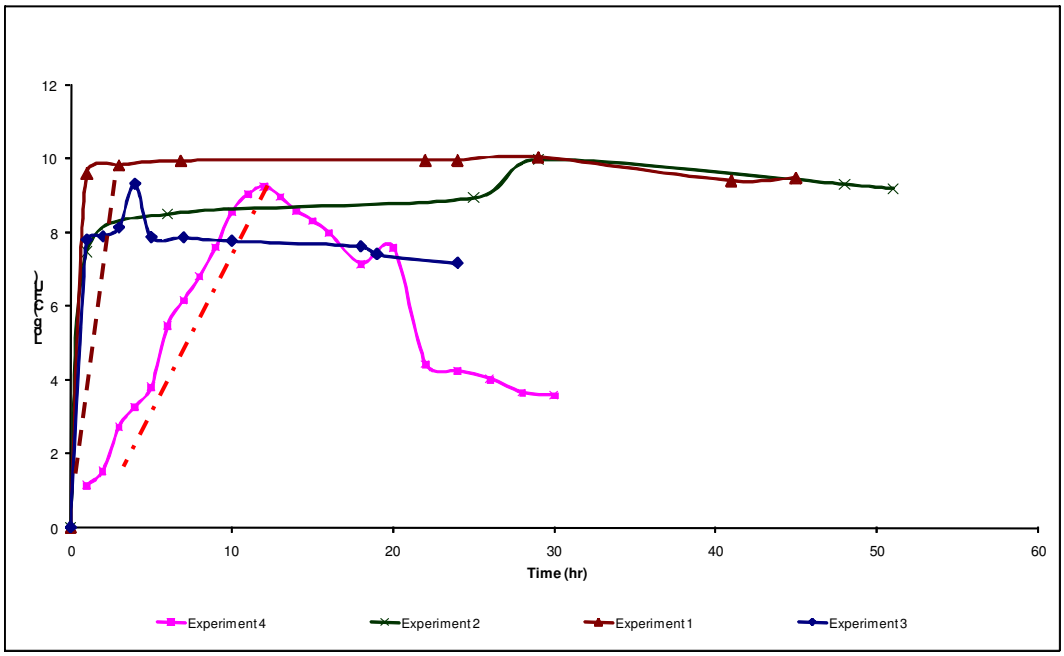

Figure 2.2: Log plot evaluation of the bacteria growth curve for *Luminescens* bacteria for the fermenter and shake flask experiments as defined in Table 2.1

– – – – Exponential phase – Fermenter (Exp 1, 2&3)

– · · — Exponential phase - Shake flask(Exp 4)

N.B:

Experiment 1: LCM in fermenter (well aerated)

Experiment 2: Egg yolk medium in fermenter (well aerated)

Experiment 3: LCM in fermenter (well aerated)

Experiment 4: LCM in shake flask (covered with foil, no aeration)

$\mu_{max} = \log_{10} 2 / t_{½}$ [4]

when μ_{max} : $\log_{10} A_{exp} - \log_{10} B_{lag}$

(Bailey and Ollis, 1986),

where A_{max} is the maximum *Photorhabdus luminescens* count at exponential phase and B_{lag} is the *Photorhabdus luminescens* count at the start of the exponential phase.The high log count in Figure 2.2 was attributable to the experimental set-up, which provided a well-aerated

system for Experiments 1–3. The reproducibility in these sets of experiment confirms the results.

The calculated doubling times for the four experiments are shown in Table 2.2.

Table 2-2: Calculated doubling time for P.luminescens bacteria for the experimental data shown in Figure 2.2

Experiment No	Doubling time
1	4.6 minutes
2	4.5 minutes
3	3 minutes
4	20 minutes

The doubling time in a well-aerated fermenter is 3–4 minutes. The shake flask count (Experiment 4) was 33.22 cell generation in 11 hours, which gives a similar doubling time to that described by Nyamboli (2008), making it consistent with the doubling time obtained with other strains of bacteria in shake flasks and under similar conditions The doubling time for Experiment 4 is also in the same region as that given by Bowen and Ensign (2001) using the same experimental apparatus.

In our research, the shake flasks were covered with foil to prevent contamination of the medium. This meant that there was a limited supply of air. Experiments 1 to 3 were carried out in fermenters or reactors, where air was pumped into the system. There was a sufficient supply of air, which was well distributed to ensure good mixing. This was the main explanation for the faster doubling time (3–4 minutes). The difference in results between experiments 1–3 and Experiment 4 confirms that the experimental set-up can affect the growth achievable for the *P.luminescens* bacteria.

This result is very important because it shows that the growth of the bacteria in the correct conditions is very rapid, occurring in a matter of minutes and not, for example, days or weeks.Thus in designing as reactor system to grow EPNs it is very important to bear in mind that the time constant (or growth rate) for the bacteria is of the order of a quarter of an hour. Furthermore, although the growth of the bacteria could be optimised, one would need to measure the growth constants for the EPNs to see what the rate-limiting process is.

In analyzing the results of Experiment 2, presented in Figure 2.2, we asked the question: what factors limit the growth of bacteria, or encourage it, that is, lead to the maximum concentration observed? The hypothetical answer was that the reactors run out of nutrients, which retards bacterial growth. This was tested by continuing with Experiment 2 and switching from the batch to a continuous system, at the point indicated by point A in Figure 2.1. The results of this experiment are shown in Figure 2.3. Point M, shown in both figures 2.1 and 2.3, corresponds with the maximum concentration of bacteria when running in batch mode.

The reactor was taken from a batch to a continuous system after 50 hours. A calculation of the residence time for the continuous phase suggested that about 167 minutes (approximately three hours) was required in the reactor to prevent any wash-out of the bacteria at continuous phase. Following the addition of fresh sterile media to the existing experimental liquid, a high increase in the number of bacteria was observed. This indicated that the reduction in the bacteria count after 30 hours in the aerated reactor was attributable to the exhaustion of the medium substrate. These findings showed that the growth of *Photorhabdus luminescens* may be increased further by the addition of fresh feed, and provided the basis for the introduction of fresh medium into existing cultures in subsequent experiments.

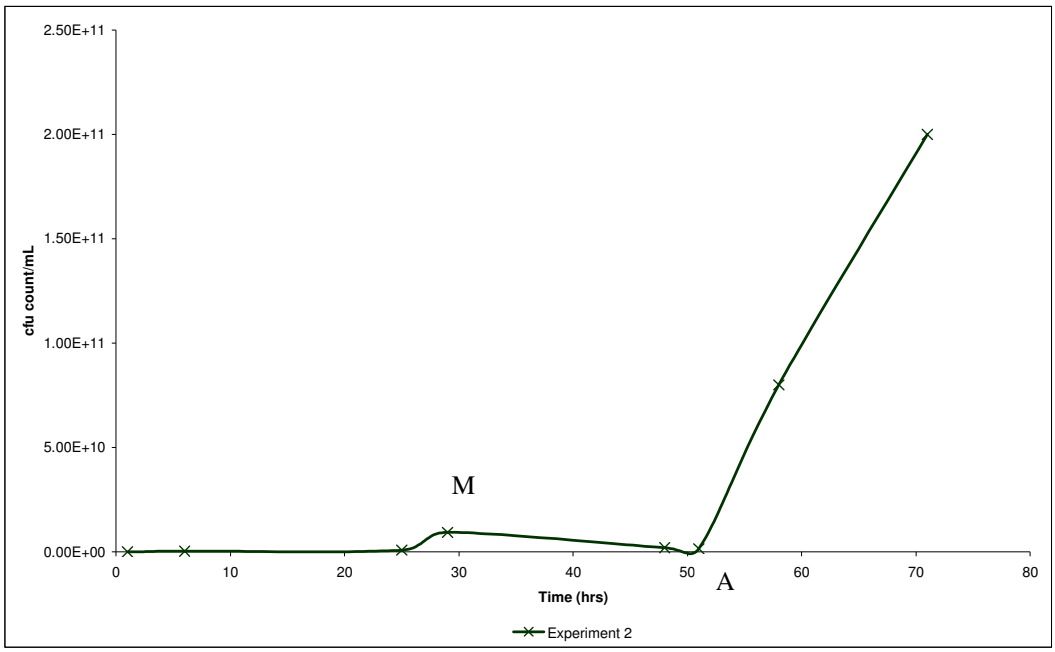

Figure 2.3: *Photorhabdus luminescens* bacteria growth curve Experiment 2, in batch operations up to point A, followed by continuous operation from point A

N.B: Experiment identified in Figure 2.1 as Experiment 2 was progressed from batch operations to continuous operation.
A: the point where continuous feed was introduced
M: the maximum concentration of bacteria in batch mode
Experiment 2: Egg yolk medium in fermenter (well aerated)

Figure 2.4 provides further information on the growth of the *Photorhabdus luminescens* bacteria by showing the growth curves for experiments 2 and 3 for both the batch operation and the switch to continuous mode. The latter, entailing the continuous addition of fresh media, was started at the onset of the death phase for the batch reactors. The two experiments differed in respect of the volumes of initial inoculums they contained, and the periods during which the bacteria were cultured. This graph shows that on the addition of fresh feed at a flow rate of about 6mℓ/min there was an increase in the growth of the *Photorhabdus luminescens* bacteria in the medium. The residence time was evaluated at about three hours at continuous phase.

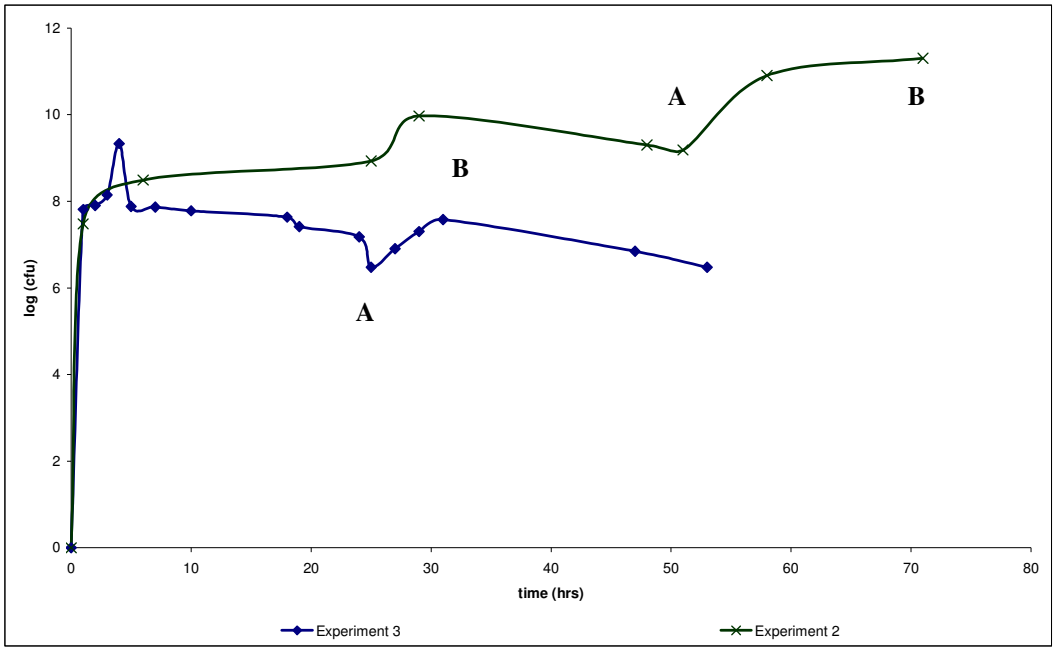

Figure 2.4: Log plot of the concentration of *Photorhabdus luminescens* bacteria as a function of time in the fermenter reactors for operation as batch reactors up to point A, and then switching to continuous operation.

N.B: Experiment identified as Experiment 3 and Experiment 2 in Figure 2.1 is progressed from batch operations to continuous operation.

A: Addition of fresh medium into reactor to proceed to continuous system

B: Peak of the short exponential phase experienced on adding fresh medium

Experiment 2: Egg yolk medium in fermenter (well aerated)

Experiment 3: LCM in fermenter (well aerated)

These results show that the concentration of bacteria that can be achieved is limited (in a situation where sufficient air is supplied) by nutrients. Thus the reactors used to grow high concentrations of bacteria firstly require enough air (but not too much, because an oversupply causes foaming and excess turbulence), and a supply of nutrients.

Figure 2.5 shows the pattern of changes measured for the pH of the media as the number of *Photorhabdus luminescens* bacteria in the reactors altered over time. The pH measured dropped to about pH 6.1 during the

exponential phase, possibly owing to the increase in production of carbon dioxide by the microbes during respiratory and metabolic activity (as shown in equation 1). After the completion of the exponential phase, the pH of the media started to increase until the death phase was reached. This may be attributed to a number of different factors, such as the formation of hydroxide compounds in the media. On visual inspection the media in the death phase appeared more viscous and cloudier than initially (at the start-up phase). This is a common observation in the course of such experiments. However, it can be inferred that the pH is not a factor that influences the growth of *Photorhabdus luminescens* to any significant extent.

This result suggests that pH could potentially be used as an online monitoring method to determine when the bacteria are growing and, more important, whether the bacteria are dying.

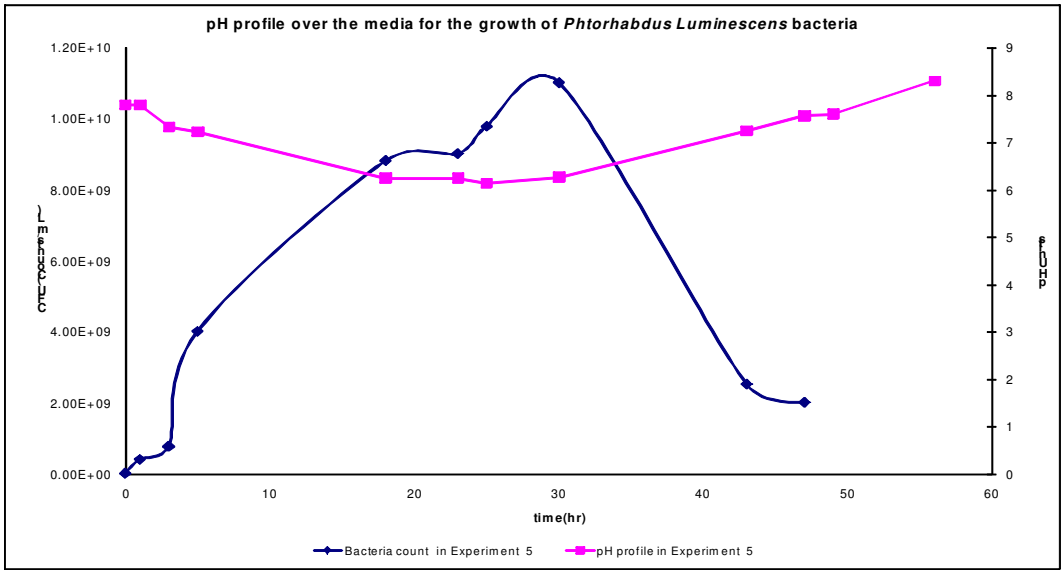

Figure 2.5: Evaluation of the pH profile in liquid culture media inoculated with *Photorhabdus luminescens* bacteria

N.B: Experiment 5: LCM in fermenter (well aerated)

Experiment 5 was conducted to determine the pH profile in the reactor over time. This was the result of

We next looked at the question of investigating whether any particular nutrient had a limiting effect on the growth of the bacteria. First we looked at the relationship between nitrogen and bacterial growth. Figure 2.6 shows the curves for both the growth of the *P. luminescens* bacteria and the associated nitrogen depletion in the centrifuged liquid and the pellets. In the centrifuged sample, the amount of nitrogen was found to increase over time. This may be explained by the enzymatic activity of the luminescence bacteria, as suggested by Shapiro et al. (2000), who measured the organic and inorganic content of *luminescens* bacteria and nematodes on solid plates. Therefore we may infer that the insoluble nitrogen in the liquid decreases as the bacteria feed and break the available nitrogen down. However this leads to a corresponding increase in the nitrogen in solution. The concentration of nitrogen was found to be highest at about 25–26 hours

A corresponding increase in the number of *P. luminescens* bacteria in solution occurs until about 29 hours have elapsed. It is assumed that the soluble nitrogen is used up by the bacteria, which are at this point approaching their maximum growth (measured at 29–30 hours). Just before that point, the nitrogen in the centrifuged sample started to reduce, and within two hours, the nitrogen measured in the centrifuged sample had decreased to just below 0.005mg/mL. Subsequently there was a slight increase in the nitrogen measured in the pellets. At batch (after 29 hours) the measurements showed a continuous drop in nitrogen concentration. This supports a study carried out by Bailey (1986) on the influence of nitrogen on bacterial growth. He suggested that nitrogen was the most important nutrient for metabolic activity in bacteria. Shapiro et al. (2000) have also published a confirmatory hypothesis that nitrogen is very important to the growth of EPN bacteria such as *P.luminescens*.

Figure 2.7 further underlines the crucial role of fresh nutrients. After adding fresh media (and hence nitrogen) continuously, we observed a

corresponding increase in bacteria counts when the system was taken to continuous operation. A parallel rise in nitrogen concentration in the centrifuged sample and the pellets was measured during this escalation in *P. luminescens* count. It can be concluded that the role of nitrogen seems to be very important to the growth of the bacteria and that therefore it would appear to limit the growth of the bacteria. This result does not prove conclusively that it is only nitrogen limiting the growth, and in further work described below we examine the role of other nutrients in the medium to see if these are also limiting.

It is important to note that at the endogenous stage of the bacterial growth the dead bacteria decay, and hence should add some nitrogen back into the medium. However, if this decay process is the only source of nitrogen for bacterial growth, it would limit the growth of the bacteria as the decay process is very slow. In this situation the growth of the bacteria would be roughly the same as the rate of decay. This is not an optimal condition for the growth of bacteria, and hence an important implication for the design of our system is that we should ensure that nitrogen-rich fresh medium is added to the fermenter to sustain the growth of the *P.luminescens* bacteria. A major limitation of our system was that measurement of the bacteria could be done only during the daytime, while the experiment ran continuously.The reading was taken at 58 hours, left for the night and resumed at 70 hours, when the researcher noticed that the feed had stopped. Figure 2.7 shows the growth of bacteria in relation to the consumption of nitrogen.The measurement of nitrogen shown at 70 hours is specific for the time after the feed had stopped.

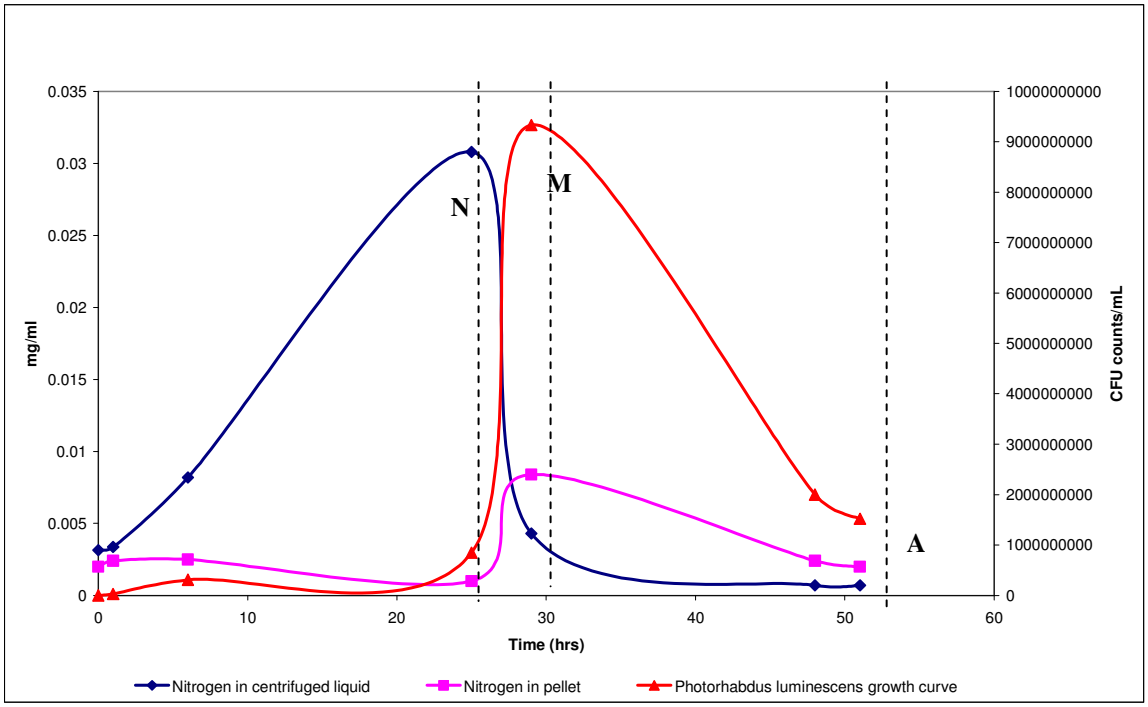

Figure 2.6: Nitrogen concentration in the liquid and pellets as a function of time and bacteria growth curves for *Photorhabdus luminescens* in a batch fermenter system for Experiment 2

N.B: The growth curve for Experiment 2 is the same as the curve described in Figure 2.1.

M: the maximum concentration of bacteria in batch fermenter

N: the maximum concentration of nitrogen substrate in batch fermenter

A: the lowest concentration of nitrogen in centrifuged liquid and bacteria in batch fermenter

Experiment 2: Egg yolk medium in fermenter (well aerated)

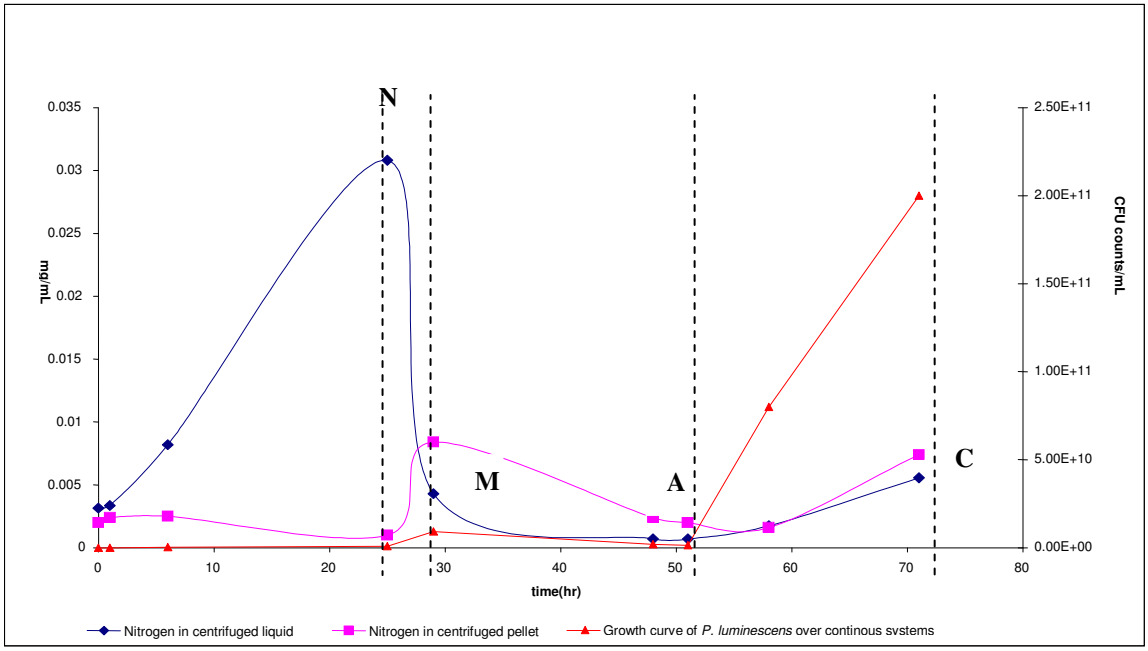

Figure 2.7: Nitrogen concentration in both liquid and solid (pellets) as a function of time and bacteria growth curves for *Photorhabdus luminescens* in Experiment 2, for batch fermenter from time 0 to point A, and switched to continuous at point A.

N.B:

M: the maximum concentration of bacteria in fermenter under batch operation

N: the maximum concentration of nitrogen substrate in fermenter under batch operation.

A: the lowest nitrogen in centrifuged liquid and bacteria concentration in fermenter under batch operation

A: Addition of fresh media into reactor to proceed to continuous system.

C: Increasing nitrogen concentration and bacteria count at continuous system

Experiment 2: Egg yolk medium in fermenter (well aerated)

The curve shown in Figure 2.8 represents the rise in COD of *P.luminescens* over time. *The results indicate that the COD is not limiting the growth of the bacteria at point A with the medium used.* Theoretically one would expect that in an aerobic system, such as the one used for the cultivation of *Photorhabdus,* the COD will be the principal substrate, but the characteristics shown in Figure 2.6 and Figure 2.8 indicate that it is nitrogen rather than COD.

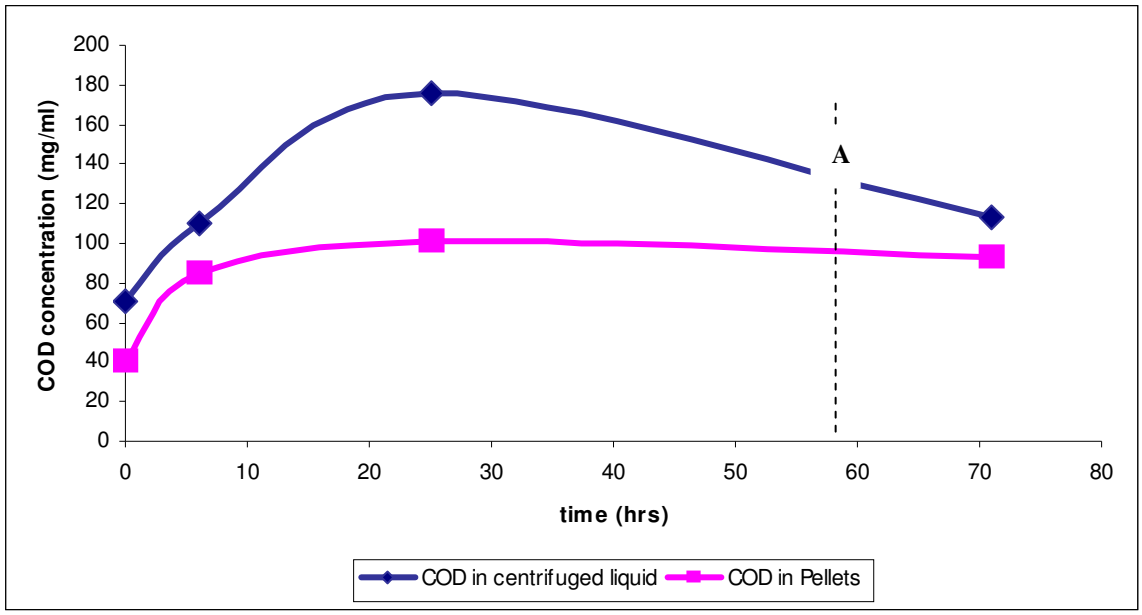

Figure 2.8: Chemical oxygen demand curve for *Photorhabdus luminescens* in Experiment 2 for a batch fermenter from time 0 to point A after which operation is switched to continuous from point A

N.B:
Experiment 2: Egg yolk medium in fermenter (well aerated)

Figure 2.9 shows the behaviour of phosphorous concentration over the batch and continuous operations in the fermenter. The initial measurement showed a slight drop in concentration of the phosphorous in the pellets, which coincided with an increase over that measured in the liquid phase. However, slight changes in concentration were observed over the phosphorous curve (which is unlike that for nitrogen in Figure2.6).

This would seem to support the finding that the growth of the bacteria in these experiments is not limited by phosphorus, as it is by nitrogen. Again the implication for the design and operation of a bacterial – EPN system is that the medium needs to be enriched by nitrogen if we wish to achieve higher bacterial concentrations in a batch reactor. Alternatively we need to consider running a fermenter on a continuous basis in order to supply the required nitrogen to the bacteria.

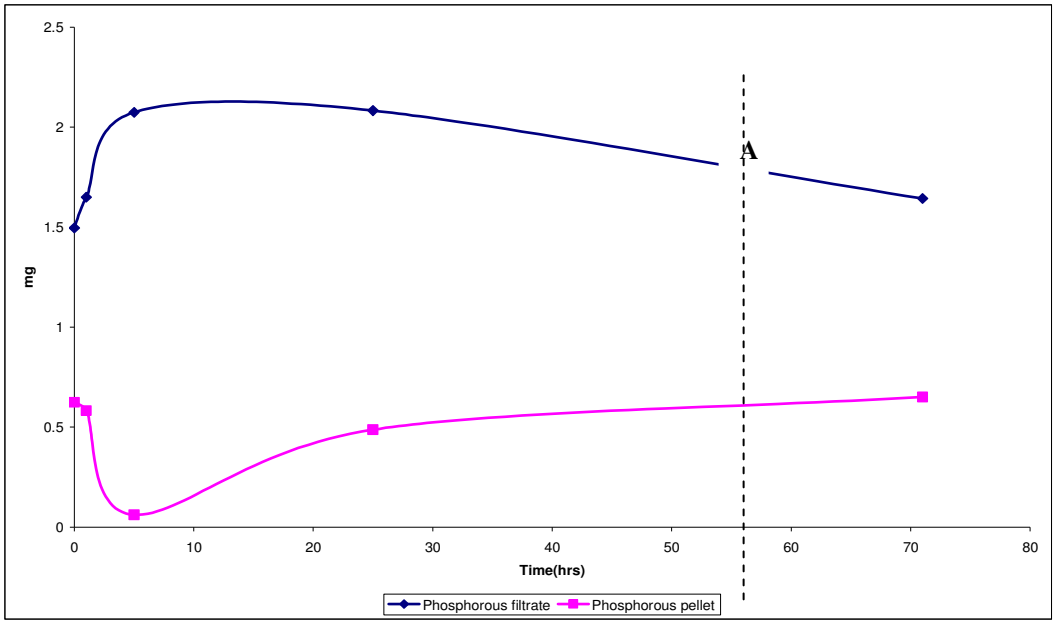

Figure 2.9: Phosphorous measurement for *Photorhabdus luminescens* in Experiment 2 for batch from time 0 to point A and switched to continuous from point A

N.B:
Experiment 2: Egg yolk medium in fermenter (well aerated)

2.4 CONCLUSION

The experiments carried out using the fermenters (experiments 1, 2 and 3), in comparison with those with the shake flask (Experiment 4) show that the lag phase for the *luminescens* bacteria can be reduced. Perhaps more important was the finding that the maximum concentration of bacteria can be increased. This was attributed to more favourable conditions in the fermenters and in particular the increased availability of air, which is bubbled directly into the fermenter. In contrast, the shake flask is sealed at the top, limiting the air supply to the reactor.

An important result in the experimental findings was the high bacterial population count achieved about an hour after the start of the experiment, and that the doubling time of the bacteria under batch conditions is about 3-4 minutes. We need to understand the growth characteristics of the EPNs to understand whether a high bacterial population is a limiting factor, and hence how necessary it is to optimise the growth rate of the bacteria when designing the overall system

Moreover, the introduction of fresh feed into the reactor on the continuous system led to an increase in number of bacteria. This led to the conclusion that the reduction in number of bacteria at a certain stage of growth resulted from exhaustion of the substrate in the submerged medium, which in turn indicates the importance of nutrients in fresh feed to the growth of *Photorhabdus luminescens* bacteria in media.

The researcher also evaluated the pH of media in a batch experimental set-up for the bioreactor, and found that pH does not really influence the growth of the bacteria because it is within a favourable pH range. However, at certain points during the experiments a lower pH was observed, mostly during the exponential phase. The reason may have been the production of carbon dioxide during respiratory and metabolic

activity. On the other hand, the pH increased towards the death phase of the bacteria, which can be attributed to a rise in cell debris and activity that might have occurred in the medium during the growth phase. A notable characteristic of the death phase is greater viscosity, possibly due to loss of volume because of evaporation in the reactor, and a corresponding increase in the concentration of salt in the medium. This implies that we might be able to use pH measurements as a simple online method for monitoring the growth of the bacteria.

The results of substrate evaluation made it evident that the breakdown of nutrients into soluble contents starts from the lag phase. However the utilisation of the soluble nutrients is highest at the exponential phase (which occurs after 30 hours), when the available nutrients were reduced to a very low point, particularly in the case of nitrogen. The growth curves for the stationary and death phases illustrate that these were the stages at which the available soluble nutrients in the media culture were reduced to a level that initiated bacterial starvation (Figures 2.6 and 2.7). This finding provides an instructive insight into how *P.luminescens* cultures can be maintained to support continuous growth. The introduction of fresh medium caused a corresponding recovery in the number of bacteria, which was accompanied by the presence of a greater amount of nitrogen in the bioreactors. Although other important substrates such as COD and phosphorous were also measured to evaluate their impact on the growth of *P. luminescens* bacteria, they were found to have less influence than nitrogen.

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3 Chapter Three

Investigating the Factors that are Important for the Production of Entomopathogenic Nematodes in Submerged Culture Using Shake Flask Experiments

ABSTRACT

An investigation into the production of *Heterorhabditis indica* (from Ireland) as well *Heterorhabditis bacteriaphora* (from South Africa) nematodes in submerged culture using shake flasks was carried out. Both types of entomopathogenic nematode (EPNs) feed on the *Photorhabdus luminescens* bacteria. A very interesting result of the experiments was that the population counts of nematodes were not reproducible, even when great care was taken to ensure that the experimental conditions were the same over repeated experiments. Typically it took between 4–5 weeks for the population count of the EPNs to reach a maximum. The *Heterorhabditis indica* nematode from Ireland was generally found to produce higher yields of juveniles than the South African strain, *Heterorhabditis Bacteriaphora*. The ratios of juvenile to adult and juvenile to dead nematodes were also evaluated, and found to vary with the different culturing media. Another experimental observation was that dead nematodes can be detected in significant numbers after about 7–8 weeks in the medium.

The author established that the decrease in nematode population after peak growth was attributable to the depletion of *Photorhabdus luminescens* bacteria in the medium, as their growth rate increased sharply when fresh feed rich in *P. luminescens* bacteria was introduced. This led to the proposal that we should examine the possibility of changing the culture medium as the life cycle of EPNs progressed. This was

followed by an investigation to determine which the most appropriate medium for each stage should be. It was found that a lipid broth would be most suitable as a start-up medium, and that this should later be switched to an egg yolk medium, in view of the latter's ability to maintain the growth of *P. luminescens* bacteria longer than any of the other media tested.

3.1 INTRODUCTION

The economic importance of the use of EPNs as a bio-agent is a by-product of the global attempt to find alternatives to chemical pesticides. A concerted effort to discover the best appropriate technology for mass-producing EPNs has been made in Australia, China, Germany and the United States. (Grewal & Georgis, 1999) The success of the early attempts has prompted similar investigations that are currently being carried out in the South African environment. The attraction of the nematode method lies in its ability to kill off an infestation within 24 hours, which makes it an effective bio-control agent. It is also possible to use a specific species of EPN to target a particular insect host, which enables it to have an even more virulent effect.

The most serious issue to be raised over the use of EPNs for this purpose concerns the quality of the biological product. Gaugler et al. (2000), for example, noted how easily bio-agents produced in the United States, particularly at cottage industry level, are influenced by environmental factors when in storage. However, efforts are being made to ensure that quality standards are developed for the industry to address these criticisms. A positive side of the EPN industry is that it can be practised on a small scale (as a cottage industry), which helps to generate employment and encourages micro-scale investment at the community level. On the other hand, it is widely believed that increasing the scale of production will reduce the capital cost. Gaugler and his co-authors suggested that the future of the production technology for EPNs lay in liquid *in-vitro* methodology. However, De la Torres (2003) suggested that this method

required further investigation that would lead to a greater understanding of the growth of EPNs in submerged culture.

The South African interest in EPNs lies in their enormous potential as an alternative to chemical pesticides. Discovering a suitable production technology for the indigenous EPNs recently found in the country was an important motivation for the investigation described in this thesis. Two strains of the same species were used in the experiments outlined below: *Heterorhabditis indica* from Ireland, and the South African *Heterorhabditis bacteriaphora*. This enabled the researcher to compare results for both strains, and determine the most important factors in culturing nematodes. Another reason for this choice was that these two EPNs, although different, share a common symbiotic bacteria, *Photorhabdus luminescens*. The investigation described here builds on the work carried out by earlier researchers such as Bedding (1984), Ehlers et al. (2000), and De la Torres (2003).

The *P. luminescens* bacteria was used as a foundation for analyzing the process of growth in EPNs and their reliance on the bacteria for food. Strauch and Ehlers (2000) indicated that bacteria symbiotic to EPNs produce a food signal into the liquid medium when this is used as a culturing base. They also noted the importance to reproduction of a sufficient food supply for the EPNs.

There are two distinct technological pathways in the production of entomopathogenic nematodes: the *in-vivo* and *in-vitro* technologies.

The *in-vitro* technology can be used with solid and liquid media. The latter have been used by some scientists such as Ehlers et al. (2000) and Surrey and Davies (1998), while one of the pioneers of the commercial production of EPNs, Bedding (1984), used *in-vitro* solid media. However, many researchers describe the liquid *in-vitro* pathway as the most

economical and effective route, and a great deal of scientific research is ongoing to develop the most efficient technology for the purpose.

Earlier techniques for producing EPNs on a large scale involved using different types of shake flask in which to grow nematodes and symbiotic bacteria at the laboratory scale. Surrey and Davies (1996) mentioned that Wouts Landcare Research New Zealand Ltd had developed a liquid medium technology, but in their journal article they described this on the basis of a private communication, without supplying precise details.

Ehlers et al. (2000) used a 200mℓ shake flask filled with about 50mℓ of medium to propagate bacteria and nematodes. The flask was constantly shaken mechanically to ensure the even circulation of oxygen in the shake flask. These scientists also reported that they had used Erlenmeyer and Spinner flasks for a similar purpose. Their research provided an important guide for choosing the appropriate technology, and developing ways to increase the scale of nematode production in submerged culture in subsequent investigations.

According to Ehlers and his co-workers, nematodes grown in liquid culture are often preferred for commercial purposes because they are easily applied in the field. Johnigk et al (2002) noted that the improvements in technology of production have led both to higher yields of nematodes in liquid media and a reduction in the cost of production of nematodes for use as bio-control agents.

In this researcher's survey of the literature on EPNs published in different journals, the medium, which provides the nutrients needed by the symbiotic bacteria, has been seen as crucial to achieving a high population density in shake flask experiments. In an earlier study, Yang et al. (1997) used different media to evaluate the potential of *S. carpocasae* EPNs, looking at issues such as body size, motility and nematode

penetration in host insects. Others, such as Friedman (1990) and Hatab and Gaugler (2001), confirmed that the nutritional composition of the medium affects the final yield of nematodes in submerged culture. The results of Hatab and Gaugler's research showed that the addition of extractible oil to the medium led to a 1.7 times increase in the production of the *Heterorhabditis bacteriaphora* compared with beef fat or lard as ingredients. Gil et al. (2002) experimented using two media, one enriched with glucose and the other with canola oil, in shake flasks, to produce *H. Bacteriaphora* nematodes. In both cases, the proportion of nematodes to liquid medium was in the range of 5 000–6 000 infective juvenile (IJ)/mℓ When the results were compared, the former (containing glucose) had a nematode yield as high as $4.25X10^5$ IJ/mℓ, while the latter (containing canola) produced about $3.62X10^5$ IJ/mℓ. Different factors influencing the growth of EPNs in liquid medium were discussed by Johngik et al. (2002), who indicated that increasing the concentration of CO_2 may lead to an increased recovery of juveniles in liquid medium. This finding is confirmed by Ehlers (2001), who had found that artificially increasing the concentration of CO_2 in a reactor had led to a higher recovery of juveniles. Johngik et al. (2002) also mentioned that when the pH in liquid medium falls below pH 6.5, it may lead to reduction in juvenile recovery.

A major constraint on the study of *Heterorhabditis spp.* is the low yield often experienced when compared with that obtained with other species, such as *S.carpocapsae* and *S. feltae spp*, according to Ehlers et al. (1998). Minimal information is available on the reason for the low yield by the *Heterorhabditis spp*., and this has limited the use of this species for commercial production.

3.2 EXPERIMENTAL METHODOLOGY

3.2.1 Preparation of the *in vivo* nematode culture

The inoculation of nematodes into galleria (insect larvae) was done under a laminar flow bed in order to ensure a sterile environment, and hence avoid contamination of the medium. The culturing of nematodes *in vivo* follows a number of preparatory steps. The galleria are washed in a 0.1% hyamine solution and then dissected with a sterile blade. The nematode sample, which has also been rinsed in a 0.1% hyamine solution, is inoculated into the dissected galleria. Both are laid inside a sterile petri dish filled with about 2ml of water treated by reverse osmosis. The nematodes in the petri dish are placed under an electron microscope to check that the nematode samples are living, and to discover whether an increase in nematode population can be observed.

3.2.2 Preparation of nutrient agar plates

The plates contain a solid medium made by combining 16g nutrient agar (Biolab) and 1 000mℓ distilled water in an autoclavable bottle. A magnetic stirrer is used to mix the contents until the nutrient agar is well dissolved in the solution. It is subsequently placed in the autoclave and sterilised at a temperature of 121^0C for about an hour. The sterilised nutrient agar solution is allowed to cool to a temperature of approximately 70^0C before being poured into petri dishes (hereafter referred to as plates) under the laminar flow bed, and left to cool and solidify before the bacteria are streaked onto them (see below).

3.2.3 Extraction of *Photorhabdus luminescens* bacteria from entomopathogenic nematodes in *in vivo* culture

After the researcher has determined that the nematodes in the petri dish are living and multiplying inside the galleria, an extraction experiment is carried out under the laminar flow bed. *P. luminescens* bacteria are taken from the nematodes by means of a streaking rod that has been sterilised by immersing it in ethanol absolute 99% and flaming it with naked fire. The rod is dipped into the cadavers of the galleria and the extracted sample is streaked onto the nutrient agar plates. These are placed upside down in the incubator at 25^0C, and are checked for growth after 24 hours.

3.2.4 Preparation of the sterile nutrient broth solution

Using a scale, the researcher measures 13g of powdered nutrient broth (Biolab) onto aluminium foil and then pours it through a funnel into a 1 000mℓ autoclavable bottle. To this is added 1 000mℓ of sterile distilled water that has been measured in a calibrated cylinder. The bottle is placed on the magnetic stirrer to ensure proper mixing, and processed until all the nutrient broth is completely dissolved in water. The solution is then divided between shake flasks in the proportion of 50mℓ of nutrient broth solution to each 200mℓ shake flask bottle, and covered with a single layer of aluminium foil. Afterwards the bottles are sterilised at about 100^0C and a pressure of 1bar in an autoclave.

Preparation of liquid culture medium

Liquid culture medium (LCM) is a formulation of different compounds adapted from Ehlers et al. (1998). For 1 000mℓ of liquid culture, the ingredients are 2.0g tryptic soy broth, 2.0g nutrient broth, 1.0g yeast extract, 0.072g potassium chloride, 0.044g calcium chloride, 1.0g sodium chloride, 1.0g bacto peptone, 6.0mℓ canola oil and 1 000mℓ distilled water. These are mixed with a magnetic stirrer to ensure a proper amalgamation before the solution is sterilised in the autoclave.

Preparation of egg yolk medium

An equivalent volume of egg yolk medium is adapted from the formulation od Surrey and Davies (1996). The ingredients comprise the following: 12.5g spray dried egg, 23g yeast extract, 2.3g sodium chloride (NaCl), 40mℓ corn oil and 1 000mℓ distilled water. The 1 000mℓ bottle is placed on the magnetic stirrer to ensure proper mixing, and processed until all the components are is properly mixed. However, it is common to see some small flecks of yolk in the bottle during mixing. The solution is then divided between shake flasks in the proportion of 50mℓ of nutrient broth solution to each 200mℓ shake flask bottle, and covered with a single layer of aluminium foil. Afterwards the bottles are sterilised at about 100^0C and a pressure of 1bar in an autoclave.

3.2.5 Preparation of *Photorhabdus luminescens* inoculum culture

The *P. luminescens* bacteria are scooped from the nutrient agar plate under the laminar flow bed using a sterilised streaking rod, and dropped into the 50mℓ nutrient broth solution in the 250mℓ shake flask bottle. The single layer of aluminium foil that is used to cover the bottle allows oxygen to penetrate it. After inoculation the sample bottle is placed in an incubating shaker and agitated at a speed of about 130rpm to enhance oxygen distribution in the shake flask, and kept at a constant temperature of 25^0C, which is optimal for the growth of *P. Luminescens*. As reported by Ehlers (2000), *P.luminescens* isolated from *Heterorhabditis indic*a nematodes can tolerate temperatures as high as 40^0C. Although the species of nematode used in these experiments grows at temperatures above 30^0C, it takes time to adjust to new temperatures. At above 36^0C, a drop occurs in the growth rate and optical density measurements. (A major characteristic of *P. luminescens* in nutrient broth or egg yolk medium is that after about 12 hours the solution takes on an orange colour.)

The bottle is left in the shaking unit for about 24 hours, after which an inoculating sample can be collected. The reason is that only after this period do the bacteria reach the primary phase (Smigielski et al., 1998), which has been identified as the optimal stage for inoculation.

3.2.6 Initial *Photorhabdus luminescens* growth experiment

The medium used initially was nutrient broth. This was subsequently changed to liquid culture and egg yolk medium, which have proved more beneficial to the growth of microbes than the nutrient broth.

For a 250mℓ shake flask, 50mℓ of the medium is measured using a calibrated cylinder and decanted into the flask before being autoclaved. The researcher then carries out an optical density measurement using a flask specially designed for the purpose. A complementary measurement of bacterial colony-forming units (CFUs) is also made to determine the number of bacteria present in the medium before the inoculum is added. Both of these measurements (optical density and CFU) are necessary preliminary steps that enable the scientist to identify the most appropriate point at which to inoculate nematodes into the liquid culture medium for production purposes. They also assist in drawing up a picture of the growth pattern of the bacteria.

The shake flask requires an inoculum volume of 2mℓ per 50mℓ of medium. The high proportion of inoculum is intended to compensate for contamination arising from handling procedures.

3.2.7 The preparation of lipid agar plates

While the volume of lipid agar solution prepared is dependent on the number of plates required, the formula for 1 000mℓ is 10g honey, 5g yeast,

2.5mℓ cod liver oil, 2g magnesium chloride (hydrated) Mg $Cl_2.6H_2O$, 25g nutrient agar and 1 000mℓ distilled water. In situations where nutrient agar is not readily available, 13g nutrient broth and 12g agar can be used instead. The mixture is sterilised in an autoclave at 121^0C with a pressure of 1bar after having been properly mixed by the magnetic stirrer. After sterilisation, the lipid agar solution is allowed to cool to about 70^0C and poured into petri dishes, filling them to the halfway mark, under a laminar flow bed in order to avoid contamination. The solution in the plates is left to cool and solidify, after which it is ready for use for the solid *in vitro* culture of nematodes.

3.2.8 The inoculation of nematodes onto lipid agar plates

(See the description of the preparation of *in vivo* nematode culture in section 3.2.1.) Distilled water is added to the petri dish containing *in vivo* cadavers infected with nematodes, to loosen them. The water is drained off, after which the nematodes in the plate are sterilised with 0.1% hyamine solution, and finally rinsed again with distilled water. A watch glass is inverted over another petri dish, and a white trap made out of filter paper is set over the watch glass. The contents of the plate containing the nematodes are then decanted into the petri dish with the white trap. After 24 hours the nematodes are expected to have migrated onto the trap. At this point the white trap is removed and dropped into another petri dish, where it is covered with just enough distilled water to form a perfect, thin layer over the filter paper. A 2mℓ sample is collected using a sterilised pipette, and introduced onto a prepared lipid agar plate that had been inoculated with 2mℓ *P. luminescens* culture 48 hours beforehand. After the inoculation of nematodes, the plates should be checked periodically to check how their growth is progressing. As a rule the nematode population reaches its peak about one week after inoculation, when big and mature adults can be found on the plates.

After completing this primary phase of *in vitro* culture, the researcher can make more *in vitro* solid plates of nematode. One or two of the original plates of nematode culture are selected (depending on the required number of new plates), and cut into rough squares about 1cm X 1cm in size, using a sterilised flat-end spatula under a laminar flow bed. The cut-out pieces are transferred under sterile conditions to lipid agar plates that have been inoculated with *P. luminescens* bacteria 48 hours earlier. It is estimated that the cut-out pieces of one lipid agar plate should be able to inoculate about 50 freshly-prepared new plates.

3.2.9 Inoculation of *Photorhabdus luminescens* bacteria and *Heterorhabditis indica* and *bacteriaphora* entomopathogenic nematodes into submerged liquid cultures

Samples extracted from the initial bacteria culture (described in an earlier section) are introduced into the liquid culture media or the egg yolk media after about 24–48 hours. The volume of the inoculum should be between 1–10% of the medium volume, depending on the environment in which the experiment is being carried out. In a laboratory situation where sterile conditions are being maintained, the container of medium is set over a naked flame under a laminar flow bed before being opened. The recommended proportion of *P. luminescens* culture inoculum is about 8–10% percent of the volume of the medium, whether egg yolk or liquid culture. After the *P. luminescens* culture has been introduced, the contents of the shake flask should be left to incubate for about 24–48 hours before the *Heterorhabditis indica* or *bacteriaphora* nematodes are inoculated into the medium.

The second process (inoculating *Heterorhabditis indica* or *bacteriaphora* nematodes into the *P. luminescens*-rich submerged medium) should be followed with scrupulous care to avoid compromising the sterile nature of the submerged medium. The means of collecting *Heterorhabditis indica*

EPNs chosen by the researcher was to harvest them from the solid lipid agar plates. Distilled water is poured onto the plate, and a swirling movement is initiated for about 30 seconds to dislodge the nematodes from the solid agar. The nematode-bearing water is poured into a 15mℓ sterile test tube, which is generally able to accommodate the 'washout' from an average of three plates. The contents of the tube are allowed to settle for about 30 minutes, during which the nematodes sink to the base of the tube. The nematodes are then collected using a sterile pipette, and squeezed out into another sterile tube. The same procedure for collecting other samples of nematodes is repeated. When the scientist has transferred sufficient nematodes to fill the sterile tube to the 5mL volume level, about 10mℓ of 0.1% hyamine solution is added to sterilise the nematodes. The tube is inverted every second for approximately 30 seconds to ensure proper mixing, after which the contents are allowed to rest for 15 minutes to allow the nematodes to settle. A sterile pipette is used to transfer the nematodes into another sterile tube. About 20 mℓ of distilled water is added, and the same mixing procedure is followed, after which the liquid is allowed to settle for about 30 minutes. The nematodes are then collected and introduced into the submerged medium by means of a sterile pipette. During this process the flask containing the medium and the *P. luminescence* culture is positioned over a naked flame and under the laminar flow bed to ensure that only sterile air enters the shake flask.

The OD and the CFU of the *P. luminescens bacteria* are determined just before the introduction of the nematodes, in order to estimate their growth in the medium. The nematodes are also counted after they have been added, to determine the number of infective juveniles and adults the medium contains. (See section 3.2.10 below on the method used.) The standard recommendation, which gives the nematodes the best chance of survival, is that the number of nematodes per millilitre of medium should be about 3 000IJ/mℓ after inoculation. Other factors that affect the survival

of nematodes in submerged media are temperature, pH, oxygen diffusion and mixing.

When a shake flask is the container of choice, the optimal temperature for the growth of nematodes in the incubator and shaker is 25^0C and the pH is about 7–8. Proper oxygen diffusion is achieved by ensuring thorough mixing of the contents of the shake flask, the mouth of which is covered with aluminium foil. The shaker is agitated at a speed of between 130–145rpm. Earlier, the mouth was covered with cotton wool and then sealed with aluminium foil, but the researcher found that the nematodes were dying and tried an alternative technique, sealing the flask with a layer of aluminium foil only. This produced a better result. The volume of the medium was also found to be very important, particularly at the shake flask level, because of the need for oxygen diffusion in the medium to foster the growth of the nematodes. The ratio of medium volume to that of the shake flask that contains it is 1:5. For example, 50mℓ of medium should be used in a 250mℓ shake flask.

3.2.10 Measurement of entomopathogenic nematode counts in a submerged medium

It is important to determine the number of nematodes that have been introduced into a particular medium, so that their subsequent survival, growth and feed rates can be monitored. The open shake flask containing liquid medium, bacteria and nematodes is placed over a naked flame under the laminar flow bed, and a sterile pipette is used to collect a sample of about 100µℓ of medium containing nematodes. This is squeezed into a sterilised eppendorf, about 900µℓ of distilled water is added, and the sample and water are mixed in a vortex machine for about 30 seconds. Thereafter a sample of about 100 µℓ is collected with a pipette and deposited on a petri dish so that it can be studied under an electron

microscope. The counting of infective juveniles (IJs) is carried out at this point. If the number of IJs exceeds 30, further dilution of the sample is necessary. In most cases the adults are counted along with the IJs.

3.3 DISCUSSION OF RESULTS

The purpose of the experiments was to evaluate the potential for mass production of EPNs. The results discussed in this chapter concern the growth of *Heterorhaditis indica* and *Heterorhaditis bacteriaphora* nematodes with the symbiotic *Photorhabdus luminescens* bacteria in liquid *in vitro* cultures in shake flasks. The media used for the propagation for these nematodes were LCM, egg yolk, lipid broth and nutrient broth.

The findings described below concern the core attributes of EPNs grown in the experimental conditions set out in the previous section.

3.3.1 Variability of results

In this section the growth patterns of *Heterorhabditis indica* and *Heterorhabditis bacteriaphora* nematodes were investigated. A number of duplicate samples were inoculated with the same population of EPNs, and their growth was monitored so their characteristics could be identified and compared. Figures 3.1 to 3.4 show four different repeats of the same experiment.

The average count of nematodes in each inoculum introduced to every shake flask at the start of the experiment was less than 20 000 infective juveniles per mL (IJ/mℓ). In Figure 3.1 we plot the concentration of *Heterorhabditis indica* IJs in egg yolk medium as a function of time. When a population count was carried out after 24–48 hours, the maximum population recorded for the four samples in Figure 3.1 was found in

Sample 1, at 2.3X 10^6IJ/mℓ. None of the other samples had an equivalent population count. This showed that the growth pattern in each sample is different from the others, despite the researcher's expectation that all the results would be similar because they had been taken from the same inoculum source, fed an identical nutrient, and subjected to exactly parallel experimental conditions. This variation suggests that other factors localised in each shake flask might have influenced the growth of the nematodes. It also indicates that the growth pattern of the nematodes is not easily reproducible. Although there is a large variability in the maximum population count ($1X10^5$- $2.5X10^6$), there is less variability in the time at which this maximum occurs, which in all four experiments is between 4–5 weeks.

Figure 3.2 shows the growth pattern of the adult *Heterorhabditis indica* for the four samples, using egg yolk medium in the experiment. The maximum concentration of adults that was achieved was ($1X10^4$-$2X10^5$). This illustrates the variations in the adult population, which led to the conclusion that the results were not reproducible. There is also variability in the time when the maximum concentration occurs as well as the number of maxima that occur during the 8-week period of the experiment.

The maximum growth for juvenile EPNs is likely to occur at five weeks; however it can take place before that, as is shown in the results for the *Heterorhabditis indica* nematodes in Figure 3.1, where in Sample 1 their growth peaked at four weeks. The other three samples reached their highest point at five weeks. The adults, as shown in Figure 3.2, did not follow the same pattern as observed in Figure 3.1. Samples 1 and 4 peaked at two weeks, whereas samples 2 and 3 did so at five weeks. This further confirms the variability of the results in the nematode experiments.

The population count for *Heterorhabditis bacteriaphora*, shown on Figures 3.3 and 3.4, reveals a similar trend. The IJ population numbers achieved

in each shake flask differ despite the similarity in the initial conditions of experiment. The maximum concentration of IJs varied between ($2X10^4$ – $1.5X10^5$). The time at which the maximum in the IJ population count occurs is fairly reproducible, however, occurring at about five weeks. In this case, the adult population count (Figure 3.4) follows the behaviour of the IJ count more closely, reaching a maximum at about five weeks as well. Again there is variability in the concentration of adults at the maximum ($5X10^3$-$7X10^4$), but the trends are very similar to those of the IJs shown in Figure 3.3.

A factor that could partly explain the discrepancies between the behaviour of the *Heterorhabditis indica* and *Heterorhabditis bacteriaphora* could be their belonging to different strains of the same species of nematode. These observations are similar to the findings of Strauch and Ehlers (1998) on EPN variability. Various factors may be responsible for the variability in results. One is the availability of bacteria in the shake flask, which influences food signal production and in turn affects the growth of juveniles. The literature on the subject also mentions that the predisposition of juveniles to respond to food signals differs from one juvenile to the other, which also affects their growth. According to Ehlers (1998), the density of bacteria when the inoculation of EPNs takes place must be very high, to boost growth. If the density of bacteria differs between one flask and another it will cause variation in growth. Shapiro-lan and Gaugler (2002) noted that the composition of the medium can affect the growth and yield of EPNs because of the differences in lipid quantity, protein source and salt. The concentration of CO_2 will also have a significant effect. Also, according to Shapiro-lan and Gaugler (2002), the size of the EPNs also influences yields. This is a major factor in the difference between *Heterorhabditis indica and Heterorhabditis bacteriaphora* counts.

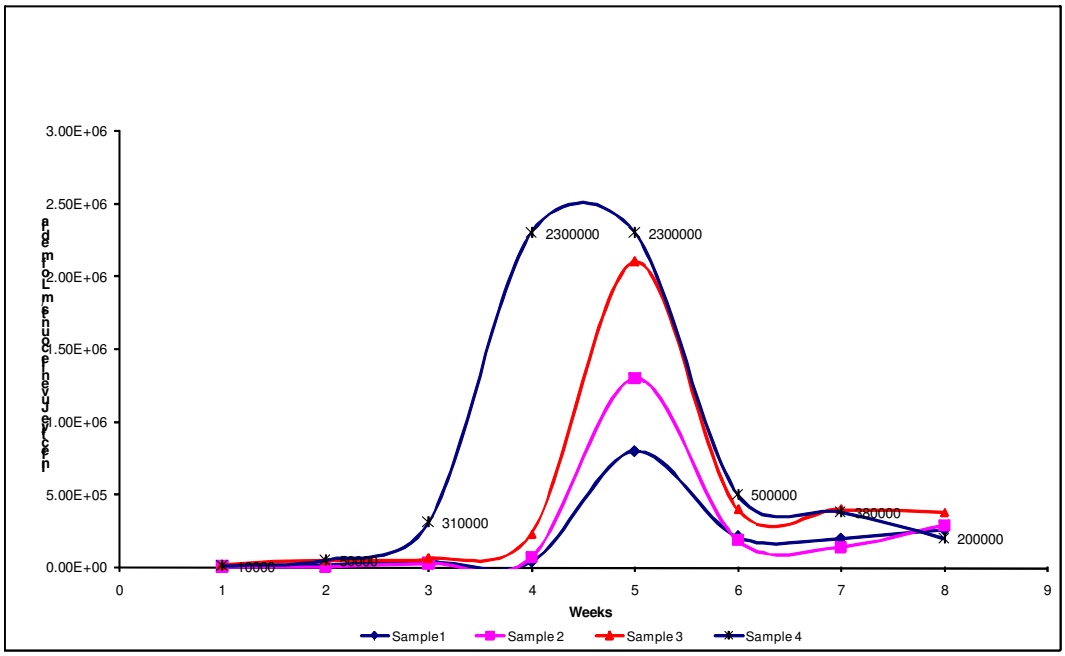

Figure 3.1: Variability in population count for *Heterorhabditis indica* infective juveniles in egg yolk medium in 4 parallel experiments

N.B

Sample 1: Experiment 1 shake flask with HI nematodes

Sample 2: Experiment 2 shake flask with HI nematodes

Sample 3: Experiment 3 shake flask with HI nematodes

Sample 4: Experiment 4 shake flask with HI nematodes

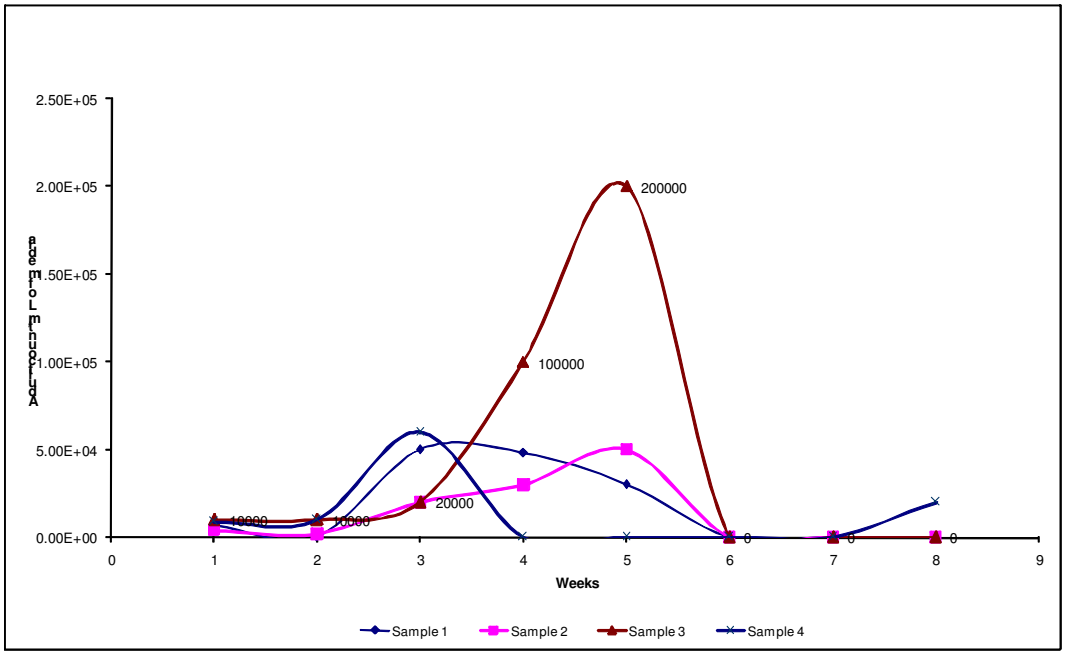

Figure 3.2: Variability in population count *for Heterorhabditis indica* adults in egg yolk medium in 4 parallel experiments

N.B

Sample 1: Experiment 1 shake flask with HI nematodes

Sample 2: Experiment 2 shake flask with HI nematodes

Sample 3: Experiment 3 shake flask with HI nematodes

Sample 4: Experiment 4 shake flask with HI nematodes

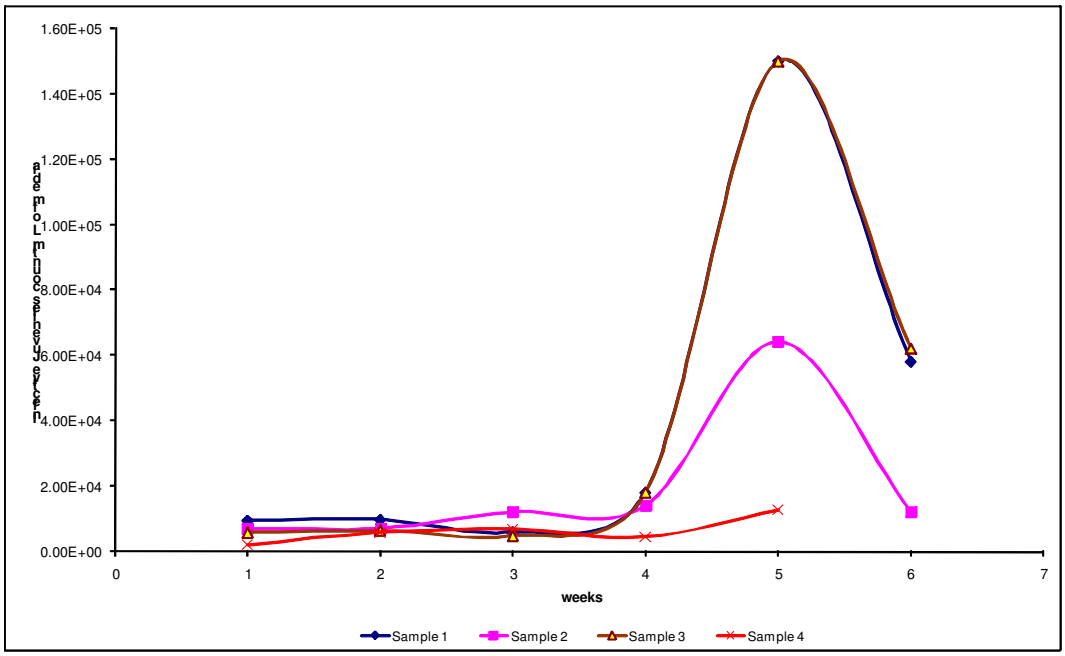

Figure 3.3: Variability in population count for *Heterorhabditis bacteriaphora* infective juveniles in egg yolk medium in 4 parallel experiments

N.B

Sample 1: Experiment 1 shake flask with HB nematodes

Sample 2: Experiment 2 shake flask with HB nematodes

Sample 3: Experiment 3 shake flask with HB nematodes

Sample 4: Experiment 4 shake flask with HB nematodes

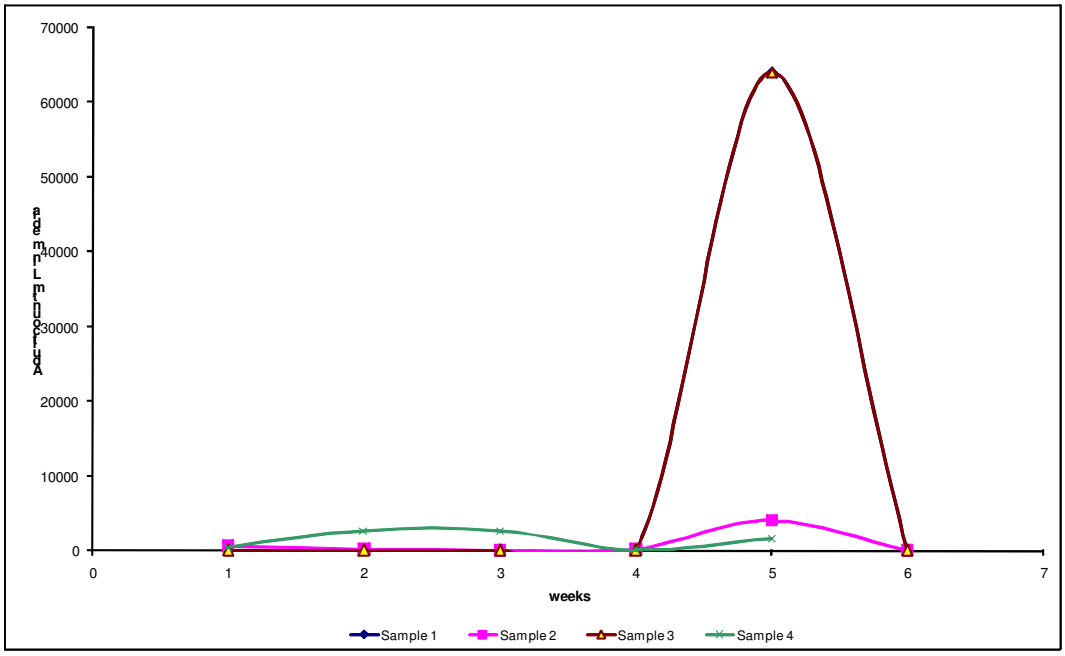

Figure 3.4: Variability in population count for *Heterorhabditis bacteriaphora* adults in egg yolk medium in 4 parallel experiments

N.B

Sample 1: Experiment 1 shake flask with HB nematodes

Sample 2: Experiment 2 shake flask with HB nematodes

Sample 3: Experiment 3 shake flask with HB nematodes

Sample 4: Experiment 4 shake flask with HB nematodes

3.3.2 Comparison of the *Heterorhabditis Bacteriaphora* (South Africa) and *Heterorhabditis Indica* (Ireland) species

In this section we will be discussing the differences between the EPNs found in South Africa and Ireland. Figures 3.1 and 3.2 map the growth of the *Heterorhabditis indica* species from Ireland, while the pattern for the South African *Heterorhabditis bacteriaphora* is presented in Figures 3.3 and 3.4.

The highest *Heterorhabditis indica* juvenile count, presented in Figure 3.1, was greater at 2.3X 10^6 juveniles /mℓ cultured in egg yolk medium than that of *Heterorhabditis bacteriaphora*, as depicted in Figure 3.3, which was 1.6X 10^5 juveniles per mℓ grown in the same medium. This may be due to a higher reproduction capability in *Heterorhabditis indica* than in *Heterorhabditis bacteriaphora*. A major factor in reproduction is the ability of the nematodes to adjust to the hydrodynamic conditions of the medium during their life stages, from the production of eggs as adults to their development through the juvenile stages. Clearly the *Indica* strain is better able to adapt, and is generally more fertile, despite the higher incidence of endokotria matricidal examples, which are more easily found in the *Heterorhabditis indica* shake flask.

Another noticeable difference between the two strains is to be observed in the body structures of the *Indica* and *Bacteriaphora* nematodes. The *Bacteriaphora* has a larger body structure when compared to the *Indica*. The young adults in *Indica* are smaller, shorter and more robust than those of the *Bacteriaphora*, which are longer and more slender. As Shapiro-lan and Gaugler (2002) mention, the size of EPNs also influences yields.

3.3.3 Nutrient depletion and replenishment in submerged media

Figure 3.5 describes the growth pattern of *Heterorhabditis indica* in egg yolk medium that is rich in symbiotic *P. luminescens* bacteria. The egg yolk medium was able to sustain the growth of nematodes in culture until the growth peaked between weeks four and five at about 2.30 X 10^6. After that point a continuous decrease was recorded in the number of juveniles counted. In the eighth week, the contents of the shake flask were divided into two and introduced into two new shake flasks (designated as 1 and 2) that contained fresh medium enriched with *P. luminescens* bacteria. This change in the growth medium is indicated by the point marked “A “in Figure 3.5.

Beyond point A, it can be seen that the two new flasks produced growth counts that surpassed those in the initial experiment, where figures as low as 2.5X 10^5 juveniles were recorded. Two weeks after the addition of new medium, the counts for shake flasks 1 and 2 were as high as 2.5 X 10^6 and 3.0 X 10^6 respectively. This is an indication that the decrease in number of nematodes in the original shake flask was attributable to the depletion of the *P. luminescens* food source in the submerged medium. It took between 2–3 weeks for the concentration of juveniles to reach a maximum after the addition of fresh bacteria-rich egg yolk medium.

Similar experiments were carried out with LCM, which also contained symbiotic *P. luminescens* bacteria. The results are shown in Figure 3.6. Similar numbers to those depicted in Figure 3.5 were recorded, although with slightly lower counts than the initial experiments in Sample 2 (the equivalent of shake flask 2, but with a different medium). However, Sample 1 did not produce favourable results, possibly owing to contamination of the sample. *It is interesting that it again took between*

2–3 weeks for the concentration of juveniles to reach a maximum after the addition of fresh bacteria-rich LCM medium,

A widely-reported challenge in experimenting with liquid culture medium replenishment is the difficulty of finding a supplement that fosters the growth of nematodes after they have started dying off in the original medium. The introduction of egg yolk medium resulted in a very favourable growth increase when used with a high concentration of *P. luminescens* bacteria as replenishment feed.

A further evaluation of the impact of introducing fresh medium was carried out with a medium that had not been inoculated with *P. luminescens* bacteria. *The finding that a culture medium that did not contain bacteria did not encourage growth in* Heterorhabditis indica *led to the deduction that the reason was the absence of the* luminescens *bacteria.* This further confirms that the suggestion of Ehlers (2000) that it is possible to reintroduce living nematodes in an existing or old medium to another that has been freshly inoculated with *P. Luminescens* is correct. It further demonstrates that the growth of the EPNs is limited by the availability of bacteria. Shapiro-Ilan and Gaugler (2002) described factors that can affect the yield, such as increasing the lipid content of the medium to compensate for any depletion of lipids that might have taken place over the eight-week period. Other nutrients that can also affect yields are glucose and yeast, both of which are components of the liquid medium. Another clear difference in yield of EPNs results from whether the medium is egg yolk or LCM. The IJ population count was higher for the egg yolk medium that for the LCM. This is a result similar to that reported by Yang et al. (1997), who explained variations in yield as attributable to the media used to rear the EPNs.

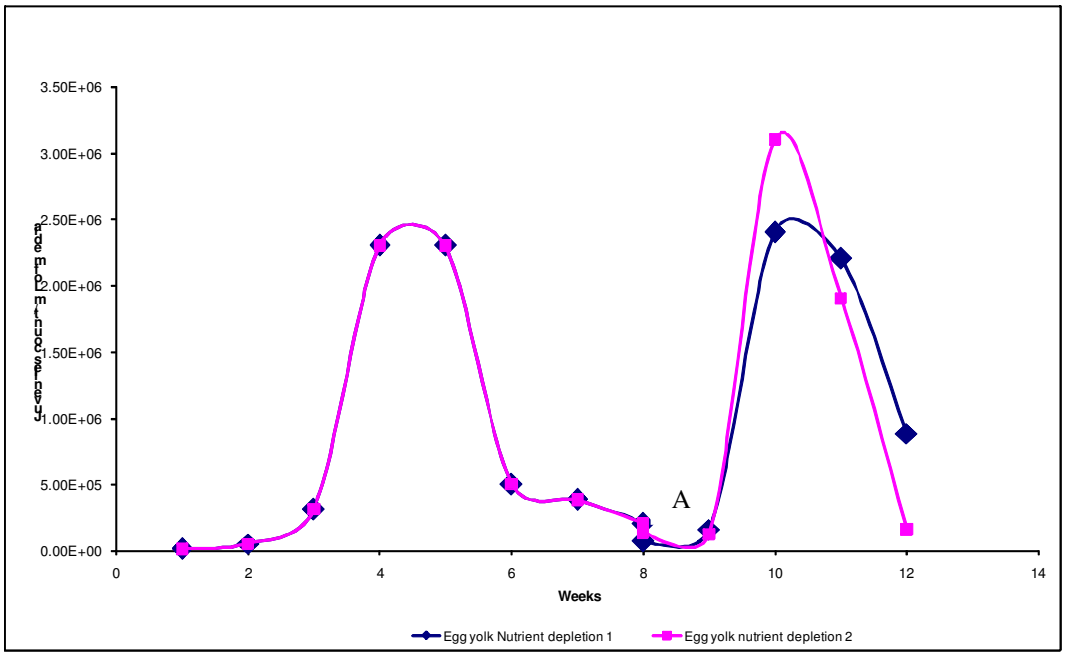

Figure 3.5: Effect of nutrient depletion and replenishment with bacteria-rich egg yolk medium on *Heterorhabditis indica* nematodes. The original sample is divided at point A and added to fresh bacteria-rich egg yolk medium.

A: addition of fresh egg yolk medium rich in Photorhabdus luminescens bacteria

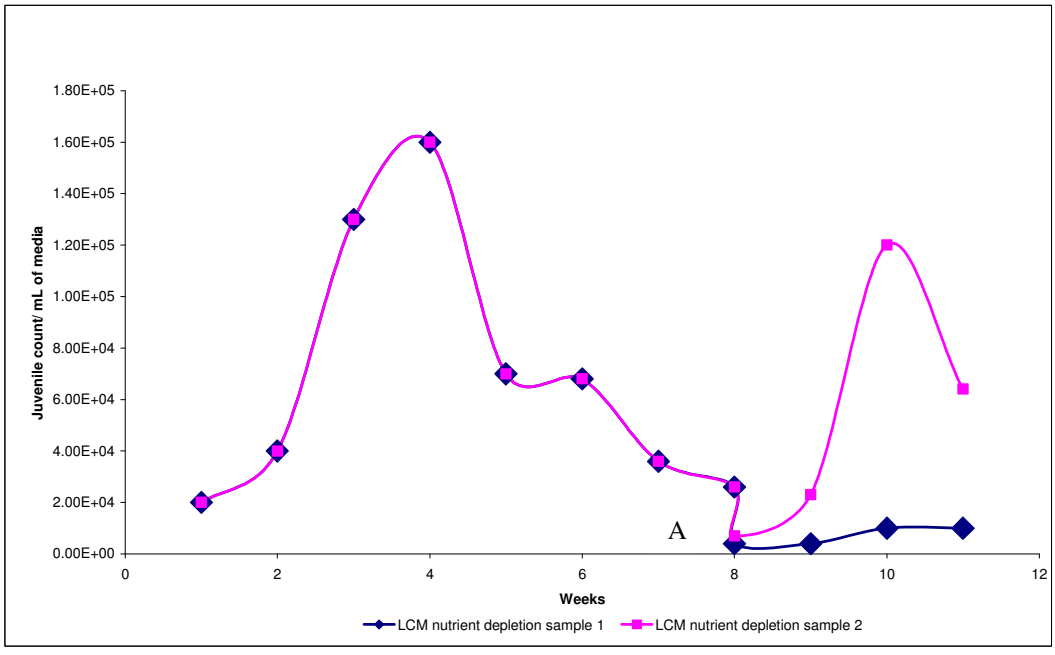

Figure 3.6: Effect of nutrient depletion and replenishment with bacteria-rich liquid culture medium on *Heterorhabditis indica* nematodes

A: addition of fresh medium rich in Photorhabdus luminescens bacteria

3.3.4 Preferences and process sustainability for different types of culture medium

An evaluation of the suitability of different media for the culture of EPNs was carried out using the *Heterorhabditis bacteriaphora* species in submerged media. The results presented in Figure 3.7 are typical of the behaviour of the nematodes in the different media analysed. The lipid broth medium was found to support faster growth of nematodes after inoculation, as is shown by the descriptive results in Figure 3.7. Maximum growth was reached within two weeks after their inoculation into a medium rich in *P. luminescens* bacteria. Similar behaviour was observed in other lipid broth medium experiments, although some reached their maximum three weeks after inoculation. The longest period to elapse before reaching maximum count in any of the lipid broth samples was more than four weeks, but this result occurred only once.

The medium that produced the second-highest maximum count of juveniles was nutrient broth, as illustrated in Figure 3.7. This peak occurred in the third week after inoculation, conforming with the same timing as nematodes grown in lipid broth. The number of juveniles counted in the nutrient broth at their peak was lower than the equivalent maximum achieved in the lipid broth cultures.

The samples grown in egg yolk medium reached their maximum juvenile count at five weeks (which is similar to previous results presented earlier in Figures 3.1 and 3.3). Although it is possible for nematodes to reach maximum growth at four weeks in egg yolk media, as shown in the experimental results, the average time taken is five weeks. This shows that the egg yolk medium has the capacity to sustain the growth of nematodes longer than any of the other media analysed in these experiments.

The results for nematodes cultured in LCM are presented in Figure 3.7. While the outcomes for *Heterorhabditis bacteriaphora* were unfavourable, those for the growth of *Heterorhabditis indica* (shown in Figure 3.6) were much more positive. However, this discrepancy could have been caused by contamination or by the accidental introduction of a lower number of *Bacteriaphora* nematodes into the shake flask experiment.

The results presented in this section show the advisability of starting a nematode experiment with lipid broth because of its ability to bring about faster and earlier growth. It is expected that after the third week, the number of juveniles in the lipid broth medium will start to decrease. At this point it might be advisable to switch to egg yolk, which is able to sustain symbiotic *P. luminescens* bacteria longer than other types of medium. The quick growth experienced in the lipid broth medium was due to the presence of lipid, which, according to Shapiro-Ilan and Gaugler (2002), helps the EPNs to mimic their natural growing environment.

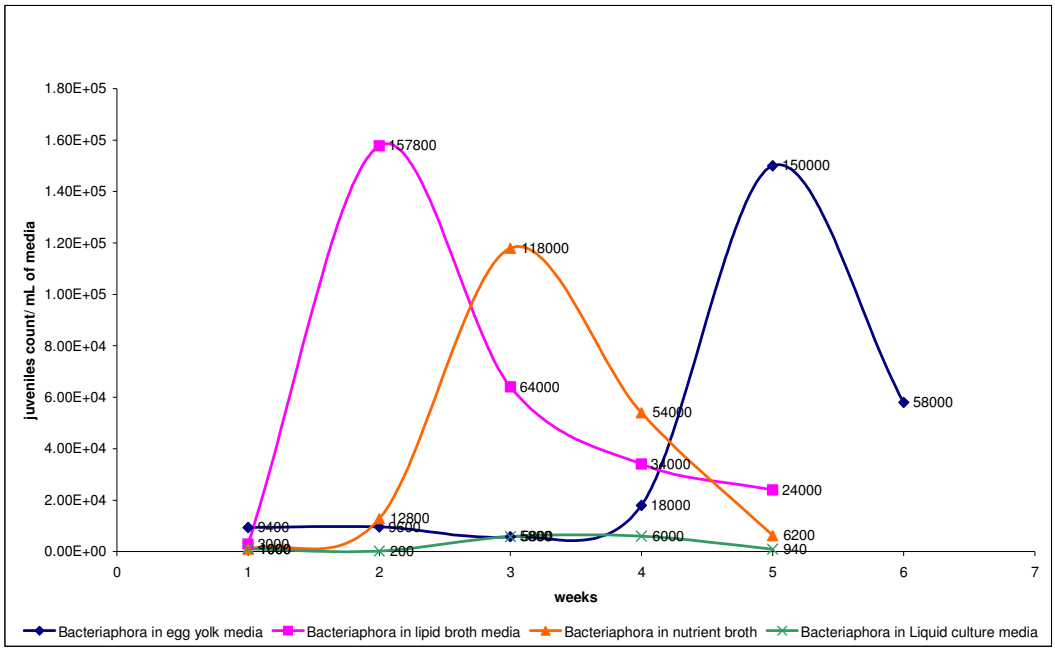

Figure 3.7: Evaluation of the growth patterns of *Heterorhabditis bacteriaphora* juveniles in different media

3.3.5 Analysis of the life stages of *Heterorhabditis indica* and *Heterorhabditis bacteriaphora* as depicted by the results of the experiments

The life span of *Heterorhabditis indicia* nematodes cultured in LCM is illustrated in Figure 3.8. The juveniles reached peak growth at four weeks. At this point the ratio of dead nematodes to living juveniles in the medium was approximately 0.5: 1, while that of adults to juveniles was 0.25:1. After the fourth week there was a decline in the growth count. Towards week eight there were more dead nematodes in the medium than juveniles and adults. This suggests a decrease in the nematodes' food source and a resultant diminution in the number of living nematodes.The dead nematodes break down and may add one or two nutrients to the medium; but the presence of dead nematode debris in the medium becomes a major problem because it encourages the growth of contaminants in formulation, as Young et al. (2002) point out. However there are now a variety of different techniques to resolve the dead nematode and related

problems: dead end filtration, gravity settling and floatation (Young et al., 2002).

Figure 3.9 describes the life span of *Heterorhabditis bacteriaphora* nematodes grown in *lipid broth medium*. The maximum count of juveniles occurred in the second week, when the ratio of dead and adult nematodes to juveniles was less than 0.1:1. This suggests that at the height of production of juveniles in lipid broth media there are fewer dead nematodes. However, the adult count indicates that for a high yield of juveniles we do not require a very great number of adult nematodes in the solution. The ratio altered after the third week, when the proportion of dead and adult nematodes in the medium rose, while that of the juveniles declined. At the beginning of the fifth week, a continual reduction in the number of adults and juveniles was observed, together with a steady rise in the proportion of dead nematodes. This confirms that depletion of their food source in the medium had caused the death of the juveniles and adults.

Figure 3.10 is a graph of the life cycle of *H. bacteriaphora* nematodes in *nutrient broth solution*. The results show a higher ratio of dead nematodes to juveniles (about 1.33:1) at the three-week stage, while that for dead to adult nematodes was 8:1. The inference is that this medium cannot sustain the growth of *P. luminescens* bacteria (which in turn provide food for the nematodes) as well as the media presented previously (LCM and lipid broth). This suggests that nutrient broth might best be used as an initial culture medium for bacteria before the introduction of secondary media such as egg yolk, LCM and lipid broth.

Figures 3.11 and 3.12 present two results for *Heterorhabditis indica* nematodes in egg yolk medium cultures that confirm its effectiveness in sustaining the growth of nematodes. At the point of peak growth, the ratio of adults to juveniles was less than 0.1:1, whereas that of dead nematodes to juveniles was even lower. In egg yolk medium the dead nematodes become visible on the graph only after the seventh week. This can be interpreted as demonstrating that egg yolk is the medium most effective in promoting the continuing growth of bacteria, in other words that it is the preferred option of all the media evaluated in this section.

It is probable that some dead nematodes occur in the medium after the sixth week, but are virtually undetectable owing to the viscosity of the medium at that point and the rapid enzyme activity of the remaining bacteria. Nematode debris resulting from the breaking down of dead nematodes by bacteria was also a factor in the evaluation of the life spans of EPNs in different cultures. An evaluation of the effect of temperature was not included in the work for this thesis. However, Nyamboli (2008) researched the effects of temperature, and found that different species of bacterial symbionts of EPNs attain optimum growth at different temperatures. However, the optimum temperature of the symbiont bacteria for both the *Heterorhabditis indica* and *Heterorhabditis bacteriaphora symbiont bacteria* is 28^0C (Nyamboli, 2008). The growth temperature of bacteria influences the growth of their host nematodes, as discussed by Ehlers et al. (2000).

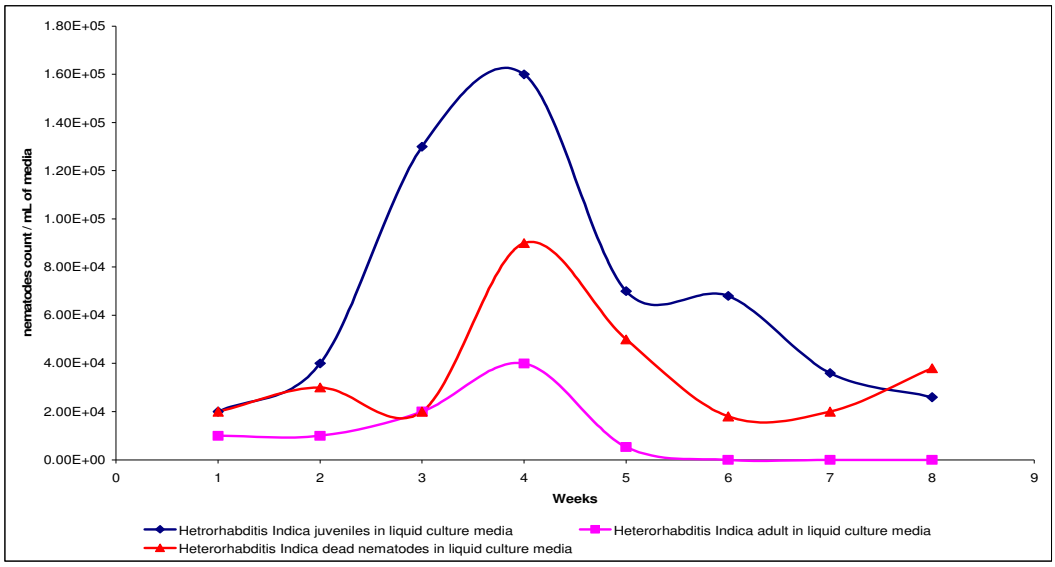

Figure 3.8: Growth patterns of *Heterorhabditis indica* in liquid culture medium

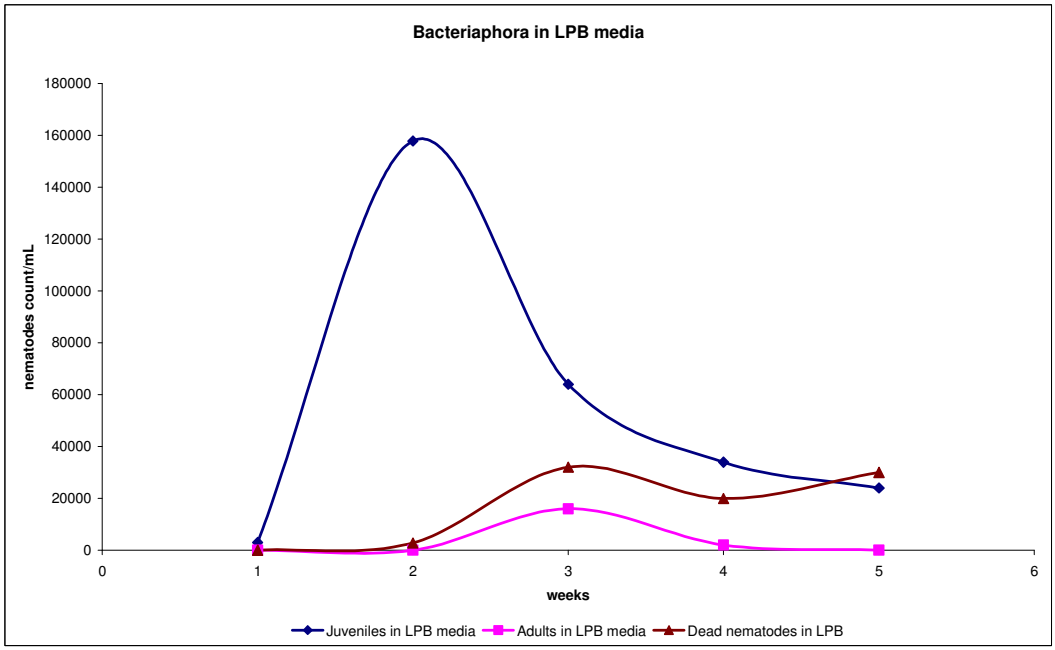

Figure 3.9: Growth patterns of *Heterorhabditis bacteriaphora* in lipid medium

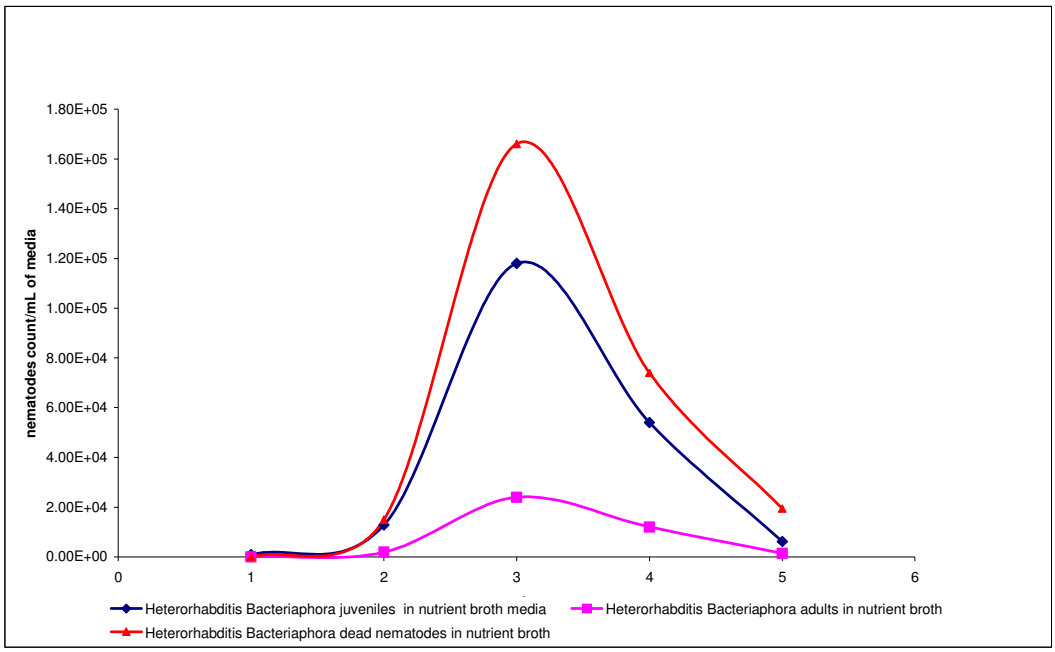

Figure 3.10: Growth patterns of *Heterorhabditis bacteriaphora* in nutrient broth medium

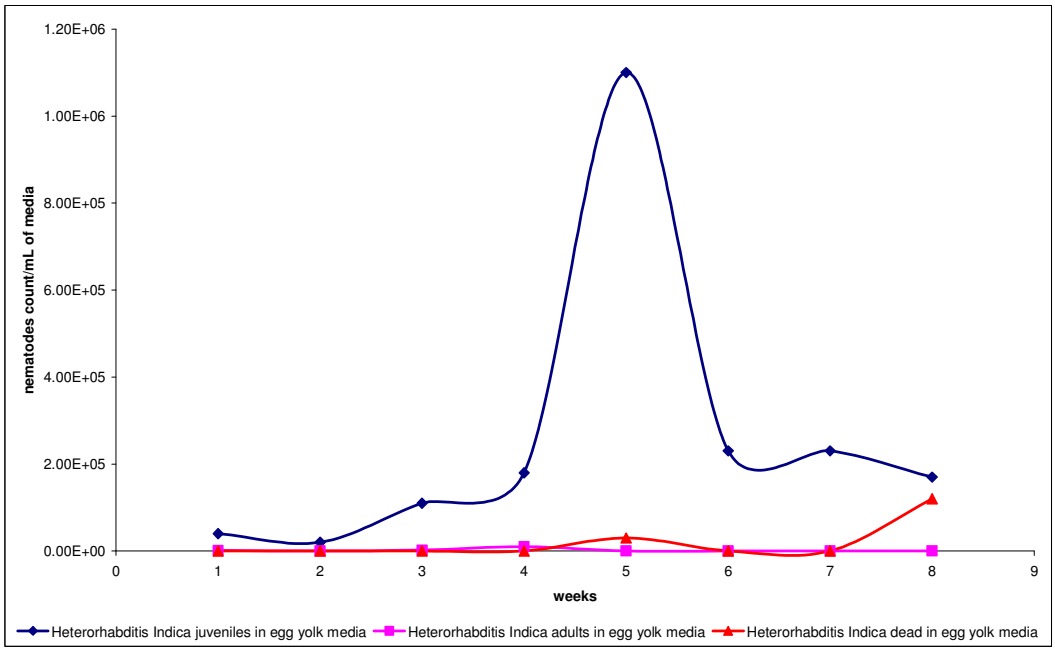

Figure 3.11: Growth patterns of *Heterorhabditis indica* in egg yolk medium

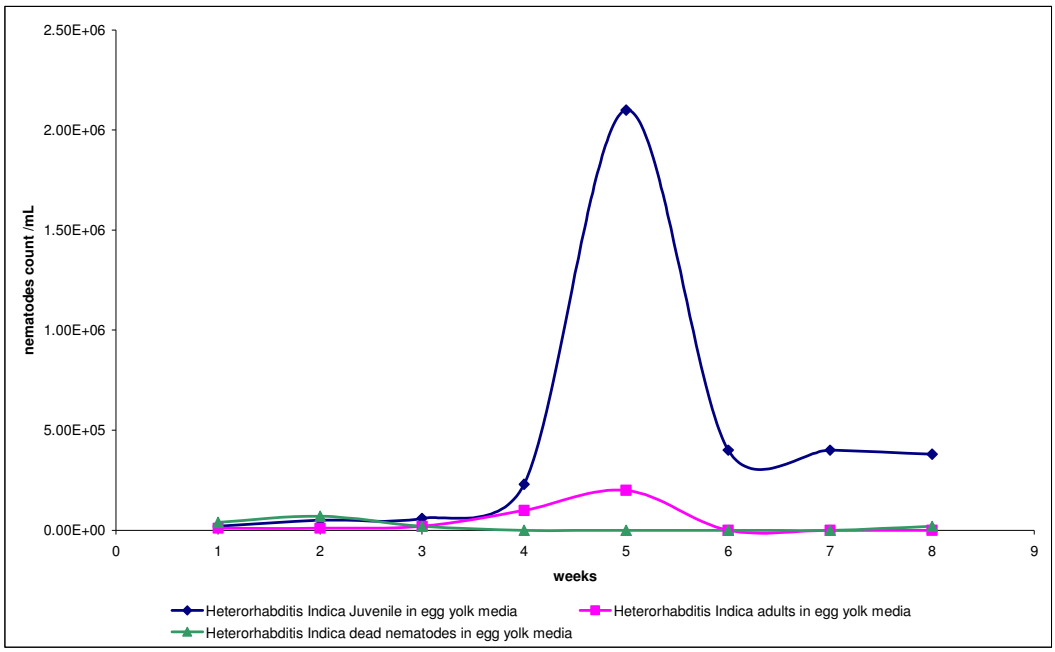

Figure 3.12: Growth patterns of *Heterorhabditis indica* in egg yolk medium

3.4 CONCLUSION

The most important findings arising from the experiments described in this chapter serve to emphasise two points. First, the growth of EPNs is dependent on factors such as food availability, environmental factors in the container for the liquid culture (in this instance shake flasks), and the types of media utilised. Second, the experimental work carried out confirmed that it is possible to grow EPNs in submerged *in vitro* media, and this opens up various opportunities for the mass production of these micro-organisms.

It was interesting to observe that experiments that appeared to replicate each other in every respect did not produce the same results. This indicates the need for further investigation of the factors that may be responsible for the variability in the experimental results. On the other hand, it was encouraging to count nematode population numbers as high 2.30X 10^6 counts/ml in some of the experimental cultures. This showed that elevated population figures can be produced in the experimental set-up.

The availability of *Photorhabdus Luminescens* bacteria locally is a major factor in experimental work on the cultivation of nematodes. The doubling time for bacteria is in the order of hours, and the bacteria reach their maximum concentration in a few days, while the nematodes take weeks to reach the maximum concentration. Thus as the bacteria and nematodes have different growth time constants, it is to be expected that the scientist will encounter some difficulty in growing the nematodes reproducibly. This could be the reason for the variability observed in the experimental results. The experiments showed that the growth of the nematodes is indeed limited by bacteria, *in that if fresh bacteria-rich medium is added to the nematodes, the concentrations of nematodes increases again and reaches a maximum in between 2–3 weeks again.* Futhermore, when

medium that contained no bacteria was added to the nematodes, no immediate increase in the number of nematodes was observed. This indicates that in the operation and design of these systems, it is very important to understand the growth characteristics of both the bacteria and the nematodes.

As far as the strain of EPN is concerned, the experiments showed that *Heterorhabditis indica*, *which is not indigenous, is higher-yielding than the South African Heterorhabditis bacteriaphora species*. Other differences, such as typical sizes and shapes, were observed. The South African species were found to be longer and slimmer in comparison with the Ireland species, which is shorter and more robust.

The ratios of juveniles to adults and of juveniles to dead nematodes for both *Heterorhabditis indica* and *Heterorhabditis bacteriaphora* grown in various mediums were evaluated at weekly intervals in the growth experiments. The ratios were found to differ with the medium. The egg yolk culture yielded the most favourable ratio in terms of juveniles to dead nematodes, because the latter are not discernible in egg yolk at five weeks, when the growth of juveniles reaches its maximum. The nutrient broth was the medium found least suitable for sustained growth (as was shown in Figure 3.10 by a dead nematode count that was higher than the number of juveniles).

Thus it has been shown that the growth of nematodes in submerged culture is indeed viable and that understanding the interrelation between the nematodes and their food source, the bacteria, is essential to successf when growing nematodes in solution.

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4 Chapter Four

Challenges in laboratory scale-up of a reactor for EPN Production

Abstract

This chapter describes the pathway followed to investigate the differences in EPN yields resulting from scaling up the experimental reactors for the production of EPNs. Three reactor types were considered, namely a shake flask, where the volume of liquid in the flask varied from 50 to 200 mℓ; a batch reactor, which was aerated, and where the volume of the liquid tested was 500 and 1000 mℓ; and finally a batch reactor with an external recycle, where the quantity of liquid varied from 1 000 mℓ to 1 500mℓ. The shake flask, the smallest container used, produced a higher population count of infective juveniles (IJs) than the larger reactors used. This was thought to be due to the slower growth and depletion of *Photorhabdus luminescens* bacteria at the shake flask level. This slower growth of bacteria in the shake flask was thought to match the growth rate of the EPNs better than was the case in the larger reactors. The result was that in the shake flask the EPNs had sufficient bacteria to feed off. In both the batch reactor systems, on the other hand, the bacteria grew faster than in the shake flask. This suggested that in these reactors the bacteria multiply quickly, reach their peak population density and then deplete the supply of nutrients in the growth medium, which results in a decline in bacteria numbers. Hence the growth rate of the bacteria in the batch reactors does not match that of the slower-growing EPNs, and consequently the population density of the EPNs is limited by the unavailability of bacteria. To test this hypothesis, the researcher switched a batch reactor in which maximum EPN density had been reached to operate as a fed batch system, by means of which fresh bacteria were

introduced to the EPNs in the reactor. This produced observable and measurable improvement in the nematode population, supporting the postulate that the growth of the EPNs in the batch reactors was limited by the amount of bacteria available as feed.

During the experiments, the supply of air was also found to be an important factor in increasing the nematode population, although in the bigger reactors turbulence had to be controlled because excessively high air flow rates hampered nematode reproduction. When the population count recorded for the external recycle reactor was compared with that of the fermenter reactor, the former was found to be higher. There was also a difference between the numbers recorded for the two different types of nematode grown in the same kinds of reactor. The *Heterorhabditis indica* strain at external reactor scale produced a population of 2.0X 10^5 infective juveniles/mℓ, while the South African *Heterorhabditis bacteriaphora* count was about 3.0X 10^4 IJ/mℓ.

The experiments described in this chapter suggest a possible route for developing an industrial process for the mass production of EPNs that could revolutionise the South African bio-pesticide industry.

4.1 INTRODUCTION

The study of EPNs has been given impetus by the discovery that they can be used as natural bio-control agents. This in turn has awoken interest in whether EPNs can be produced commercially and in large quantities. From the South African perspective, pursuing research in this field is particularly relevant because a South African strain of EPN has been discovered.

The first steps taken by the researcher to investigate the nature of this EPN strain and evaluate their potential for mass production on a

commercial level involved a study of the growth process of the nematodes, to identify the factors that influence their life cycle (Chapter Two) and the conditions under which they can best be cultivated (Chapter Three). The experiments described in this chapter arose from the next step, which entailed scaling up the process of production in progressive stages at the laboratory level, and analysing the results.

There are two possible technological pathways for the production of EPNs: *in-vivo* and *in-vitro*. (The various methods and the work of the scientists who published accounts of their research have been discussed in some detail in Chapter Two.) Many authors describe the liquid *in-vitro* pathway as the most economically viable option, and a great deal of research is being devoted to identifying the most efficient technology this method allows. De la Torres (2003) suggested that the way to ensure that quality yields of IJs were produced at a lower cost would require a greater understanding of the growth of EPNs in submerged culture.

Early researchers in this field, such as Pace et al. (1986), Ehlers (1998) and Surrey and Davies (1996), have done valuable work over the years to investigate the best growth conditions for nematodes and symbiotic bacteria in submerged culture at laboratory scale, using different types and sizes of containers (for example shake flasks and reactors). The aim of this contribution towards the body of research was to increase the nematode population yield, reduce the time spent on culture preparation, and cut the overhead costs of production in liquid culture (which is the preferred method of cultivating nematodes for commercial purposes). This led to an engineering challenge: identifying or designing a bioreactor that would satisfy the oxygen requirements of the bacteria inoculums and allow the EPNs in the same reactor to mate. The effects of the bioreactor system on other factors influencing the kinetics of nematode growth, such as shear stress and hydrodynamics, were also investigated.

Over the years different reactor designs have been tried out by a range of scientists and research groups in an attempt to discover which type would ensure the optimum production of EPNs in submerged liquid media.
Pace et al. (1986) tried a reactor continuously stirred with blade impellers for the cultivation of *S. feltiae* nematodes, and reported that the nematodes were seriously affected by shear stress induced by the impellers. Surrey and Davies (1996) employed bubble column fermenters for laboratory production of *Heterorhabditis Bacteriaphora* nematodes. The advantage of the bubble column was found to be the low-shear mixing that occured inside the reactor, which was favourable to nematode growth, as was shown by the high number of females identified in the culture. This conclusion was further confirmed by De la Torres (2003), who claimed that females are the nematodes most susceptible to death by shear stress in liquid culture during mixing.

Ehlers et al (1998) used laboratory-scale internal loop bioreactors for the culture of *Heterorhabditis megidis* nematodes. Each of these incorporated a turbine that stirred the contents from the bottom. Shapiro-Ilan and Gaugler (2001) noted that vulnerability to shear stress also varied with the different developmental stages of the nematodes, and suggested various innovative solutions to address the shearing problem. However, they warned that in attempting to control shear in liquid cultures, researchers might cause an imbalance in the level of oxygen supply, which would lead to greater viscosity in the medium. They proposed that air bubbling should be introduced through a downward sparger, causing a slow mixing that reduced the viscosity.

Other approaches in which reactors were used for the production of EPNs included a combination of solid and liquid medium for production. Gaugler et al. (2001), for example, used a solid-base medium in a LOTEK bioreactor for the cultivation of EPNs, and liquid medium to aid the recovery of IJs from the reactor.

Neves et al. (2000) used an external loop airlift bioreactor for the production of EPNs, with the aim of creating zones in which flow rates were differentiated in order to promote sexual contact between the male and female nematodes in the *in-vitro* liquid medium. Strauch and Ehlers (2000) used a set-up similar to that of Ehlers et al. (1998), working with an internal loop bioreactor, to analyse the impact of the aeration rate on both the production of IJs and their reproduction rates. They reported that the aeration of the medium had not affected the yield of IJs, and, interestingly, that the production of IJs was higher at 24 days than the earlier count at 16 days. This suggested that the rate of filial generation during the eight days between 16–24 was much greater than previously. Chavaria-Hernandez et al. (2007) used an airlift internal loop reactor for the production of *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophilia*. This experiment was carried out using two different mixing techniques: a standard internal draft tube and a static mixer. The effects of the two different techniques were evaluated in terms of rheological properties and hydrodynamic conditions.

In the study described in this thesis, the researcher used shake flasks as a start-up apparatus before experimenting with larger-scale vessels, to investigate what difference the change in size and geometry of the reactors made to the growth process. The reactors used were a fermenter and an external recycle reactor. This variation in scale made it possible to compare methods of producing EPNs in submerged culture across a range of volumes. The section that follows discusses the experimental procedure followed.

4.2 EXPERIMENTAL SET-UP AND METHODOLOGY

In this series of experiments, the possibilities of producing EPNs in liquid media were investigated at different scales of media volume and of container. A variety of sizes of shake flask, and different types and sizes of reactor were used, containing a range of liquid volumes and media types. Table 4.1 shows the experimental apparatus and media volumes used for the study.

Table 4-1 The apparatus and media volume for the EPN scale-up study

Apparatus	Apparatus volume	Medium volume
Shake flask	250mℓ	50mℓ
Shake flask	500mℓ	100mℓ
Shake flask	1000mℓ	200mℓ
Reactor (fermenter)	1300mℓ	500mℓ
Reactor (fermenter)	1300mℓ	1000mℓ
Reactor (external recycle)	2500mℓ	1000mℓ
Reactor (external recycle)	2500mℓ	1500mℓ

Each **shake flask** was covered with aluminium foil and seated in a shaker set at a speed of about 133–136rpm. The feed was decanted into the shake flask and then autoclaved inside the flask. Afterwards the inoculums of *Photorhabdus luminescens* bacteria were introduced, followed by the EPNs, under laminar flow conditions. No air was introduced into the shake flasks.

The **fermenters** were glass reactors fitted with a stainless steel lid fitted with flexible tubing to allow the feed to be pumped into the reactor. Air was introduced from the top through a stainless steel pipe that ran from the lid to the bottom of the reactor, and then distributed by means of a sparger. This was done by means of an Air cadet pump made by Barnant Company (Birmingham, Illinois 60010), operated at a maximum of 50Kpa. (Once this pressure level is exceeded there is likelihood that medium content will be lost owing to excessive pressure, which causes foaming and a subsequent overflow containing nematodes. The air distribution system used in these experiments was made at the University of the Witwatersrand laboratory, with air pore sizes of less than 1mm on the stainless steel pipe. This is discussed in greater detail in 4.3.3 below.) The pump head was a Master Flex, model 77201-62. The air was sterilised using a Cole-Palmer syringe filter, sterile G.D PTFE membrane and PP housing. The rotameter that was fitted was capable of measuring an air pressure of up to approximately 100mm of air. The temperature in the reactors was maintained at 26^0C by means of a stainless steel pipe that ran through the reactor and opened through the lid, where flexible tubing connected to the opening was fitted to a Jutabo water bath.

The **external recycle reactors** were constructed from Perspex transparent material which was surrounded by an external recycle and water jacket connected to a water bath, which maintained the temperature inside the reactor at 26^0 C. The feed was introduced through the top of the reactor, using peristaltic pumps (models 77201-62, 230VAC, 80Hz 1Hp, 0.75Amp) with autoclavable master flex tubing. Air was introduced by an Air cadet pump made by Barnant Company (Birmingham, Illinois 60010) from the bottom via an air distributor (a fish tank sintered stone aeration system—an off-the-shelf purchase), to ensure an even spread of fine bubbles inside the medium, and sterilised using a Cole-Palmer syringe filter, sterile G.D PTFE membrane and PP housing.

The external recycle reactors were sterilised using autoclaved water containing a concentration of about 10% of hypochlorite liquid, which was left to stand for about 24 hours. The vents of the reactors were fitted with autoclaved flexi tubings connected at the air disengaging end to a syringe with plastic tubing filled with glass wool. This was a precaution against both the entry of drosophila flies (which feed on the nematodes), and their laying any of their eggs on the site (because the abrasive texture of the wool would destroy them). The exteriors of the reactors were cleaned twice a week with hypochlorite diluted in water to repel drosophila flies from alighting on the reactors. The laboratory was also equipped with a fly trap to reduce the number of drosophila Foaming problems occasionally occurred in the external reactors, for example because of build-up of pressure inside them caused by the blockage of a vent or an air pressure exceeding 15Kpa. As previously noted, foaming is undesirable because it causes some of the liquid medium containing nematodes to overflow and collect in the air disengager..

The fermenter and external recycle reactors were equipped with three monitoring devices (Eutech probes), for pH, temperature and dissolved oxygen.

The range of media used was intended to allow for comparison of the effects of different kinds of medium (liquid culture, egg yolk and lipid broth) on the growth of EPNs.

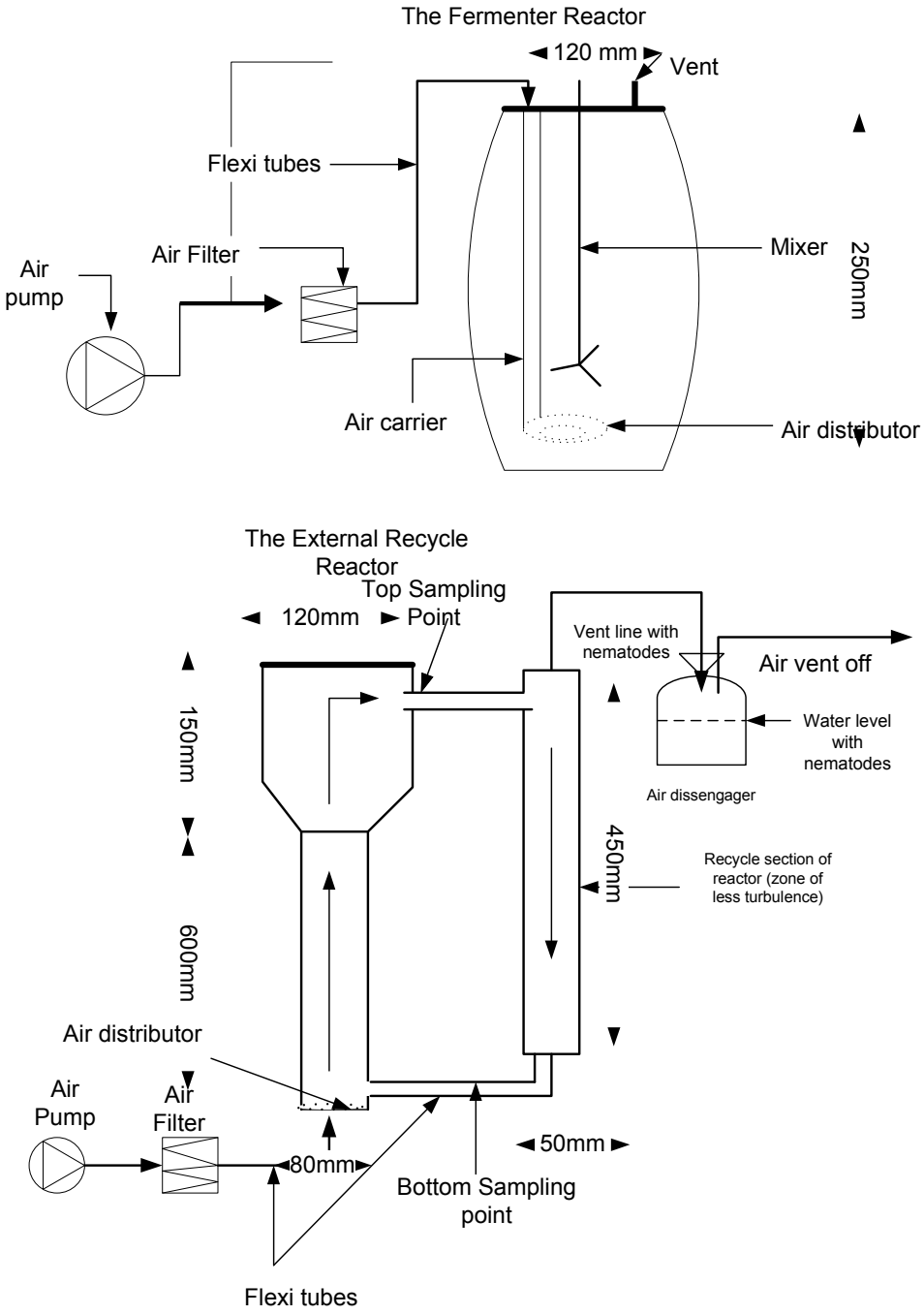

Figure 4.1: Schematic drawing of the fermenter reactor and the external recycle bioreactor systems

4.3 RESULTS AND DISCUSSION

Table 4.2 shows the range of results recorded throughout the scale-up experiments, to allow for comparative analysis based on the parameters selected.

Table 4-2 Information on the experimental parameters and summary of the observed experimental results for the scale-up process

Container volume	Medium volume (mℓ)	Air pressure	IJ/mℓ@ start up	Infective juvenile/ mℓ	Days to max count	Strain *	Note
Shake flask (250mℓ)	50	None	5000-20000	$1.5 \times 10^5 - 2.3 \times 10^6$	21-35	HI	
				$1.5 \times 10^5 - 8.0 \times 10^5$		HB	
Shake flask (500mℓ)	100	None	16000	6×10^4	30	HB	
Shake flask (1000mℓ)	200	None	11000	11.6×10^5	21-35	HB	
Reactor/ fermenter (1300mℓ)	500	15kpa	9000	2.0×10^4	13	HB	Batch
Reactor/ fermenter (1300mℓ)	1000	10kpa	5000	2.1×10^4	6	HB	Batch
Reactor (External recycle (2500mℓ)	1000	15kpa	5000	2.0×10^4	7-15	HI	Batch
Reactor (External recycle (2500mℓ)	1500	10kpa	10000	2.0×10^5	9	HI	Experiment stopped owing to contamination
Reactor (External recycle (2500mℓ)	1500	10kpa	5000	3.0×10^4	15	HB	Fed Batch
Reactor (External recycle (2500mℓ)	1500	15Kpa	5400	1.2×10^4	15	HB	Fed Batch

*HI: *Heterorhabditis indica* (Ireland)
*HB: *Heterorhabditis bacteriaphora* (South Africa)

4.3.1 The impact of medium volume on the growth of entomopathogenic nematodes

The overall results indicated that as the volume in the shake flasks was scaled up, so the number of IJs counted per millilitre of sample generally declined. This indicated that factors such as the increase in volume related to the availability of sufficient air for the bacteria (which provide food for the EPNs) might have inhibited the growth of the nematodes. Figure 4.2 shows the results of samples taken at five-day intervals from the 250, 500 and 1 000mℓ shake flasks, all of which had been agitated at the same speed in the shaker. It was found that the smaller the shake flask (and the amount of medium contained), the greater the likelihood that the nematodes would grow in it. The researcher concluded that probably the major reason was that the smaller the volume of medium in the flask, the greater surface area there was in contact with air.

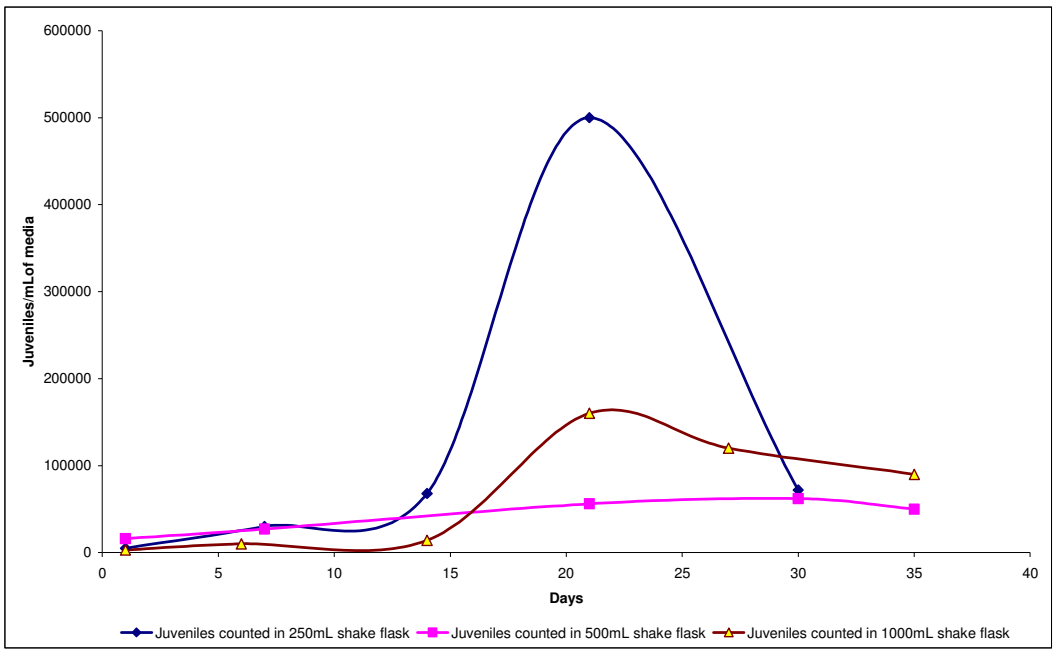

Figure 4.2: Comparison of juvenile growth in different shake flask sizes for *Heterorhabditis bacteriaphora* in egg yolk medium

Production Rate

250mℓ shake flask at maximum production is 25 000000 IJ at 21 days

500 mℓ shake flask maximum production is 7 000000 IJ at 30 days

1000 mℓ shake flask maximum production is 30 000000 IJ at 21 days

Although air was supplied in abundance in the reactors, the samples showed a lower number of nematodes than the counts recorded for the shake flasks. This triggers a relevant question: how much difference is there between the air requirements of EPNs and those of *P. luminescens* bacteria?

In contrast to the shake flask experiments, those using the external recycle reactor indicated a different, and critical, role for the amount of medium. It was found that volumes of 1 500mℓ in the external recycle reactors produced a higher count of juveniles than that containing a smaller amount (1 000mℓ). The advantage of having the higher volume was that the liquid level was above the recycle exit line. This translated into greater ease of transition into the recycle section because of a reduced dependence on air to push the medium through the connection. In the case of a medium volume of 1 000mℓ, air is necessary to propel the medium through the recirculation line, which might lead to turbulence in the reactor. This is the most likely explanation of what made the EPNs vulnerable to shearing.

As previously mentioned, a higher number of IJ counts was obtained from the shake flasks than the reactors. This is an indication that the nematodes adapted better to conditions in the shake flasks than in the reactors. However, the number of days taken for EPNs to reach peak growth was smaller in the reactors than in the shake flasks. This can be attributed to the faster growth of *P. luminescens* bacteria in the well-aerated reactor environment, and the consequent availability of feed for the nematodes. However, this more rapid multiplication of nematodes was

accompanied by an earlier onset of feed depletion in the reactors than in the shake flasks. This assumption was tested by withdrawing samples from the reactors and culturing them in shake flasks, adding some fresh feed (bacteria-rich medium), and allowing the experiment in the reactors to continue under the same conditions as before. The results showed an increase in the number of juveniles in the shake flasks, as can be seen in Figure 4.3. This experimental finding prompted the decision to change the batch system previously used in the reactors to a fed batch system. This is also in line with the results of Chavaria Hernandez et al., 2011, who discussed the influence on nematode yields in submerged culture of their shapes, sizes and geometry. However, these researchers carried out their experiments in two internal loop reactors that were geometrically dissimilar.The reactor with a higher inertia force encourage more production when comapared with the one that is less, because this may help encourage mating among young female and young male which are more virulent.However in our setup, the reactor environment is more active and higher inertia force than the shake flask, this would have also help to boost the fast growth experienced at a very short period for the EPNs.

Due to the fast growth of EPNs in the reactor their was a quick depletion of bacteria, hence this necessitate that fresh bacteria feed is added to sustain the EPNs growth in reactor.Contamination of the samples proved more of an issue in the reactors than the shake flasks during the scale-up experiments. The wider surface area of the reactors made the risk of contamination higher than in the smaller containers.

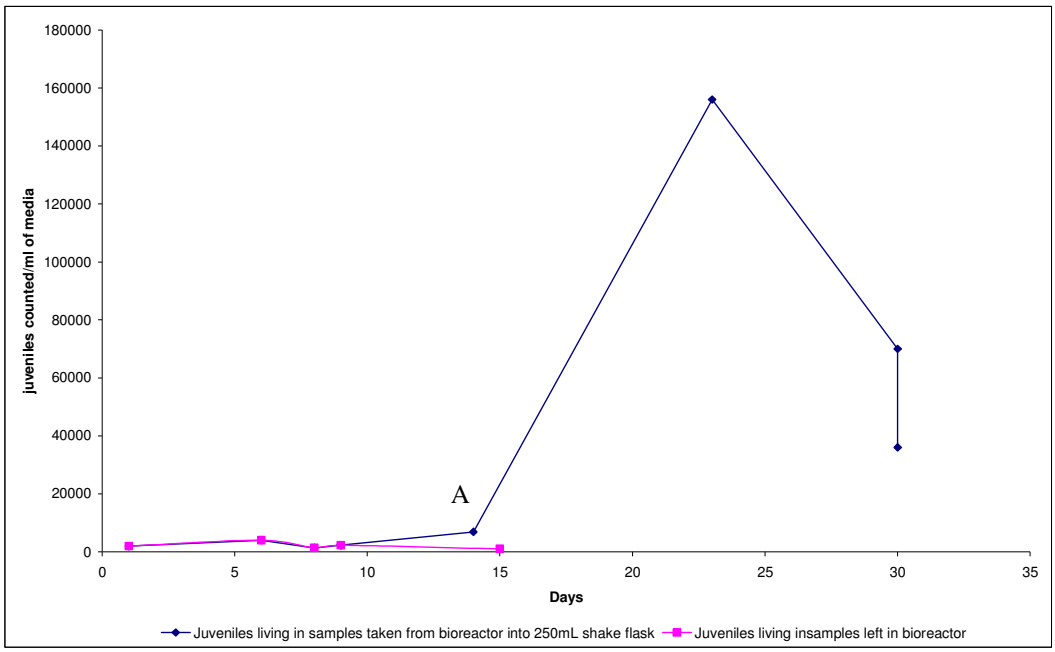

Figure 4.3: Comparison of growth levels after the introduction of a reactor sample into shake flask sample

A: This is the point at which samples were withdrawn from a reactor and put into a shake flask. The graph indicates the basis for changing to a fed batch reactor system.

Production rate

Reactor at maximum production is 6 000 000 IJ at 6 days.

250mℓ shake flask at maximum production is 8 000 000 IJ at 23 days.

4.3.2 The influence of *Photorhabdus luminescens* bacteria growth on nematodes in culture

Our earlier research described in Chapter 2 investigated the growth of the *Photorhabdus luminescens* bacteria and compared the growth rates in shake flask and reactor experiments. It was found that the measured growth rate of the bacteria in the shake flasks was much slower than that measured in the reactors, as shown in Figure 4.4 below. The *Photorhabdus luminescens* bacteria grew faster in the reactors than in the shake flasks, and it was found that on average their growth in the reactors would peak within 1–5 hours. The ratio of bacteria inoculum volume to that of the medium was the same in the shake flasks and

reactors (1:10), and other conditions, such as temperature and sterile environment, were replicated in all cases, except that air was supplied in the reactors but not in the shake flasks. Thus the slower growth of the bacteria in the shake flasks as compared with the reactors was attributed to the limitation of air supply in the shake flasks,

In contrast, as we see in Table 4.2, the EPNs reached a higher maximum concentration in almost all the shake flask experiments than in the reactors (10^5 as opposed to 10^4 in most of the reactor experiments). On the other hand, the reactors reached their maximum concentration of EPNs more quickly than the time needed for their maximum concentration in the shake flasks (7–15 days in the reactors as opposed to 20–30 days in the shake flasks).

We postulated that the improved growth of EPNs in the shake flasks was due to the higher availability of bacteria as feed in the shake flasks, which in turn was attributable to the slower growth rate of the bacteria in the shake flasks. We further assumed that the growth of the bacteria in the shake flasks was limited initially by air, and thus occurred more slowly, which in turn retarded the depletion of nutrients in the shake flask medium. Hence the EPNs, which require a longer growth time than the bacteria, have bacteria available to feed on and hence are able to reach relatively large concentrations, albeit not rapidly, in the shake flasks. Thus in these vessels, where the growth rates of the bacteria are limited by less air, there is a closer match between the growth rate of the bacteria and the nematodes, which leads to higher concentrations of EPNs.

In the reactors, the growth rate of the bacteria was boosted by the supply of air and thus reaches a maximum within hours, after which it drops progressively to low values as the nutrients in the medium are depleted. Hence the nematodes (which grow much more slowly than the bacteria), do not have a high concentration of bacteria available to them. This means

that although they reach their (lower) maximum value more quickly in the reactors, the EPNs do not reach high concentrations.

As already discussed in Chapter 3, we found that when bacteria were introduced into a shake flask in which the number of EPNs had already passed through the maximum value stage, the introduction of fresh bacteria into an existing shake flask culture resulted in increased EPN counts. This proved that the EPN growth rate had declined because of a shortage in the bacteria.

To test the hypothesis that the growth of nematodes in the reactors was also limited by the amount of bacteria available, we added a supply of fresh bacteria to the nematodes in the whole range of containers, to see how the growth rate of nematodes responded

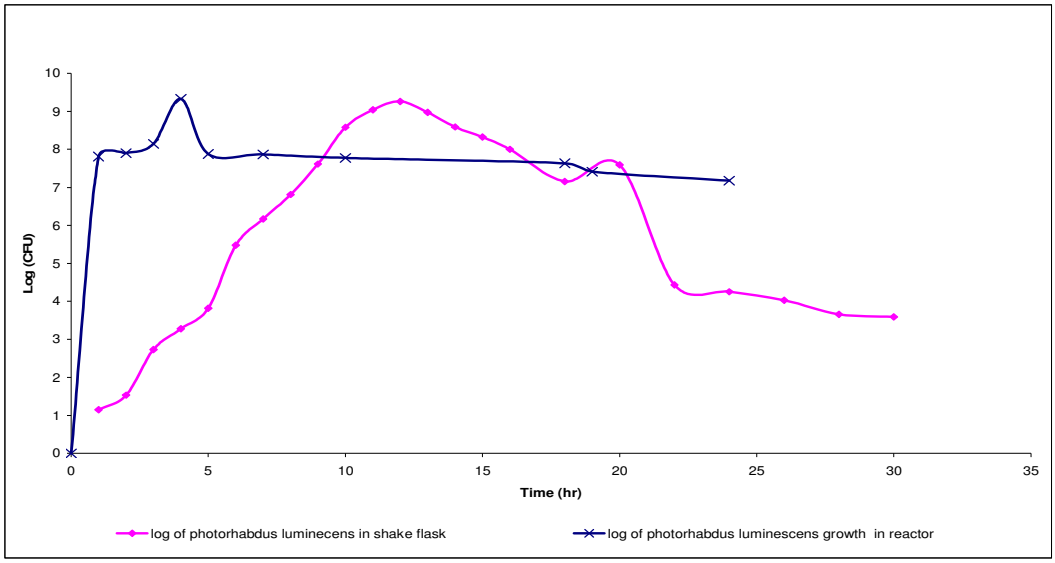

Figure 4.4: Comparison of bacterial growth in the shake flask and fermenter reactor systems

4.3.3 The impact of adding fresh feed into existing experimental cultures

The technique followed for adding bacteria and nutrient-rich supplements to the contents of the shake flasks was to allow the EPN count to drop

before introducing fresh medium. This led to an increase in the number of EPNs counted until the bacteria they fed on became depleted, after which the number of EPNs declined again. These effects are graphically represented in Figure 4.5. However, in the reactors it proved difficult to bring about an equivalent recovery in the nematode count through replenishment with fresh bacteria-rich medium. This led to the adoption of a different technique: adding about 50mℓ of the fresh feed every five to seven days, before addition of the 50mℓ fresh feed sample same volume (50mℓ) is first removed. This technique, which is referred to as the fed batch system, proved effective, because it made sustained as it made growth of the EPNs possible over time (see Figure 4.6). The addition of fresh medium also helped to strengthen the resistance of the *Photorhabdus Luminescence* population in the reactors to any foreign contaminant. *This result is outstanding because it shows us that by replenishing the feed for nematodes we are able to increase their growth in submerged culture.*

In most of the reactor experiments in which fresh feed was not added, it was difficult to initiate or sustain growth in the reactor systems, as can be seen in Figure 4.7. This was attributed to either depletion of the bacteria food source or contamination of the medium. A co-researcher conducted the following experiment to explore liquid medium contamination further. A sample of the liquid medium was streaked onto solid agar plates and cultivated. A Polymerase Chain Reaction (PCR) technique involving PCR tubes (Whitehead Scientific 0,2mℓ thin wall PCR® tube with domed cap) was used to identify the dominant bacteria in the medium. Based on the findings of Nyamboli (2008), the experimental conditions were as follows: 25µℓ of 2X PCR Master Mix(promega) 1µℓ of each primer (sense and anti sense) 20.5µℓ nuclease free water (Promega) and 2.5µℓ nuclease free water (Promega). An addition of 2,5 µℓ of template DNA was made to limit contamination. A control speciment of *Eschericia coli* (E coli) was made ready for use in the experiment, which made use of all the components

except the template, which was replaced with nuclease-free water. The PCR tubes were centrifuged for one minute at a speed of 10 000 rpm, and the ensuing cycling file was used for amplification of the DNA Gene Amp® PCR express 2700 thermocycler. (Nyamboli, 2008) The oligonucleotide primers are as shown on Table 4-3.

The following were specified by Nyamboli, 2008:

1. Initial denaturation: 92^0C for 2 min
2. 35 cycle amplification series:
    - 95^0C for 30 secs (denaturation)
    - 57^0C for 30 secs (reannealing)
    - 72^0C for 1 min (extension)
3. Final extension: 72^0C for 10 min

Table 4-3 Oligonucleotide primers for amplification of bacteria

Oligonucleotide primers used for amplification of a region of the bacterial 16S gene based on Nyamboli, M (2008)		
Oligonucleotide	Sequence	Tm(^{0}C)
Sense primer *E.coli* numbering 6-25	5'-GGAGAGTTAGATCTTGGCTC-3'	62
Anti-sense primer *E.coli* numbering 1540-1521	5'-AAGGAGGTGATCCAGCCGCA-3'	66

In Table 4.2 the growth results in the reactors that showed a peak in about 10 days were mostly explained by rapid depletion of the food source or contamination of the media in the external and fermenter systems. The *Photorhabdus luminescens* bacteria demonstrate some antibiotic activity when the population is very high in the medium; but when their numbers become depleted it becomes easier for competing microbes such

as *E coli* to contaminate the medium, which kills the nematodes. In Figure 4.8 we compare the growth in batch versus fed batch systems to illustrate that the addition of fresh medium helps to extend the life cycle of EPNs cultivated in the reactor.

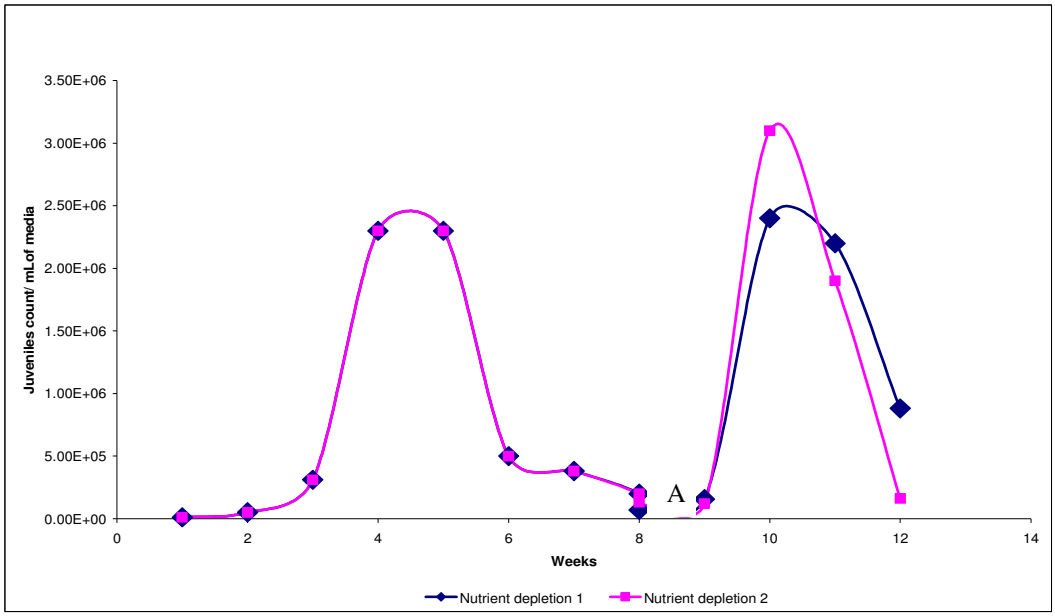

Figure 4.5: Impact of adding *Photorhabdus luminescens*-rich fresh egg yolk medium to a shake flask containing *Heterorhabditis Indic*a nematodes

Point A: The samples are divided in two, and fresh feed rich in Photorhabdus luminescens bacteria added.

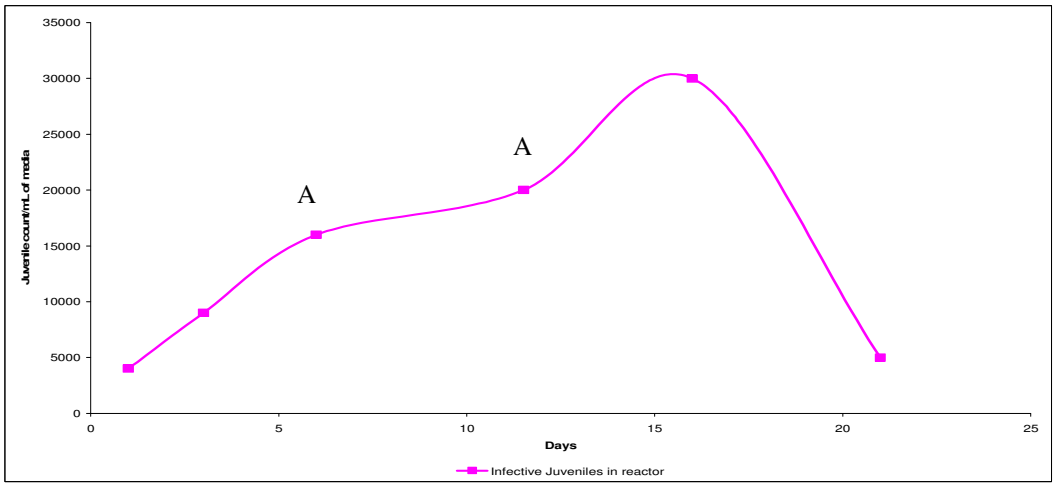

Figure 4.6: Increase in population of infective juveniles using the fed batch system in an external recycle reactor, *Heterorhabditis bacteriaphora* and lipid broth medium

A: Point of addition of bacteria rich lipid broth medium external recycle reactor containing the Heterorhabditis bacteriaphora nematode.

Production rate

Reactor maximum number attained is 45 000 000 IJ at 18 days

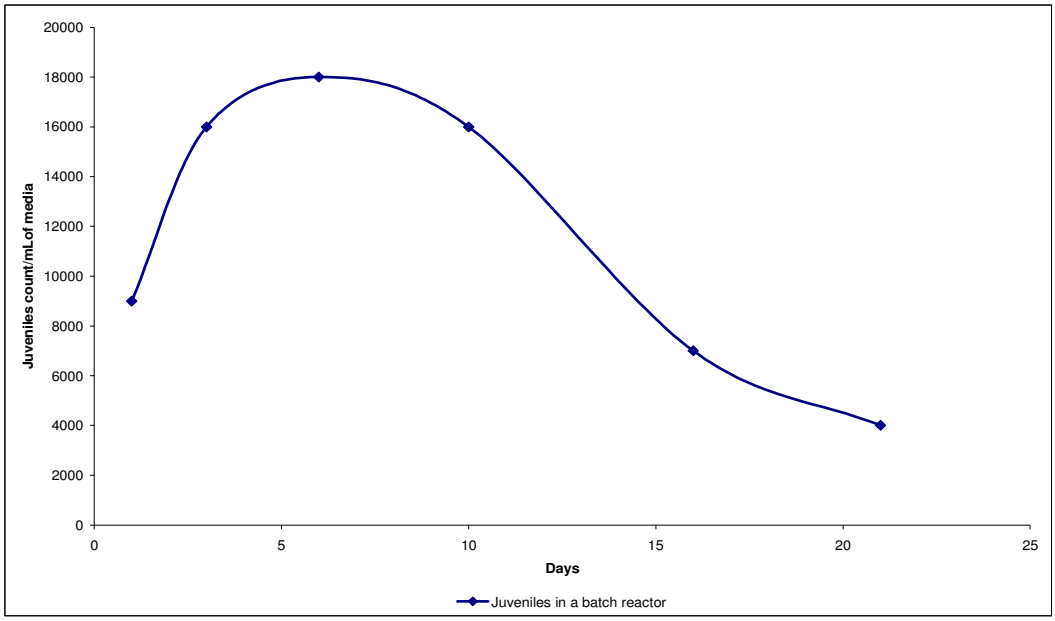

Figure 4.7: Population of infective juveniles of *Heterorhabditis bacteriaphora* count over a batch external recycle reactor in a lipid broth medium

Production rate

Reactor experiment maximum production rate attained is 27 000 000 IJ at 6 days

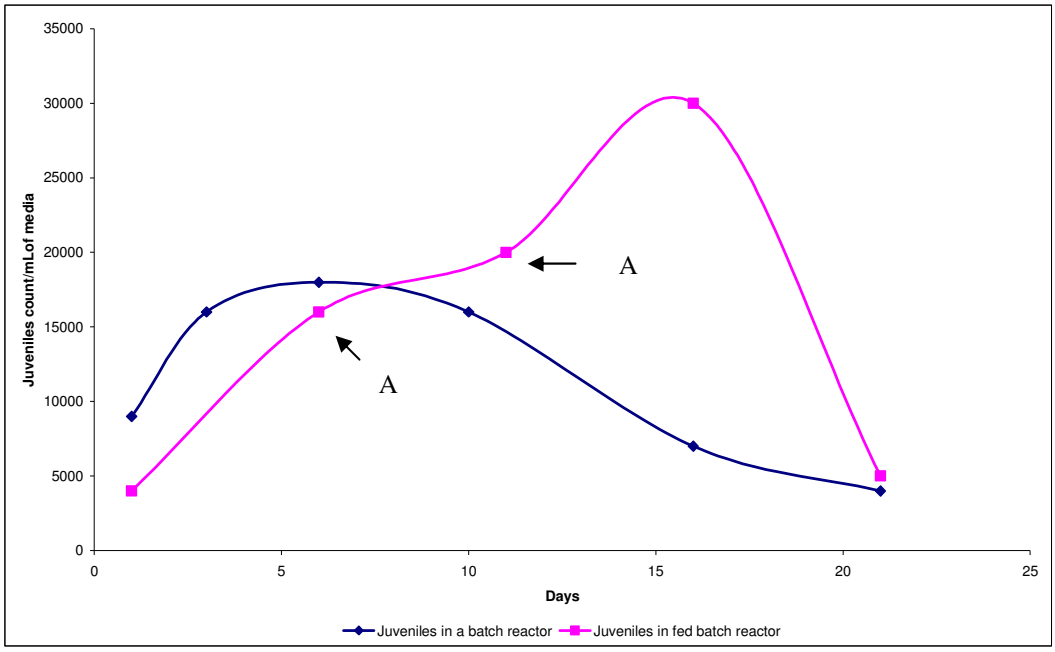

Figure 4.8: Comparison of infective juveniles counted over a batch and fed batch external recycle reactor system

A: Point of addition of bacteria rich fresh feed.

Production rate

Juveniles in batch reactor maximum is 27 000 000 IJ at 6 days

Juveniles in fed batch reactor at first point of fresh feed addition 24 000 000 IJ over 6 days.

Juveniles in fed batch reactor at second point of fresh feed addition 30 000 000 IJ at 11 days

Juveniles in fed batch reactor at maximum is 45 000 000 IJ at 18 days

4.3.4 The impact of air pressure and its influence on entomopathogenic nematodes in reactors and shake flasks

The effects of mixing were less noticeable in the shake flask experiments than in the reactors, because the latter were thoroughly mixed by a continuous supply of air. However, mixing does occur in the shake flask due to the side-to-side movement of the shaker, which may be less disturbing to the nematodes, allowing them to follow their normal cycles, such as mating and reproduction, without inhibition. The hypothesis on the results of mixing in the reactors is that the unsettling effects of continual bubbling of air and mixing of the material could limit the ability of EPNs to mate and reproduce. In the fermenter reactor there is no area that is conducive to mating, and this might constrain nematode reproduction. De la Torre (2003) suggested that this hydrodynamic effect is critical to an understanding of what affects the growth of EPNs in submerged culture. However, the results presented in Table 4.2 show that the impact of mixing on the reproduction and growth of nematodes is less harmful than that caused by the depletion of bacteria, which constitute the food source for the nematodes in the experiment.

The recycle section of the external recycle reactor, on the other hand, is well suited to the mating and reproduction of EPNs because it is an area of lower turbulence and mixing. This led to the postulation that this was why the number of IJ nematodes recovered from the external recycle reactor was higher than that from the fermenter. In order to verify the postulated advantage for mating offered by the external recycle section of the reactor, the researcher took samples from the top (top left-hand chamber in Figure 4.1) and the return lower section of the recycle (bottom right-hand chamber in Figure 4.2) and counted the number of nematodes (see Table 4.3). In most instances the results of the sample counts confirmed that the external recycle did encourage mating, because the

nematode count at the bottom sampling point was higher than that for the top sampling point, as shown in Table 4.3. This indicates that the external recycle portion of the return lower section offers the advantage of a mating zone for the nematodes.

Table 4-4 The sampling for nematode count in the external recycle reactor at the top and bottom of the recycle section

Top IJ	1	1	2	1	1	2	2	3	3	4
Top Adult	4	2	3	1	2	2	1	1	3	1
Top Dead	2	1	2	1	-	-	-	-	-	-
Bottom IJ	12	12	10	10	13	11	10	10	7	18
Bottom Adult	2	1	2	2	1	1	1	-	-	-
Bottom Dead	1	-	-	-	-	-	-	-	-	-

N.B: Samples are diluted to 10^2

IJ: Infective Juveniles

Another consequence of air pressure was observed in terms of content loss in the reactors. When an air pressure exceeding 10 kPa was pumped into the reactor, the medium contained tended to foam, leading to an overflow of the reactor's contents into the disengager. At the initial stage (before the addition of the disengager) there were spills to the side of the reactor, which led to the loss of EPNs in the medium, as shown in Figures 4.9 and 4.10. This was more evident in the external recycle reactors when pressure built up inside them and the liquid content rose far above the recycle exit level. After the disengaging unit was installed it was used as a liquid trap in which the medium was retained. The nematodes in the

medium were therefore able to continue to grow in the disengager. The presence of this unit made it possible to collect and recover the nematodes after the medium had been allowed to settle. This discovery was serendipitous, and may offer a means to recover IJs from operating reactors. The results also indicates that, based on the samples collected from the air disengager, the nematodes lost from the reactors are more likely to be IJs than adults

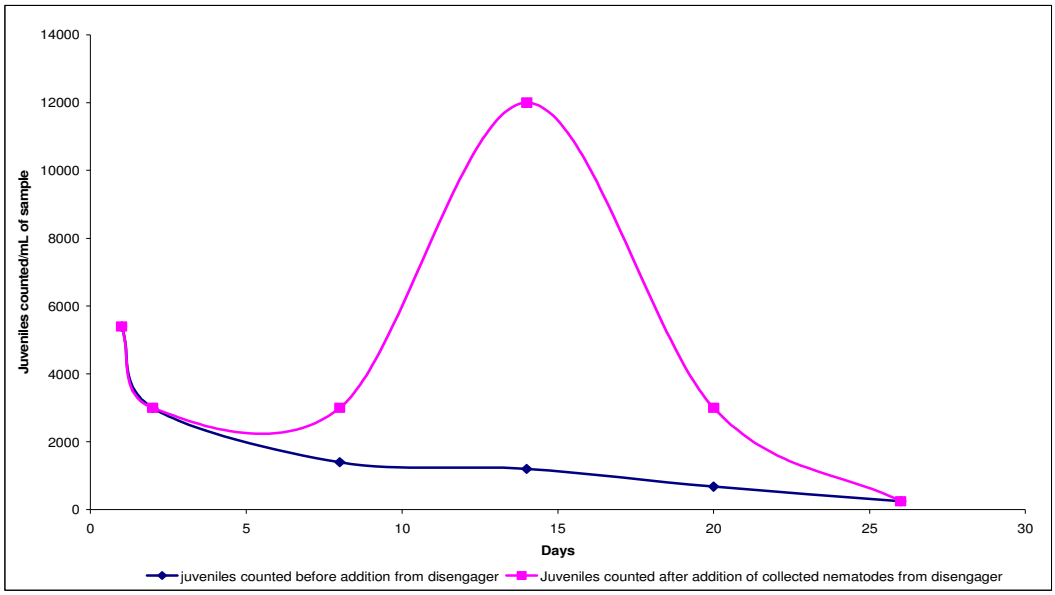

Figure 4.9: Comparison of the juvenile count before and after the disengager was used.

This result shows that IJs can be collected in the disengager

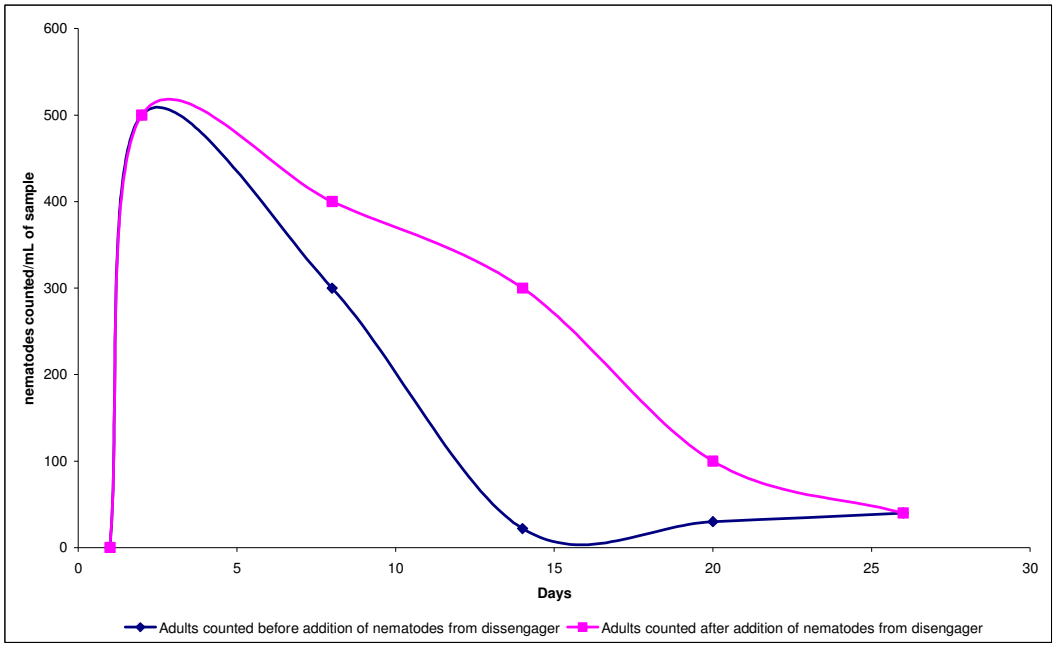

Figure 4.10: Comparison of the adult count before and after the disengager was used.

The evaporation of medium content is a critical problem. It is often more pronounced in the fermenter reactors because of their smaller volume. Fermenters were found to require the periodical addition of autoclaved water, which is preferred because it does not add to the salt load in the reactor system, whereas the introduction of fresh medium does so. An oversupply of salt could threaten the survival of the EPNs. However, although the ambit of this study did not directly include an evaluation of the impact of salt load on the EPNs, it was possible to conclude that the quantity of salt contained in the amounts of fresh media did not have a harmful effect on the EPNs.

Another important point is that the air pressure required for the growth of nematodes is lower than that needed to cultivate *P. luminescens* bacteria. Although the bacteria are able to grow at an air pressure of about 15Kpa, it is important that the air pressure should be reduced to about 10Kpa in the external reactor systems on the introduction of nematodes, to prevent the loss of medium content and the early death of EPNs. At air pressures

of 10Kpa there is less foaming into the disengager. However, air pressure it is still necessary in the event of any upset or blockage in the air vent. The disengager was not used in the fermenter reactor, which meant that in cases of high air pressure the content of the fermenter would be lost.

4.3.5 Establishing ideal conditions for the cultivation of entomopathogenic nematodes

In order to ensure higher EPN growth as the experiment was scaled up, we attempted to provide the favourable conditions to be found in the 250mℓ flask in the larger vessels. These included varying the air supply pressure. When the air supply pressure in the reactors was reduced to less than 10 kPa, the medium started to ferment, the nematodes died, and the contents gave off a foul odour. More research will be required to determine the exact balance between the amount of air introduced (for mixing as well as the growth of bacteria and nematodes) and the need to maintain an environment of zero turbulence.

As has been noted, when samples were collected from the bioreactors and transferred into the shake flasks for culturing, they responded by producing a greater number of IJs in the new environment than in the reactors. Again, having set up a control experiment in a shake flask at the same time as the nematodes were introduced into the reactors, the researcher found that the sample taken from the control flask produced higher population numbers than those recorded from the reactors. These findings confirm the favourability of the shake flask environment for nematodes. The question remains how these conditions can be replicated on a larger scale.

4.3.6 Other important results

The experimental results produced in the external recycle reactors demonstrated that the *Heterorhabditis Indica* species is a higher-yielding type of nematode than the South African *Heterorhabditis bacteriaphora*. This finding was further confirmed by the outcome of the shake flask experiments. More research is required to investigate the causes.

Two other factors were isolated, both of them raising engineering questions that have implications for the design of reactors that are to be used for the cultivation of EPNs. At present the recovery (harvesting) of EPNs is done by collecting mixed samples. The different classes (that is, the males/females, juveniles/adults and dead nematodes) are then identified in each sample before counting can take place. It should be possible to choose, based on a selected stage of the nematode life-cycle, the type of nematode the researcher wishes to recover (for example only adults or juveniles). Currently, the reactors are engineered to produce EPNs; but in the future, they should include recovery equipment (like the disengaging bottle on the external recycle reactors) designed to collect nematodes on a selective basis, such as size or a particular life stage.

The second shortcoming in the current design of the bioreactors is that they expose the EPNs to the direct force of the air that is pumped in. In future the design blueprint should incorporate means to reduce the disturbance to the nematodes caused by the air. This may involve adding solid material into the reactor, which would provide the nematodes with a solid surface to grow on and also act as a buffer that absorbs the impact of the air. These solid materials should also be suspended in the liquid medium in the reactor to help reduce turbulence.

Another important suggestion is the culturing of the bacteria and the nematodes in two different reactors, each of these sized so as to match

the growth characteristics of the species. This would have the advantage of ensuring that the conditions required for the growth of both bacteria and nematodes are satisfied in the different reactor compartments, and take into account the finding that bacteria have a faster growing rate (in hours) than nematodes (in weeks). This approach would give us greater control over the growth of both the EPNs and their symbiotic bacteria.

It is important, however, to explain at this point that the aim of our experiments was to retain the EPNs in the reactor. In this research nematode production was carried out over a batch and not a continuous process, with the continuous addition of bacteria-rich feed. Also, the growth of nematodes could not be measured on an hourly basis because the life cycle of EPNS requires a longer time span (weeks rather than hours). We have included in our results the production rate at maximum for the reactor experiments based on the number of days and the total number of EPNs in the medium volume. This shows that the production rate differed from one reactor experiment to the other. The most important point elicited from these experiments was that *the addition of fresh feed into reactor can indeed increase the growth rate of nematodes in submerged culture*. The loss of media is through evaporation via the vent and liquid through the disengager.The liquid collected over the dissengager is decanted periodically.The nematode in the decanter is then returned into the reactor as discussed in section 4.3.4

4.4 **CONCLUSION**

In this chapter, a production process for EPNs was investigated by means of experiments carried out in a range of vessels on an ascending scale of capacity. One of the most significant findings was the difference in the number of EPNs produced at the smaller (shake flask) scale and those recorded in the larger containers used in the production process. A gradual reduction in the number of juveniles counted in the medium was observable as the process was scaled up. In the larger shake flask, the falling-off in population counts was attributable to various factors, such as the availability of sufficient air for the *Photorhabdus luminescens* bacteria (the food source for the EPNs). In the reactors, air was used both to sustain the growth of the bacteria and for mixing the reactors' contents. However, the introduction of air to the reactors required monitoring. If the supply was excessive (for example, at a pressure above 15 kPa), a tendency towards foaming could be observed in the reactor. This also caused shearing and inhibited the reproduction of the nematodes. At too low a supply pressure, (say less than 10 kPa) the air supply was insufficient, causing the contents of the reactor to ferment. Evaporation of the medium was also found to be an issue, because it exposed the EPNs to the threat of an increase in the salt load. This situation was rectified by the addition of sterilised water into the medium.

It was found that *Photorhabdus luminescens* bacteria grew faster in the reactors, which in turn led to more rapid depletion of their numbers than in the shake flasks, where the nutrients in the medium lasted longer. In order to sustain good growth in the reactors, it became necessary to adopt a two-pronged approach. Fresh feed samples rich in bacteria were introduced at five-day intervals in the reactor experiments (a fed batch process, which replaced the earlier batch system), while replenishment of the shake flasks was provided only every five to six weeks. This indicates that bacteria require a regular supply of nitrogen, as shown in chapter 2,

although in the endogeneous phase there is a breakdown of the dead bacteria, which will release some nitrogen into the medium. However, this is a slow process that does not produce sufficient nitrogen adequate to maintain bacteria growth in the reactor.

In the external recycle reactors, the medium level was found to be critical to achieving a high population number. The highest counts of EPNs were recorded in cases where the medium volume rose above the recycle tubing line, which assisted recirculation The advantage of a recycle section is that it creates a zone of low turbulence where mating and reproduction can occur. This explains why the experiments in the fermenter reactors, which create conditions of high turbulence, did not yield high EPN counts. The ideal environment for nematode reproduction was found to be that in the 250mℓ shake flask, which prompted our attempt to find a means to replicate those conditions on the larger scale.

The production of a high count of juveniles in the reactors was significant to exploring the possibility of producing EPNs on a large scale for commercial purposes. However, the type of EPN seems to play a major role in the number of counts achievable. In this study, the *Heterorhabditis indica* were found to be yield higher population counts across the entire range of culturing vessels, from the shake flasks to the reactors, than the indigenous *Heterorhabditis bacteriaphora*. This leads to the speculation that it may be more suitable to grow the bacteria and nematodes in two different vessels and to introduce the bacteria as feed to the nematode-containing vessel as required, to ensure optimum and sustainable conditions for EPN growth.

The factors affecting the growth of EPNs in the South African context have been discussed in terms of the research carried out in the experiments described in this thesis, but require more study to enable researchers to further engineer the life of EPNs. More sophisticated methods involving

harvesting selected classes of nematode for counting purposes are also needed. Both are important to effective commercial production of EPNs, although they fall outside the scope of this work.

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5 Chapter Five

5.1 Conclusions and recommendations

The purpose behind the research outlined in this thesis was to explore the possibility of using EPNs as bio-control agents in South Africa, and to do so on a commercial scale. In order for this to be feasible, it is necessary to be able to produce EPNs on a commercial scale. In this work we investigated the possibility of producing the EPNs in submerged culture.

An essential component of the attempt to produce the EPN species of *Heterorhabditis Indica* (from Ireland) and *Heterorhabditis bacteriaphora* (originating in South Africa) was the parallel study of the symbiotic bacteria *Photorhabdus luminescens*, which was carried out at the same time. This made it possible to identify the principal factors that influence the growth of both the *P. luminescens* bacteria and the nematodes.

The conclusions described below were arrived at by means of the experiments carried out by the researcher.

5.1.1 Cultivation of *P. luminescens* bacteria

The availability of nutrients is important for the optimum growth of *P. luminescens* bacteria, because when the nutrients required by the bacteria became depleted, the death phase sets in. The experimental results identified nitrogen as the limiting substrate in the medium used for propagating the *P.luminescens* bacteria. When fresh medium rich in nitrogen was introduced into the culture at the death phase, the bacteria recovered and their concentration increased. Although it would normally be expected that at the endogenous phase the decay of the bacteria would create additional nitrogen, it was clearly not the case. The

explanatory hypothesis is that either the above process is very slow, or that it adds insufficient nitrogen: hence the need to add fresh nutrients rich in nitrogen. Another issue that was found to address the problem of nutrient depletion was to co-ordinate the rate of growth with the frequency of fresh feed supplementation. The decision to change from a batch to a continuous system proved favourable for the growth of the bacteria.

The availability of air was found to be significant for the growth of *P. luminescens*, as was shown by a comparison between bacteria cultivated in the shake flasks and those grown in the reactors. Because the shake flasks are sealed at the top, no or very little air is introduced; whereas in the larger-scale experiments, air is bubbled into the reactors. The faster exponential phase in bacterial growth experienced in the bioreactors was attributable to the more favourable conditions they offered, which included a greater supply of air. Also, in the reactors the air is used to mix the contents, while in the shake flask experiments this is achieved by the swirling action of the shaker. This helps to explain the findings that bacteria grow faster in the reactors than in the shake flasks.

5.1.2 Comparison of the conditions of cultivation of the bacteria and the EPNs

The growth curve of the bacteria, compared with that of the EPNs, shows that that the former grew faster than the latter: the doubling time of bacteria is about 3-4 minutes in reactor and 18 minutes in the shake flask, whereas that for the EPNs is in the order of days or even weeks. The challenge therefore was to find a way to enable the growth curves of the two types of organism to correspond more closely, both in the reactors and the shake flasks. The answer appeared to lie in supplying sufficient nutrients to the EPNs to sustain population increase. This explains why it is important that nematodes, whether cultivated in reactors or other kinds of container, are given food (bacteria-rich) supplements on a fairly regular

basis. In the experimental study the existing culture was replenished with fresh bacteria medium, after 6–8 weeks in the shake flasks experiment and after 5–7 days in the reactors, to ensure the sustainable growth of nematodes in submerged culture

In the shake flask experiments, the growth of both species of EPN (*Heterorhabditis Indica* and *Bacteriaphora*) was found to depend on a number of different factors, such as food availability, environmental conditions within the container, and the type of medium used. The results of the experiments conducted in this study confirmed the type of nutrient (enriched by *P. luminescens* bacteria) essential to the growth of nematodes, and the need to maintain population increase by introducing fresh bacteria-rich medium into existing experiments. *A comparison of the two species of nematode used in the experiments revealed that the Heterorhabditis indica is higher-yielding than the South African Heterorhabditis bacteriaphora.* Considerable differences were also found in the size and shape of the two species.

An interesting result was that repetition of experiments, for example using the same inoculum in different shake flasks that were subject to identical conditions, did not produce the same outcomes. It would appear that the reason lies in the variability of environmental factors, which may be important determinants of the growth of nematodes and bacteria in liquid media.

The results of the experiments also indicated that a fed batch process is the most appropriate for the production of EPNs in submerged media. It is, however, advisable that the production process of the bacteria and the EPNs be kept separate. The bacteria should be grown in one reactor and the nematodes in another, before the bacteria are introduced into the nematode reactor as feed to ensure the continuous growth of the nematodes. There is a difference in air requirements between bacteria and

EPNs, as well as difference in growth rates. Sufficient air to cause turbulence in the bacteria reactor can promote good and faster growth, while in the nematode reactor only enough air to sustain the bacteria as feed for the nematodes is required. The quantity of air nematodes need to remain alive was not determined by this series of experiments, as it fell outside the scope of the investigation. However, the researcher established that in order to overcome the impact of turbulence in the nematode reactor, which was found to interfere with various stages of the nematode life cycle, a solid medium immersed in the reactor helped to reduce the deleterious effects. It is proposed that in future a two-reactor system be considered, in which the bacteria and nematodes are grown in different reactors and the bacteria are fed to the nematode reactor at a controlled rate.

The production of a high count of juveniles in the reactor proved particularly relevant to the aim of the research because it opened up a possible means of exploiting EPNs commercially. However, the researcher found that the species of EPN seems to be significant to the number of population counts achievable. In this study, the *Heterorhabditis indica* was found to yield higher numbers of juveniles across the whole range from shake flask to the reactor scale than the South African *Heterorhabditis bacteriaphora*.

5.1.3 Results of applying a Mathematical Model

The mathematical model set up was based on the following set of partial differential equations, which model the specific growth cycle of nematodes.

$$\frac{dA}{dt} = K_4 A I J_4 \longrightarrow KA - DA \quad [5]$$

$$\frac{dIJ_1}{dt} = K_A\text{-}0 \text{ const } A \longrightarrow K_{12} IJ_1 \quad [6]$$

$$\frac{dIJ_2}{dt} = K_{12}\, IJ_1 \longrightarrow K_{23}.IJ_2 \quad [7]$$

$$\frac{dIJ_3}{dt} = K_{23}\, IJ_2 \longrightarrow K_{3\text{-}4} IJ_3 \quad [8]$$

$$\frac{dIJ_4}{dt} = K_{34}\, IJ_3 \longrightarrow K_{4A}\, IJ_4 - KIJ_{D4} \quad [9]$$

Applying the set of equations in the model we see that some series can be established for growth: for example adults produce IJ_1, which then progress to IJ_2 to IJ_3 to IJ_4, to living adult (A) and dead adult (DA).

However we note that the quantitative results are very variable in respect of the time at which the population reaches maximum in terms of the model, and the maximum based on experimental results. This led us to conclude that EPN growth was controlled by the availability of bacteria in feed, and that that of the bacteria was also nutrient-related, for example controlled by the availability of nitrogen.

5.2 Recommendations

In view of the findings described above, a number of recommendations can be made for subsequent investigation unto the commercial viability of mass production of EPNs. Further research into the factors affecting the growth of *Heterorhabditis bacteriaphora* nematodes in the South African context needs to be done. Also, the recovery of EPNs is an important aspect of commercial production that has yet to be addressed. A careful study of how to further engineer the life of EPNs to make it possible to choose the optimal stage at which they should be recovered is still required.

However this work has shown that it is indeed possible to grow EPNs in submerged culture, and this is perhaps a first small step towards commercializing this concept.

Appendix A

A1:Experiment 1
Medium: Liquid Culture Medium
Reactor: Fermenter
Size: 1000mℓ
Air flow rate:15kPa

time	counts/mℓ
0	1.00E+00
1	3.98E+09
3	6.70E+09
6.84	8.77E+09
22	9.00E+09
24	9.17E+09
29	1.10E+10
41	2.51E+09
45	3.00E+09

A2:Experiment 2
Medium: Egg Yolk
Reactor: Fermenter
Size: 1000mℓ
Air flow rate:10-15kPa

Time(hrs)	Counts/mℓ	Centrifuged Nitrogen Concentration in Liquid (mg/mℓ)	Centrifuged Nitrogen Concentration Pellet (mg/mℓ)	Centrifuged COD Concentration in Liquid (mg/mℓ)	Centrifuged COD Concentratio In Pellet (mg/mℓ)
0	1	0.00315	0.002	70	40
1	3.00E+07	0.00336	0.0024	Not Measured	Not Measured
6	3.10E+08	0.00819	0.0025	110	85
25	8.50E+08	0.0308	0.001	178	110
29	9.33E+09	0.0043	0.0084	Not Measured	Not Measured
48	2.00E+09	0.0007	0.0024	Not Measured	Not Measured
51	1.52E+09	0.0007	0.002	Not Measured	Not Measured
58	8.00E+10	0.00175	0.0016	Not Measured	Not Measured
71	2.00E+11	0.005565	0.0074	118	98

(Batch and Continuous data)
(Experiment 2 Continued…..)

Time(hrs)	Centrifuged Phosphorous Concentration in Liquid (mg/mℓ)	Centrifuged Phosphorous Concentration In Pellet (mg/mℓ)
0	1.5	0,6
1	1.65	0,59
6	2.10	0.1
25	2.10	0.5
29	Not Measured	Not Measured
48	Not Measured	Not Measured
51	Not Measured	Not Measured
58	Not Measured	Not Measured
71	1.64	0.65

(Batch and Continuous data)

A3: Experiment 3
Medium: Liquid Culture Medium
Reactor: Fermenter
Size: 1000mℓ
Air flow rate:10-15kPa

time	Counts/ml
0	1
1	6.50E+07
2	8.00E+07
3	1.40E+08
4	2.14E+09
5	7.60E+07
7	7.40E+07
10	6.00E+07
18	4.30E+07
19	2.60E+07
24	1.50E+07
25	3.00E+06
27	8.00E+06
29	2.00E+07
31	3.80E+07
47	7.00E+06
53	3.00E+06
71	1.60E+07

A4: Experiment 4
Medium: Liquid Culture Medium
Reactor: Shake flask
Size: 250mℓ
Shaker rate:133rpm

time	Counts/mℓ
1	1.37E+01
2	3.30E+01
3	5.37E+02
4	1.89E+03
5	6.40E+03
6	3.00E+05
7	1.46E+06
8	6.47E+06
9	4.13E+07
10	3.85E+08
11	1.13E+09
12	1.83E+09
13	9.40E+08
14	3.90E+08
15	2.11E+08
16	1.02E+08
18	1.45E+07
20	3.89E+07
22	2.68E+04
24	1.78E+04
26	1.06E+04
28	4.50E+03
30	3.70E+03

A5 Experiment 5
Medium: Liquid Culture Medium
Reactor: Fermenter
Size: 1000mℓ

Time(hrs)	Counts/mℓ	pH
0	1.00E+00	7.8
1	4.00E+08	7.8
3	7.60E+08	7.32
5	4.00E+09	7.23
18	8.80E+09	6.24
23	9.00E+09	6.24
25	9.77E+09	6.13
30	1.10E+10	6.26
43	2.51E+09	7.24
47	2.00E+09	7.56

A6:Photorhabdus Luminescence growth Experiment

Medium: Liquid Culture Medium
Reactor: Fermenter and Shake flask
Size: 1000mℓ and 70mℓ
Air flow rate:10-15kPa
Medium pH: 6.15

		Bioreactor					shake flask		
time	hrs	OD1	OD2	Average	CFU		OD1	OD2	Average
10am	0	0	0	0	1	0	0	0	0
11am	1	0.335	0.502	0.4185	650000	5.81291336	0	0	0
12am	2	0.515	0.515	0.515	8000000	6.90308999	0.28	0	0.14
1pm	3	0.69	0.734	0.712	150000000	8.17609126	0.644	0.385	0.5145
2pm	5	1.189	1.239	1.214	2140000000	9.33041377	0.609	0.609	0.609
3pm	6	0.9	0.9	0.9	76000000	7.88081359	0.655	0.66	0.6575
5pm	7	1.044	1.01	1.027	74000000	7.86923172	0.439	0.43	0.4345
8pm	10	0.894	1.047	0.9705	60000000	7.77815125			0.44
5am	18	1.655	1.697	1.676	43000000	7.63346846	0.441	0.468	0.4545
6am	19	1.964	1.997	1.9805	26000000	7.41497335	0.563	0.533	0.548
10am	24	4.35	4.1	4.225	15000000	7.17609126	0.28	0.539	0.4095
11am	25	0.143	0.87	0.5065	6000000	6.77815125			0.799
1pm	27	0.124	0.157	0.1405	8000000	6.90308999	1.704	1.862	1.783
3pm	29	0.206	0.21	0.208	1200000000	9.07918125			1.77
5pm	31	0.173	0.157	0.165	38000000	7.5797836			1.65
7am	43	0.569	0.545	0.557	7000000	6.84509804	1.503	1.727	1.615
9pm	45	0.62	0.62	0.62	32000000	7.50514998	1.367	1.616	1.4915
9am	57	1.28	1.243	1.2615	16000000	7.20411998	0.677	0.818	0.7475

A7: Photorhabdus Luminescence growth Experiment
Medium: Liquid Culture Medium
Reactor: Fermenter
Size: 1000mℓ
Air flow rate:10-15kPa

time	hrs	OD1	OD2	Average	CFU	logCFU
12pm	0	0	0	0	1	0
1pm	1	7.11	6.53	6.82	400000000	8.602059991
2pm	3	6.665	7.04	6.8525	760000000	8.880813592
5pm	5	2.33	2.17	2.25	4000000000	9.602059991
9am	18	4.32	4.42	4.37	8800000000	9.944482672
11am	23	4.16	3.82	3.99	9000000000	9.954242509
1pm	25	4.96	4.98	4.97	9770000000	9.989894564
6pm	30	10.102	10.013	10.0575	11000000000	10.04139269
7am	43	6.6	7.65	7.125	2510000000	9.399673721
11am	47	8.8	8.82	8.81	2000000000	9.301029996

A8: Photorhabdus Luminescence Experiment in reactor
Medium: Liquid Culture Medium
Reactor: Airlift Reactor
Size: 1000mℓ
Air flow rate:10-15kPa

Experiment with Pure Photorhabdus Culture				
Innoculum used		40mℓ		
Volume of media(LCM)		1000mℓ		
Time	Spec Avg	number	Dilution	pH
1	0.682	39800000000	10	7.5
3	0.44	67000000000	10	7.34
5	0.225	87700000000	10	7.27
21	0.437	89000000000	10	6.24
23	0.399	90000000000	10	6.24
25	0.497	67700000000	10	6.13
30	1.014	1.1E+11	10	6.26
43	0.713	25100000000	10	7.24
47	0.881	40000000000	10	7.56
49	0.963	57200000000	10	7.5
68	1.0015		20	8.3

A9: Photorhabdus Luminescence Experiment in reactor
Medium: Liquid Culture Medium
Reactor: Airlift Reactor
Size: 1300mℓ
Air flow rate:10-15kPa

1.3Litre reactor A		
Innoculume used		20mℓ
Vol of Media(LCM)		700mℓ
Time	OD Spec Avg	
0	0	
1	0	
2	0	
3	0	
4	0.019	
5	0.021	
21	0.13	
23	0.305	
23.5	0.355	
24	3.428	
24.5	0.384	
25	0.388	
26	0.366	
27	0.344	
28	0.431	
29	1.243	

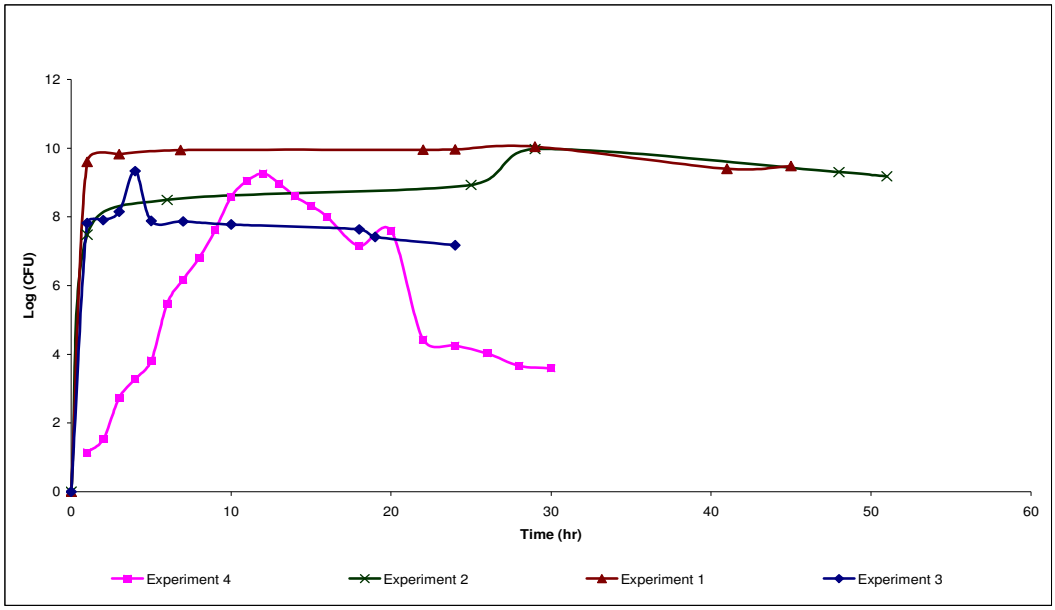
A10: Photorhabdus Luminescence Experiment in reactor
Reactor: Airlift Reactor
Size: 1300mℓ
Air flow rate:10-15kPa

1.3Litre reactor B		
Innoculume used		20mℓ
Vol of Media(LCM)		700mℓ
Time	Spec Avg	
0	0	
1	0	
2	0	
3	0	
4	0.019	
5	0.021	
21	1.113	
23	1.221	
23.5	1.317	
24	1.364	
24.5	1.38	
25	1.444	
26	Above range reading	

A11: Photorhabdus Luminescence Experiment in reactor
Reactor: Airlift Reactor
Size: 1300mℓ
Air flow rate:10-15kPa

Experiment with Pure Photorhabdus Culture			
Innoculum used		10mℓ	
vol of media(LCM)		1000mℓ	
Time	Spec Avg	number	
0	0		
1	0.736		
3	1.343		
5	1.413		
21	1.676		
22	1.981		
23	0.477		
24	0.51		
25	0.255		
26	0.27		
27	0.31		
28	0.334		
29	0.364		
36	1.1335		

A.12 Calculation for Doubling time Figure 2.2



Doubling time from the equation: $\log_{10} N_{Exp} - \log_{lag} + g \log_{10} 2$

Exp 1 calculation

$= (\log_{10} N_{exp} - \log_{lag} N_o) / \log_{10} 2$

$= (9.8 - 1.9)$ h / 0.301

= 26.24 generations in 2 hours

= 2 / 26.24 = 0.0762 hours

= 0.0762 x 60minutes = 4.57 minutes

Exp 2 calculation

$= (\log_{10} N_{exp} - \log_{lag} N_o) / \log_{10} 2$

$= (8.1 - 2)$ h / 0.301

= 20.3 generations in 1 hours

= 1 / 20.3 = 0.0493 hours

= 0.0493 x 60minutes = 3 minutes

Exp 3 calculation

$= (\log_{10} N_{exp} - \log_{lag} N_o) / \log_{10} 2$

$= (9.8 - 2)$ h / 0.301

= 25.91generations in 2 hours

= 2 / 25.91 = 0.0772 hours

= 0.0772 x 60minutes = 4.63 minutes

Exp 4 calculation

$= (\log_{10} N_{exp} - \log_{lag} N_o) / \log_{10} 2$

$= (9.8 - 1.5)$ h / 0.301

= 27.57generations in 8.4 hours

= 8.4 / 27.57 = 0.3047 hours

= 0.3047 x 60minutes = 18.3 minutes

Appendix B

B1:Mean counts for Figure 3.1
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

	Exp 1	Exp 2	Exp 3	Exp 4
week 1	8000	8000	20000	10000
week 2	20000	20000	50000	50000
week 3	40000	40000	60000	310000
Week 4	40000	40000	230000	2300000
week 5	800000	800000	2100000	2300000
week 6	210000	210000	400000	500000
week 7	200000	200000	400000	380000
week 8	260000	260000	380000	200000

B2:Mean Counts for Figure 3.2
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

	Exp 1	Exp 2	Exp 3	Exp 4
week 1	7000	4000	10000	8800
week 2	0	2000	10000	10000
week 3	50000	20000	20000	60000
week4	48000	30000	100000	0
week 5	30000	50000	200000	0
week 6	0	0	0	0
week 7	0	0	0	0
week 8	0	0	0	20000

B3:Mean counts for Figure 3.3
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

	Exp 1	Exp 2	Exp 3	Exp 4
week 1	9400	7000	5800	2000
week 2	9600	7000	6400	5800
week 3	5800	12000	4800	6800
week4	18000	14000	18000	4400
week 5	150000	64000	150000	12800
week 6	58000	12000	62000	

B4; Mean counts for Figure 3.4
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

	Exp 1	Exp 2	Exp 3	Exp 4
week 1	0	600	300	400
week 2	0	200	100	2600
week 3	0	0	0	2600
week4	0	200	100	0
week 5	64000	4000	34000	1600
week 6	0	0	0	

B5: Nutrient Depletion Experiment using egg yolk Media in Figure 3.5
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

Period	1	2
week 1	10000	10000
week 2	50000	50000
week 3	310000	310000
week 4	2300000	2300000
week 5	2300000	2300000
week 6	500000	500000
week 7	380000	380000
week 8	200000	200000
week 8	70000	130000
week 9	60000	120000
week 10	2400000	3100000
week 11	2200000	1900000
week 12	880000	160000

B:6Nutrient Depletion Experiment using Liquid culture Media in Figure 3.6
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Liquid culture media

Period	1	2
week 1	20000	20000
week 2	40000	40000
week 3	130000	130000
week 4	160000	160000
week 5	70000	70000
week 6	68000	68000
week 7	36000	36000
week 8	26000	26000
week 8	4000	7000
week 9	4000	23000
week 10	10000	120000
week 11	10000	64000

B7: Media evaluation of the growth patterns in Figure 3.7
Average shaker speed: 133rpm
Temperature: 26^0C

Period	Lipid broth Media	Nutrient broth media	Liquid culture media	Egg yolk media
week 1	3000	1000	1000	9400
week 2	157800	8800	200	9600
week 3	64000	118000	5800	5800
week4	34000	54000	6000	18000
week 5	24000	6200	940	150000
week 6				58000

B8: *Heterorhabditis Indica* in liquid culture medium in Figure 3.8
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Liquid culture media

Period	Juveniles	Adults	Dead nematodes
week 1	20000	10000	20000
week 2	40000	10000	30000
week 3	130000	20000	20000
week4	160000	40000	90000
week 5	70000	2000	50000
week 6	68000	0	18000
week 7	36000	0	20000
week 8	26000	0	38000

B9:*Heterorhabditis Bacteriaphora* in lipid broth medium in Figure 3.9
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Lipid broth media

Period	Juveniles	Adults	Dead nematodes
week 1	3000	0	0
week 2	157800	0	0
week 3	64000	16000	32000
week4	34000	2000	20000
week 5	24000	0	30000

B10:*Heterorhabditis Bacteriaphora* in nutrient broth medium in Figure 3.10
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Nutrient broth media

Period	Juveniles	Adults	Dead Nematodes
week 1	1000	65	0
week 2	8800	400	4000
week 3	118000	24000	166000
week4	54000	12000	74000
week 5	6200	1400	19400

B11: *Heterorhabditis Indica* in egg yolk in Figure 3.11
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

Period	Juveniles	Adults	Dead nematodes
week 1	40000	1000	0
week 2	20000	0	0
week 3	110000	2000	0
week 4	180000	10000	0
week 5	1100000	0	30000
week 6	230000	0	0
week 7	230000	0	0
week 8	170000	0	120000

B12: *Heterorhabditis Indica* in egg yolk in Figure 3.12
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

Period	Juveniles	Adults	Dead nematodes
week 1	20000	20000	40000
week 2	50000	50000	70000
week 3	60000	60000	20000
week4	230000	230000	0
week 5	2100000	2100000	0
week 6	400000	400000	0
week 7	400000	400000	0
week 8	380000	380000	20000

B13:Heterorhabditis Bacteriaphora in lipid broth medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Lipid broth media

	Juveniles	Adults	Dead nematodes
week 1	5000	75	0
week 2	90000	8000	0
week 3	68000	0	0
week4	700000	0	100000
week 5	72000	0	48000

B14:Heterorhabditis Bacteriaphora in lipid broth medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Lipid broth media

	Juveniles	Adults	Dead nematodes
week 1	5000	75	0
week 2	5600	600	0
week 3	156000	4000	0
week4	70000	0	10000
week 5	36000	0	20000

B15:Heterorhabditis Bacteriaphora in lipid broth medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Lipid broth media

	Juveniles	Adults	Dead nematodes
week 1	3000	0	0
week 2	157800	0	0
week 3	64000	16000	32000
week4	34000	2000	20000
week 5	24000	0	30000

B16: Heterorhabditis Bacteriaphora in lipid broth medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Lipid broth media

	Juveniles	Adults	Dead Nematodes
week 1	3000	0	0
week 2	30000	0	0
week 3	30000	14000	8000
week4	44000	0	36000
week 5	34000	0	10000

B17: Heterorhabditis Bacteriaphora in Egg yolk medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

	Juveniles	Adults	Dead Nematodes
Week1	35000	1500	250
Week 2	17500	2500	0
Week 3	77500	2250	0
Week 4	122500	10000	7500
Week 5	482500	2500	17500
Week 6	172500	0	0
Week 7	120050	0	1050
Week 8	160000	0	60000

B18:Heterorhabditis indica in Liquid culture medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Liquid culture medium

	Juveniles	Adults	Nematodes
week 1	20000	10000	20000
week 2	40000	10000	30000
week 3	130000	20000	20000
week4	160000	40000	90000
week 5	70000	2000	50000
week 6	68000	0	18000
week 7	36000	0	20000
week 8	26000	0	38000

B19:Heterorhabditis indica in Liquid culture medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Liquid culture medium

	Juveniles	Adults	Nematodes
week 1	70000	10000	0
week 2	10000	0	40000
week 3	40000	0	70000
week4	10000	10000	90000
week 5	4000	0	25000
week 6	1600	0	12200
week 7	1000	0	19200

B:20: Heterorhabditis indica in Liquid culture medium shake flask
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Liquid culture medium

	Juveniles	Adults	Nematodes
week 1	20000	10000	0
week 2	30000	0	0
week 3	80000	0	0
week4	30000	0	0
week 5	4000	0	2000
week 6	12000	0	0
week 7	2800	0	0
week 8	1200	0	1000

Appendix C

C1: Comparison of Juveniles in different shake flask volumes in Figure 4.2
Average shaker speed: 133rpm
Temperature: 26^0C
Medium: Egg yolk

Days	250mL(IJ/mℓ)	Days	!000mL(IJ/mℓ)	Days	500mL(IJ/mℓ)
1	5000	1	3000	1	1000
7	30000	6	10000	7	27000
14	68000	14	14000	21	56000
21	500000	21	160000	30	62000
30	72000	27	120000	35	35000
		35	90000		

C2:The introduction of a reactor sample into a shake flask sample in Figure 4.3
Average shaker speed: 133rpm
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

Days	Juveniles/mℓ (Reactor)	Days	Juveniles/ mℓ (Shake flask)
1	2000	1	2000
6	4000	6	4000
8	1400	8	1400
9	2300	9	2300
15	1000	9	2300
		14	6900
		23	156000
		30	70000
		30	36000

C3: The increase in population of infective juveniles using the fed batch system in an external recycle reactor in Figure 4.4
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

Days	Juveniles/mℓ
1	4000
6	16000
11	20000
16	30000
21	5000

C 4: The population of Infective juveniles count over a batch external recycle reactor
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

Days	Juveniles/mℓ
1	9000
3	16000
6	18000
10	16000
16	7000
21	4000

C5: Comparison of infective juveniles counted over a batch and fed batch external recycle reactor system.
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

Days	Juveniles/mℓ	Days	Juveniles/mℓ
1	4000	1	9000
6	16000	3	16000
11	20000	6	18000
16	30000	10	16000
21	5000	16	7000
		21	4000

C6:The Juveniles collected in water disengager over external recycle reactor.
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

day	Juveniles/mℓ	Juveniles/mℓ
1	5400	5400
2	3000	3000
8	3000	1400
14	12000	1200
20	3000	680
26	250	250

C7: The Adult collected in water disengager over external recycle reactor
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

day	Adult/mℓ	Adult/mℓ
1	0	0
2	500	500
8	400	300
14	300	22
20	100	30
26	40	40

C8: Reactor Experiment Using Bacteriaphora EPNs in lipid broth medium
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

Day	Juveniles/mℓ	Adult/mℓ
1	5000	1000
3	9000	0
6	12000	1000
9	20000	1000
16	12000	27
21	1000	0
24	1000	62

C9: Reactor Experiment using Bacteriaphora in Egg yolk medium
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Egg yolk medium

day	Juveniles/mℓ	Adult/mℓ
1	5500	0
2	3000	500
9	3000	300
9	3000	400
16	4000	0
16	12000	300
22	300	30
29	250	40

C10: Reactor Experiment using Bacteriaphora using LCM
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Liquid culture medium

day	Juveniles/mℓ	Adult/mℓ
1	3000	500
3	2900	1300
4	2400	800
10	530	600
17	4000	1500
24	2900	700
33	1900	1700
33	12000	1000
35	2000	0

C11: Reactor Experiment using Bacteriaphora using Lipid Broth medium
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

day	Juveniles/mℓ	Adult/mℓ
1	9000	2000
3	16000	2000
6	18000	2000
10	16000	2000
16	7000	300
21	4000	2000

C12: Reactor Experiment using Bacteriaphora using Lipid Broth medium
Temperature: 26^0C
Air flow rate: 10KPa
Medium: Lipid broth medium

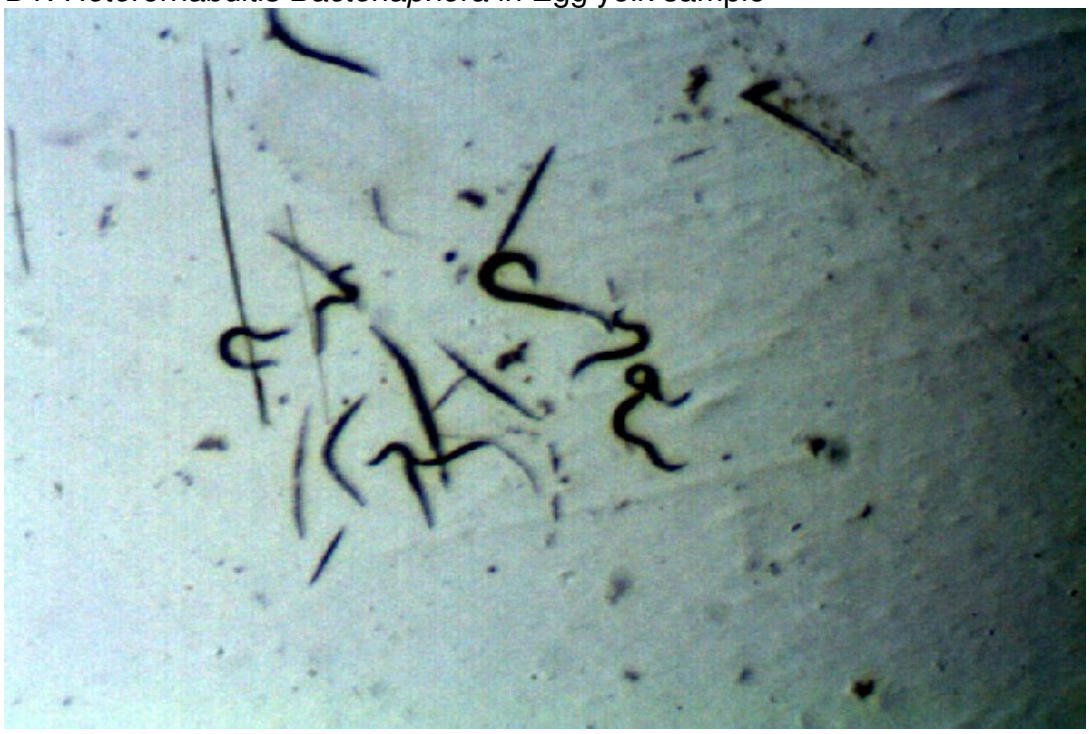
day	Juveniles/mℓ	Adult/mℓ
1	4000	2000
6	16000	300
11	20000	0
16	30000	0
21	5000	0

C13:1000mℓ shake flask Experiment using Bacteriaphora using Lipid Broth medium
Temperature: 26^0C
Medium: Lipid broth medium
Container: Shake Flask
Volume: 1000 mℓ

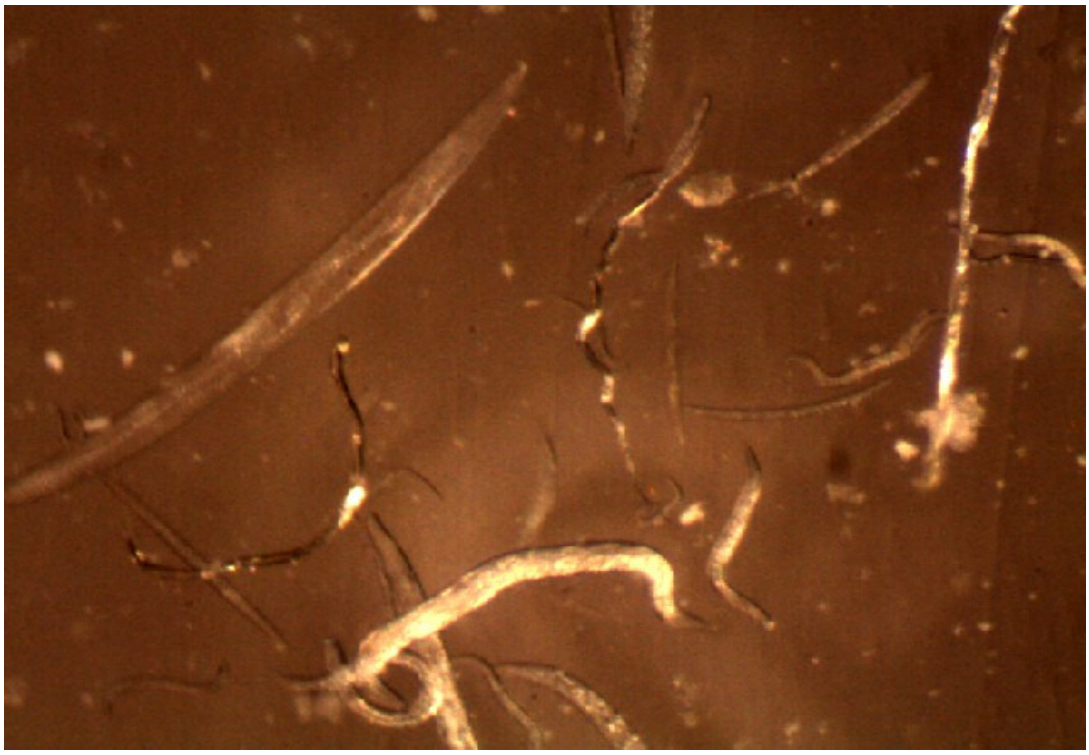
day	Juveniles/mℓ	Adult/mℓ
1	10000	2000
9	14000	10000
16	160000	20000
22	120000	10000
31	90000	0

Appendix D

D1: *Heterorhabditis Bacteriaphora* in Egg yolk sample



D2*:Heterorhabditis Bacteriaphora* in egg yolk sample



D3:*Heterorhabditis Bacteriaphora* in Egg yolk sample



D4: *Heterorhabditis Bacteriaphora* in lipid broth Sample



D5:*Heterorhabditis Bacteriaphora* in lipid broth Sample



D6:*Heterorhabditis Bacteriaphora* in lipid broth Sample



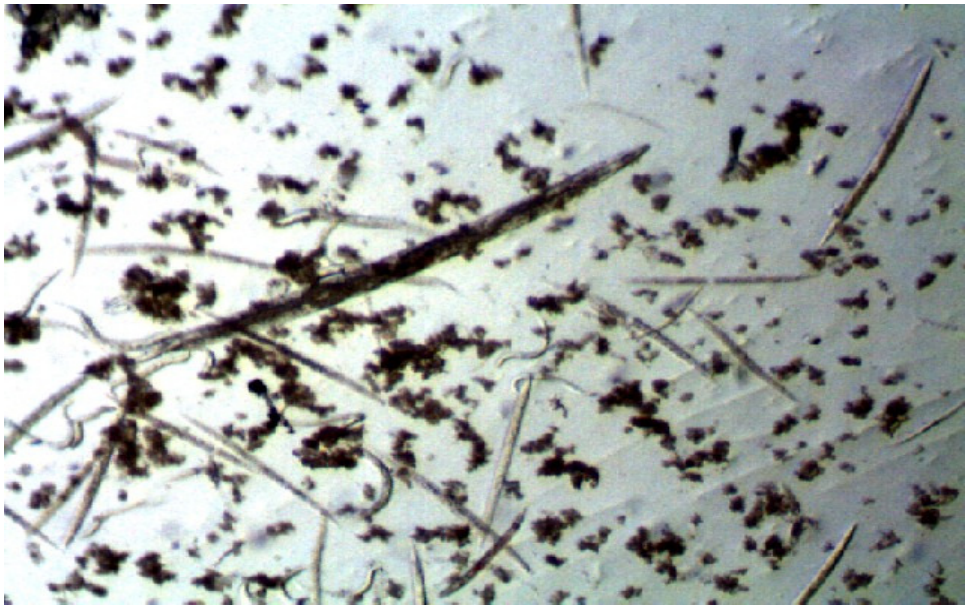
D7: *Heterorhabditis Bacteriaphora* in lipid broth Sample



D8: *Heterorhabditis Bacteriaphora* in lipid broth Sample



D9: *Heterorhabditis Bacteriaphora* in Egg yolk Sample



D10:*Heterorhabditis Bacteriaphora* in liquid culture sample



D11:*Heterorhabditis Bacteriaphora* in Nutrient broth Sample

